LIMBURGS UNIVERSITAIR CENTRUM
Departement Medische Basiswetenschappen
Dienst Fysiologie

CELLULAR MECHANISMS OF K^+ TRANSPORT IN MALPIGHIAN TUBULES OF *FORMICA*

Anne LEYSSENS

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Eindwerk aangeboden
tot het behalen van
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II

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SAMENVATTING

Het verkrijgen van een beter inzicht in het transport van ionen en water in epitheelcellen en van hun antwoord op diverse stimuli uit de omgeving, vormt een belangrijk onderzoeksdomein van de hedendaagse fysiologie. Het doel van het hier voorgestelde werk was de mechanismen te achterhalen die verantwoordelijk zijn voor het transcellulair transport van K^+-ionen in geïsoleerde tubuli van Malpighi uit de volwassen werkster behorende tot de soort *Formica polyctena* (rode bosmier). De eerste stap in de urineproduktie van insekten is de aktieve secretie van zouten, gevolgd door osmotisch gekoppelde vloeistofsecretie. In het hier bestudeerde preparaat wordt K^+ gesecreteerd tegen een aanzienlijke electrochemische gradient; de aktieve stap is gelokaliseerd ter hoogte van de apikale membraan. Cl^- fungeert als tegenion om de electroneutraliteit te verzekeren. De secretiesnelheid is voornamelijk afhankelijk van de K^+- en Cl^--concentratie in de hemolymfe. In deze studie werden de transportsystemen in de basale en apikale celmembraan bestudeerd evenals het regelmechanisme waardoor de K^+-concentratie van de hemolymfe de secretiesnelheid kan moduleren. Het uitvoeren van secretieëxperimenten en van electrofysiologische metingen in verschillende experimentele omstandigheden resulteerde in de volgende bevindingen.

De hoge K^+-permeabiliteit van de basale celmembraan.
De basale celmembraan, in direct contact met de hemolymfe, wordt gekenmerkt door een hoge K^+-permeabiliteit. Het basale membraanpotentiaalverschil (V_{bl}) is zeer gevoelig voor veranderingen van de basale K^+-concentratie (K_{bl}) maar zeer weinig voor veranderingen van de basale Na^+ - (Na_{bl}) en Cl^- - (Cl_{bl}) concentratie (artikel 1). K^+-selectieve metingen in verschillende K_{bl} en Cl_{bl} toonden aan dat V_{bl} de evenwichtspotentiaal voor K^+ over de basale membraan (E_K^{bl}) altijd zeer dicht benadert (artikel 3). De gevoelige reductie van de secretiesnelheid, het verlies van de K^+-sensitiviteit van de basale membraan en het verhogen van de basale membraanweerstand in aanwezigheid van de K^+-kanaalblokker Ba^{2+} wijzen eveneens op het bestaan van K^+-kanalen in de basale celmembraan (artikel 2).

De intracellulaire K^+-concentratie varieert, afhankelijk van de K^+-concentratie in de hemolymfe.
In tegenstelling tot de algemene bevinding dat de intracellulaire K^+-concentratie (K_c) in eukaryotische cellen gewoonlijk relatief onveranderlijk is en varieert tussen 120 en 140 mM, reageert in de hier bestudeerde tubulescellen K_c op een snelle en reversiebele wijze op veranderingen van K_{bl}. Afhankelijk van K_{bl} varieerde K_c tussen 29 en 123 mM. Deze wijzigingen in K_c komen tot uiting in transiënte veranderingen van V_{bl} bij het wijzigen van K_{bl}. De luminale K^+-concentratie (K_l) verandert ook in functie van K_{bl} maar in veel geringere mate (artikel 3).

De intrinsieke regeling van de K^+-secretie door de K^+-concentratie in de hemolymfe.
De hoge K^+-permeabiliteit van de basale celmembraan en de veranderingen van K_c in functie van K_{bl} spelen beiden een belangrijke rol in de intrinsieke regeling van de K^+-secretie door K_{bl} : hoe hoger de K^+concentratie in het medium, hoe groter de secretiesnelheid, wat op zijn beurt kan resulteren in een verlaging van de omgevende K^+-concentratie, m.a.w. in de hemolymfe.
Als een eerste regelmechanisme kan een verhoging van K_{bl} de secretie stimuleren door de hoogte van de electrische barrière te verminderen, die overwonnen moet worden door het K^+-transport ter hoogte van de apikale membraan. Dit is mogelijk omdat de verhouding van de apikale (R_{ap}) over de basale (R_{bl}) membraanweerstand hoog is (ongeveer 40) en de basale membraan gekenmerkt wordt door een hoge K^+-conductantie, zodat V_{bl} enerzijds $E_K{}^{bl}$ benadert, en anderzijds zijn waarde oplegt aan de apikale membraan. Dit laatste is te begrijpen uit de toepassing van het electrisch equivalent schema van het epitheel (artikel 1).
Als een tweede regelmechanisme bepaalt K_{bl} ook de intracellulaire hoeveelheid K^+ die beschikbaar is, en dus ook de concentratiestap die overwonnen moet worden voor de extrusie van K^+ naar het lumen (artikel 1 en 3).
Het bestaan van beide regelmechanismen wordt bevestigd door het omgekeerde verband dat werd gevonden tussen de apikale electrochemische gradient voor K^+ en de secretiesnelheid. De gradient zelf is des te kleiner naarmate K_{bl} groter is (artikel 1 en 3).

Een H^+-pomp van het vacuolaire type in de apikale membraan .
In onze studie wordt het bewijs geleverd dat een electrogene V-type H^+-pomp aanwezig is in de apikale membraan van tubuli van Malpighi van *Formica*. De metabole inhibitor monojodo-azijnzuur (MIA) en twee V-type H^+-ATPase inhibitoren, N-ethylmaleimide (NEM) en bafilomycine (Baf.A1), verminderen significant de secretiesnelheid. De hoge verhouding van R_{ap} over R_{bl} heeft echter tot gevolg dat de effecten van de pompinhibitie op de membraanpotentiaalverschillen klein zijn en enkel zichtbaar aan de apikale zijde. De toediening van Ba^{2+} daarentegen resulteert in een verlaging van de verhouding R_{ap} over R_{bl} en in een hyperpolarizatie van V_{ap} en V_{bl}. Deze hyperpolarizatie suggereert de aanwezigheid van een electrogene pompaktiviteit in de apikale membraan die kan geïnhibeerd worden door MIA, NEM en Baf.A_1 (artikel 2).

K^+-extrusie naar het lumen wordt gerealiseerd door de combinatie van een electrogene H^+-pomp en een electroneutrale K^+/H^+-uitwisselaar.
We tonen verder aan dat de apikale celinwaarts gerichte H^+-concentratiegradient, die opgebouwd wordt door de electrogene H^+-pomp, de drijvende kracht is in de aktieve transfer van K^+ naar het lumen via een electroneutrale K^+/H^+-antiporter.

Voor elke K_{bl} was de celinwaartse H^+-concentratiegradient groot genoeg om K^+ via de K^+/H^+-antiporter naar het lumen te transporteren op een electroneutrale wijze (artikel 3).

Het verband tussen de apikale celinwaarts gerichte H^+-concentratiegradient enerzijds en K^+- en vloeistofsecretie anderzijds wordt verder verduidelijkt door het gebruik van de K^+-kanaalblokker Ba^{2+} en van de protonofoor 2,4-dinitrofenol (DNP). Beide stoffen verhinderen, op een zeer verschillende wijze, de opbouw van de apikale H^+-concentratiegradient: Ba^{2+} doordat de electromotorische drijfkracht van de pomp hoofdzakelijk gebruikt wordt in het opbouwen van een groot electrisch potentiaalverschil over de apikale membraan, en DNP door de activiteit van de H^+-pomp te blokkeren via depletie van ATP, en/of door het te niet doen van het H^+-concentratieverschil over het membraan via een protonofooreffect. Het verminderen van de apikale celinwaarts gerichte H^+-concentratiegradient door Ba^{2+} en DNP resulteert in een ongunstige verhouding van de apikale H^+- over K^+-concentratiegradient , wat gecorreleerd is met een reductie van de vloeistofsecretie. Alleen, indien de electroneutraliteit van de K^+/H^+-antiporter werd verondersteld, waren de berekende drijfkrachten voor de K^+/H^+-antiporter in de af- en aanwezigheid van DNP in overeenstemming met de gevonden waarden voor K^+- en vloeistofsecretie in de respectievelijke condities (artikel 4).

De intrinsieke regeling van de K^+-secretie door de K^+-concentratie in de hemolymfe (zie hoger) kan eveneens begrepen worden in het licht van boven beschreven model, d.i. een electrogene H^+-pomp in parallel met een K^+/H^+-antiporter. Als K_{bl} toeneemt, vermindert de apikale H^+-concentratiegradient of blijft hij ongewijzigd, en depolarizeert V_{ap}. Beide effecten zullen de snelheid van transfer van H^+ van de electrogene H^+-pomp verhogen. De afname van de apikale H^+-concentratiegradient wordt gecompenseerd door een gevoelige daling van de apikale K^+-concentratiegradient (K_c stijgt immers). Het resultaat is een grotere verhouding van de apikale H^+- over K^+-concentratiegradient en dus het vergemakkelijken van de extrusie van K^+ via de K^+/H^+-antiporter (zie "outline of personal work" en artikel 3).

Transportmechanismen voor de opname van K^+ doorheen de basale membraan.

In de basale membraan moeten er transportsystemen bestaan, die een voldoende opname van K^+ verzekeren voor het onderhouden van de secretie en die de aanpassing van K_c aan de K^+-concentratie in de hemolymfe kunnen verklaren.

Voor zover de basale electrochemische K^+-gradient gunstig is, kan de influx van K^+ gebeuren via de K^+-kanalen. De hyperpolarisatie van V_{bl} die ontstaat in aanwezigheid van Ba^{2+} bij elke K_{bl}, is een aanduiding dat passieve K^+-fluxen praktisch altijd inwaarts gericht zijn. Anderzijds zijn de berekende electrochemische K^+-gradienten zeer klein en minder inwaarts gericht naarmate K_{bl} kleiner is (artikel 3).

De aanwezigheid van alternatieve transportsystemen wordt gesuggereerd door het feit dat in aanwezigheid van Ba^{2+} de secretiesnelheid gevoelig gereduceerd wordt, maar

niet volledig verdwijnt (artikel 2), en dat in deze omstandigheden K_c nog steeds varieert in functie van K_{bl} (artikel 5).
De aanwezigheid van een primair aktief systeem voor K^+-opname, via een Na^+/K^+-ATPase of K^+/H^+-ATPase kon niet worden aangetoond. In lage K_{bl} resulteert het weglaten van Na^+ uit de badoplossing in een vermindering van de secretiesnelheid maar de Na^+/K^+-ATPase-blokker, ouabaine, heeft geen effect op de secretie, noch op electrische potentiaalverschillen of op K_c. Evenmin beïnvloedt de toediening van de K^+/H^+-ATPase blokker, Sch28080, de secretiesnelheid. Een inhiberend effect van vanadaat, een mogelijke blokker van beide aktieve transportsystemen, op de vloeistofsecretie werd waargenomen, maar kan echter het best worden toegeschreven aan een inhiberend effect op de apikale H^+-pomp (artikel 5).
In hoge K_{bl} blijkt de vloeistofsecretie Na^+-onafhankelijk te zijn en ongevoelig voor bumetanide, een blokker van secundair aktieve transportsystemen voor K^+ (,Na^+) en Cl^-. In meer fysiologische omstandigheden wordt het functioneren van een K^+/Cl^--cotransportsysteem gesuggereerd door de eerder lage gevoeligheid van de secretiesnelheid voor bumetanide, en door het stimulerend effect van Cl^--substitutie door Br^- op de vloeistofsecretie. In lage K_{bl} wijzen het inhiberend effect van Cl^--substitutie door Br^-, de Na^+-afhankelijkheid en de hoge bumetanide-gevoeligheid van de secretiesnelheid op een mogelijk belangrijke rol van een $Na^+/K^+/2Cl^-$-cotransporter in de basale K^+-influx (artikel 5).

Transportmodel voor transcellulair K^+-transport.
Onze experimentele bevindingen kunnen worden voorgesteld in een transportmodel voor transcellulair K^+-transport bij verschillende basale K^+-concentraties (Fig. I) (artikel 5).
Aan de apikale zijde kan de extrusie van K^+-ionen naar het lumen gerealiseerd worden via de combinatie van een electrogene H^+-pomp in parallel met een electroneutrale K^+/H^+-antiporter.
De influx van K^+ doorheen de basale celmembraan blijkt te verlopen via kanalen en secundair aktieve transportsystemen, en hun relatief belang lijkt te varieren afhankelijk van de basale K^+- (en Na^+-) concentratie. Primair aktieve systemen voor K^+-opname, met name een Na^+/K^+- of K^+/H^+-ATPase, lijken niet in belangrijke mate bij te dragen tot het transepitheliaal ionentransport of tot de instandhouding van het intracellulaire K^+- (en Na^+-) niveau. In een hoog K^+, Na^+-vrij medium, t.t.z. 113 mM K_{bl}, lijkt een gekoppelde influx van K^+ en Cl^- geen grote rol te spelen; de K^+-opname gebeurt via basale K^+-kanalen en wordt dan gedreven door een gunstige electrochemische gradient. In meer fysiologische omstandigheden, t.t.z. 51 mM K_{bl}, wanneer de basale electrochemische K^+-gradient minder inwaarts is, wordt de K^+-opname via kanalen minder belangrijk en een K^+/Cl^--cotransportsysteem speelt dan een significante rol in de K^+-influx. In een laag K^+, hoog Na^+ medium, t.t.z. 5 of 10 mM K_{bl}, is er weinig K^+ beschikbaar voor transepitheliaal transport; de basale

electrochemische K^+-gradient is nog kleiner dan in 51 mM K_{bl}, en mogelijks zelfs uitwaarts. In deze omstandigheden is een $Na^+/K^+/2Cl^-$-cotransportsysteem verantwoordelijk voor een deel van de basale K^+-opname.
De verschillende K^+-transportsystemen in de basale membraan kunnen met een verschillende snelheid functioneren afhankelijk van de basale K^+- (en Na^+-) concentratie en kunnen zo de hoeveelheid K^+ bepalen die beschikbaar is voor apikaal transport via de K^+/H^+-antiporter. De toename van K_c als antwoord op een toegenomen K_{bl} kan gebeuren via K^+-kanalen of via een K^+/Cl^--cotransportsysteem.

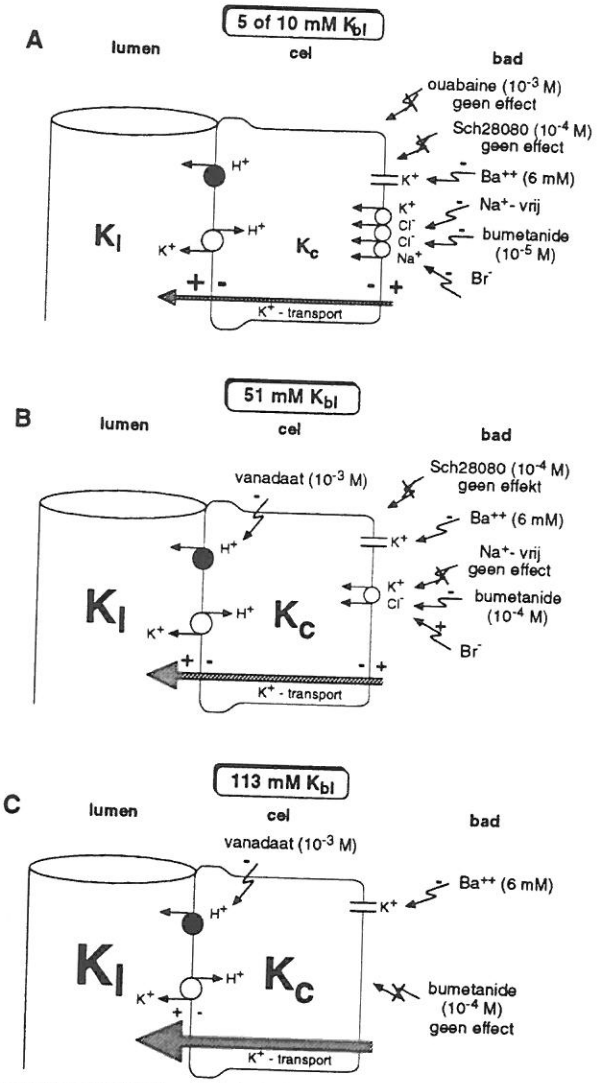

Fig. I. Model voor transcellulair K^+ transport in buisjes van Malpighi van *Formica* in 5 of 10 (A), 51 (B) en 113 (C) mM K_{bl} (143 mM Cl_{bl}).
Er wordt getoond dat :

1) K^+ transport toeneemt (grotere pijlen in de figuur) in een hogere K_{bl}
2) de apikale en basale membraanpotentiaalverschillen afnemen (kleinere + en - tekens in de figuur) als K_{bl} stijgt
3) K_l en K_c stijgen (grotere symbolen in de figuur) als K_{bl} stijgt
4) een vacuolaire H^+-pomp aanwezig is in de apikale membraan in parallel met een K^+/H^+-antiporter
5) afhankelijk van K_{bl}, K^+-kanalen, een K^+/Cl^- en/of een $Na^+/K^+/2Cl^-$-cotransporter functioneren aan de basale zijde.

Het effect van verschillende inhiberende substanties, toegediend aan de badzijde, op de verschillende transportsystemen wordt eveneens getoond.

GENERAL INTRODUCTION

A major topic in modern physiological research is the analysis of ion and water movements in secretory tissues and their response to various environmental signals. As control is exerted at many different levels, the mechanisms responsible for the regulation and fine tuning of secretory processes are very complex.

Research in our laboratory is concentrated on ion transport mechanisms and their regulation in insect epithelial tissues. To this end we used insect Malpighian tubules of adult worker ants of the species *Formica polyctena* .

Malpighian tubules of *Formica* are well suited for the physiological investigation of secretory processes (see Fig. II). The blind-ending tubules are attached to the intestine

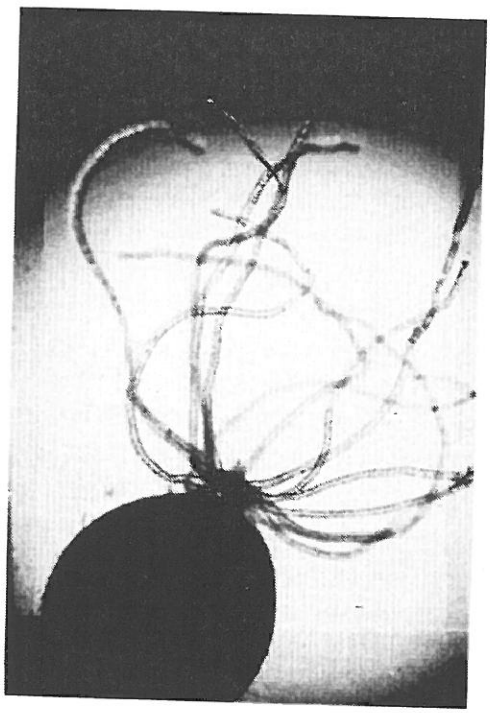

Fig. II. Part of the gastrointestinal tract with the Malpighian tubules attached to it.

between the midgut and the hindgut and are in direct contact with the haemolymph of the insect. Each consists of a single layer of cells arranged as a hollow tubule. Their basic function is to carry out ion and fluid transport in order to form an iso-osmotic primary excretory fluid. Reabsorption of water, ions, sugars, amino acids and other useful substances from the primary excretory fluid occurs further down in the system, to some extent in the anterior hindgut, but more importantly in the rectum. The first step in urine production of insects is the active secretion of salts, followed by osmotically coupled fluid secretion. The pioneering work of Ramsey (1954, 1958)

established for a number of insects that urine flow was generated by the active transport of K^+ ions from the haemolymph into the tubule lumen. Cl^- was proposed as a counterion to ensure electroneutrality. The rate of fluid secretion in isolated tubules is usually proportional to the external K^+ concentration, and the fluid produced is characterized by high K^+ levels. However it is known that feeding behaviour may influence Malpighian tubule function. The ability of Malpighian tubules of some insects to secrete Na^+ or to secrete either Na^+ or K^+ might indicate an evolutionary switch, from K^+-based secretion, as used by plant feeding insects, to a Na^+-based secretion in blood-sucking insects (for review see Phillips, 1981; Nicolson, 1993). In this view a study of the physiology of the Malpighian tubule system of the order *Hymenoptera* (subclass *Holometabola*) and in particular of the species *Formica polyctena* could contribute to a clear understanding of the ionic mechanisms involved in fluid secretion in insect Malpighian tubules.The feeding behaviour of *Formica* species is omnivorous. They feed on other insects, but also indirectly on plants, as they have a trophobiotic relationship with plant-sucking aphids (Körner, 1981). The Na^+ content of the haemolymph of adult *Formica* was found to be much larger than the K^+ content. To eliminate the excess K^+ the tubules primarily transport KCl and concentrate both K^+ and Cl^- in the lumen (Van Kerkhove *et al.*, 1989).

In view of a better understanding of the cellular transport mechanisms in both basal and apical membranes in Malpighian tubules, secretion rate measurements and the quantitative determination of the ionic content of the secreted fluid were combined in recent years with different electrophysiological techniques. Nicolson (1993) recently reviewed the data available (including part of the work presented here). Based on these data, the author proposed a general model for transepithelial ion transport in insect Malpighian tubules (see Fig. III). It includes an apical proton pump with a K^+/H^+ antiporter or Na^+/H^+ antiporter in parallel, instead of the K^+ pump once thought to be unique to insect epithelia, as well as Cl^- channels. At the basal membrane various transport mechanisms may be present: ion channels, electroneutral cotransport systems, and a Na^+/K^+ ATPase. A paracellular route, or other cell types in parallel to those which transport cations, may be significant in Cl^- movements.

Although the relative importance of these mechanisms differs markedly in different species and in the same species in different circumstances, a clear picture is beginning to emerge now on how Malpighian tubules function. The present work is aimed at better characterizing some essential transport mechanisms.

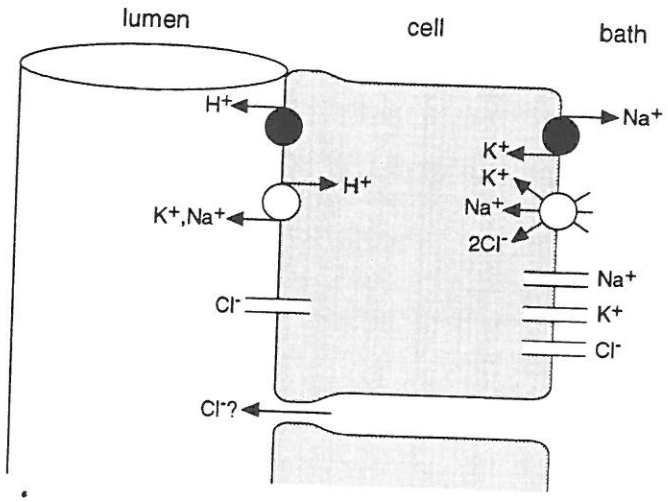

Fig. III. General model for transepithelial ion transport in insect Malpighian tubules (adapted from Nicolson, 1993).

OUTLINE OF PERSONAL WORK

Objectives and experimental approach

The present study is based on an extensive pilot study where, for the first time in Malpighian tubule research, tubules of adult worker ants of the species *Formica polyctena* were studied (Weltens, 1988; Van Kerkhove *et al.*, 1989). In this preparation, the rate of fluid secretion was found to be primarily dependent on the surrounding K^+ and Cl^- concentration. K^+ transport was always against its gradient and hence had to occur transcellularly. The active transport step was assumed to be located at the apical membrane (Van Kerkhove *et al.*, 1989).

The **aim of the present study** was to further investigate the ionic mechanisms underlying the transcellular K^+ movements in unstimulated *Formica* Malpighian tubules. An attempt has been made to reveal the K^+ transporting mechanisms in both the basal and apical membranes. The hypothesis of the existence of an apical H^+ pump and its role in establishing the driving force for transepithelial K^+ transport was investigated. Moreover the regulatory mechanisms by which the surrounding K^+ concentration can modulate the fluid secretion rate were analyzed.

To reach these objectives different **experimental approaches** were used.
As a first approach, fluid secretion rate (Fig. IVA) was measured in tubules with a low absolute rate of urine formation (Van Kerkhove *et al.*, 1989). Some series of fluid secretion experiments, carried out by other investigators, were integrated in the present study: S.-L. Zhang tested the effects of Ba^{2+} and N-ethylmaleimide (see paper 2); S. Dijkstra performed the Na^+ substitution experiments (see paper 5); P. Pirotte assisted to complete some series of experiments.
Secondly, electrophysiological techniques were applied to spontaneously secreting Malpighian tubules. Electrical potential differences across the epithelium and the cell membranes were measured using conventional microelectrodes. Intracellular and luminal K^+ activities were determined using double-barrelled K^+-selective electrodes (Fig.IVB).
Transcellular K^+ transport has been demonstrated to be linked to H^+ movements across the apical membrane; the intracellular and luminal H^+-selective measurements were carried out by S.-L. Zhang (see papers 3 and 4).

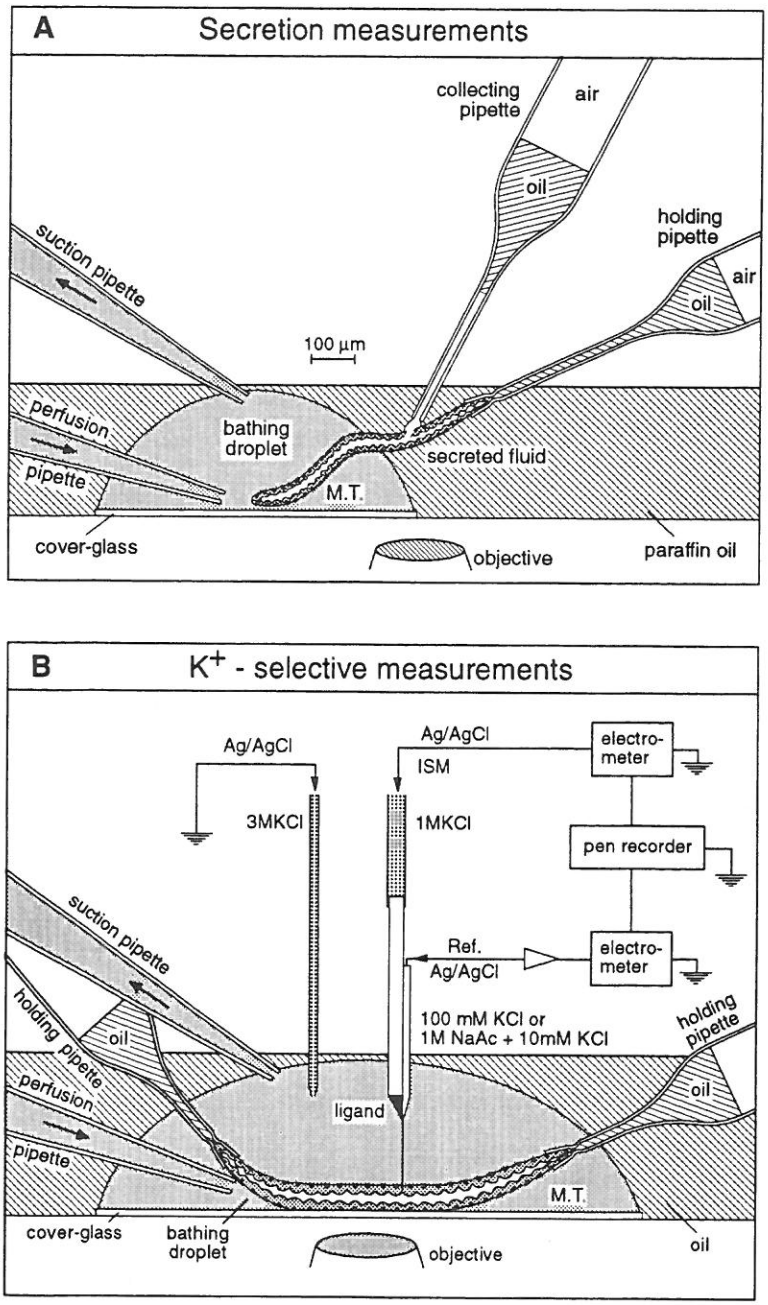

Fig. IV. Experimental set-up for fluid secretion (A) and K^+-selective measurements (B). More detailed information is given in the "Materials and Methods" section of the papers (Appendix).

Electrical resistance measurements on luminally perfused tubules, performed by P. Steels, allowed a better understanding of the pathways for, and the regulation of transcellular K^+ transport (see papers 1 and 2).

The different parts of our work have been presented as papers. The following pages are intended to briefly review the main results.

Paper 1: Intrinsic regulation of K^+ transport in Malpighian tubules (*Formica*): electrophysiological evidence.

In Malpighian tubules of *Formica*, intrinsic regulation of transepithelial K^+ transport seems to be present: the higher the K^+ concentration in the hemolymph (ranged from <5-70 mM, see Van Kerkhove *et al.*, 1989), the higher the rate of transport, resulting in turn in a lowering of the surrounding K^+. A first attempt was made to reveal this regulatory mechanism by investigating the properties of both the basal and apical membrane with convential microelectrodes.
The basal membrane potential difference (V_{bl}) demonstrated a high sensitivity to changes in the bath K^+ concentration (K_{bl}) and was only slightly influenced by changes in the Na^+ and Cl^- concentration in the bath.
Also the apical membrane potential difference (V_{ap}) was sensitive to K_{bl} : the voltage changes in V_{ap} were 60-70 % of those in V_{bl}. Analysis of the electrical equivalent model, taking into account the high resistance ratio (ca 40) of the apical over basal membrane estimated from measurements in luminally perfused tubules, explained the cross-talk between both membranes. An important role for the high basal K^+ conductance in the intrinsic regulation of K^+ transport by the surrounding K^+ concentration was proposed: increasing K_{bl} depolarizes V_{bl} and hence decreases the electrical gradient to overcome by apical K^+ translocation.
Transient changes in V_{bl} following a K_{bl} jump and the non-ideal (i.e. <58 mV/decade) relationship between V_{bl} and log K_{bl} suggested a response of the intracellular K^+ concentration (K_c) to a change in K_{bl}. A second mechanism of regulation of the fluid secretion rate by K_{bl} was proposed: the bath K^+ concentration may determine the amount of intracellular K^+ available for apical K^+ transport.
It was concluded that the active step in transcellular K^+ transport in *Formica* Malpighian tubules was located in the apical membrane. The inverse relationship between K^+ transport and V_{ap} suggested the presence of an electrogenic pump in this membrane.

Paper 2: Unmasking of the electrogenic H^+ pump in isolated Malpighian tubules (*Formica polyctena*).

The transport system that is responsible for the active transfer of cations across the apical membrane of insect epithelial cells was long thought to be an electrogenic K^+ pump or "common cation pump" since other alkali metal ions were transported as well (Maddrell, 1971). In searching for the molecular correlate of the insect K^+ pump, an ATPase activity was demonstrated in the goblet cell apical membrane of *Manduca sexta* midgut (Wieczorek *et al.*, 1986) and was subsequently purified and characterized as a vacuolar type (V-type) H^+ ATPase (Wieczorek *et al.*, 1991). This finding was unexpected because V-type ATPases are usually situated in endomembranes of acidic organelles. Along with P-type ATPases of plasma membranes and the sarcoplasmatic reticulum and F-type ATPases of mitochondria, chloroplasts and bacteria, V-type ATPases form the third family of primary active ion pumps (Forgac, 1989; Nelson and Taiz, 1989; Nelson, 1991). Finally, transport studies with goblet cell apical membrane vesicles demonstrated the exclusive H^+ specificity of the V-type H^+ pump and identified, in parallel an ATP-independent K^+/nH^+ antiporter in the membrane (Wieczorek *et al.*, 1989; Wieczorek *et al.*, 1991). Based on these results, the authors proposed a V-type H^+ ATPase and a K^+/nH^+ antiporter to represent the functional elements of the electrogenic K^+ pump in *Manduca sexta* midgut. We verified whether this model could also describe the active K^+ transport in *Formica* Malpighian tubules. Assuming the presence of an active electrogenic H^+ pump in the apical membrane, the effect of the metabolic inhibitor monoiodo-acetic acid (MIA; 2.10^{-4} M) and of two V-type ATPase blockers, i.e. Bafilomycin A1 (Baf.A_1; 5.10^{-7} - 5.10^{-6} M) and N-ethyl maleimide (NEM; 5.10^{-4} M), was tested on fluid secretion and on V_{ap}. As expected, fluid secretion was strongly inhibited by these compounds. However, only mild depolarizations of V_{ap} were observed. It was concluded that the effect of pump inhibition on V_{ap} was masked due to the high resistance ratio of the apical over basal membrane. The K^+ channel blocker Ba^{2+} was applied effectively to lower the resistance ratio and to demonstrate now a clear depolarization of V_{ap} in the presence of MIA, NEM and BAf.A_1, thus unmasking the presence of an apical H^+ ATPase. A remarkable effect of the addition of Ba^{2+} however was the hyperpolarization of both the apical and basal cell membrane potential differences. This hyperpolarization was strongly reduced after the addition of MIA, NEM or Baf.A_1.

At the same time, the existence of basal K^+ channels was suggested by the strong reduction of the fluid secretion rate, by the loss of the K^+ sensitivity of V_{bl}, and by the increase of the basal membrane resistance in the presence of Ba^{2+}.

Paper 3: Measurement of intracellullar and luminal K^+ concentration in a Malpighian tubule (*Formica*). Estimate of basal and luminal electrochemical K^+ gradients.

Using double-barrelled K^+-selective electrodes, intracellular and luminal K^+ concentrations were measured in different bath K^+ concentrations, in order to investigate the possibility of K^+ entry through K^+ channels and K^+ exit to the lumen via the combination of a proton pump and a K^+/H^+ antiporter , and to further characterize the intrinsic regulatory mechanism of transepithelial ion transport by the surrounding K^+.

The basal membrane potential was almost equal to the equilibrium potential for K^+ in all bath K^+ and Cl^- concentrations tested. The presence of a favourable electrochemical K^+ gradient was investigated. Two methods were used to estimate the K^+ gradient : (1) the gradient was calculated as the difference between V_{bl} and the equilibrium potential for K^+, (2) the gradient was recorded directly from the tracing of the ion-selective barrel on impaling a cell. Results obtained with both methods were critically evaluated. It was concluded that the first method gives the best estimate of the real basal electrochemical K^+ gradient. The calculated electrochemical gradient for K^+ across the basal membrane was found to be very small , i.e. inward, zero or even outward in low bath K^+. It tended to become more inward as the bath K^+ increased. On the other hand, following another approach to determine the direction of the basal K^+ gradient, the hyperpolarization of V_{bl} by Ba^{2+}, observed for each K_{bl} (see paper 2), seemed to favour the idea that passive K^+ movements are practically always in the inward direction.

As was expected by the transient changes in V_{bl} observed following a change in K_{bl}, it was found that K_c rose on increasing K_{bl}. Luminal K^+-selective measurements demonstrated that K_l varied as well, but to a smaller extent, on changing K_{bl}. A discrepancy was observed between the K_l values obtained with the latter method and the "total" luminal K^+ "concentrations" obtained from X-ray analysis of secreted droplets (Van Kerkhove *et al.*, 1989).

The inverse relationship between the measured apical electrochemical K^+ gradient and the fluid secretion rate was assessed. An increase in the bath K^+ concentration reduced both the electrical and concentration step to be overcome for K^+ secretion across the apical membrane, proving, as predicted (see paper 1) the double role for the bath K^+ concentration in the regulation of K^+ secretion.

The data for K_c and K_l combined with these obtained for the intracellular (H_c) and luminal (H_l) H^+ concentration provided evidence for the model of apical K^+ extrusion consisting of a H^+ pump and a K^+/H^+ antiporter in parallel. For an electroneutral exchanger, the concentration gradient for H^+ (H_l/H_c) driving K^+ extrusion should be larger than K_l/K_c. Therefore, the ratio of the H^+ (mean value, data taken from Zhang *et al.*, submitted), over the K^+ gradient (mean value) was calculated for each

K_{bl} in 57 mM Cl_{bl} (inset Fig. V). The ratio is favourable for K^+ extrusion via an electroneutral exchange system, except in 4 mM K_{bl}. But the variability in the results is large and the experiments have been done in a separate series of tubules. Therefore, for each K_{bl}, we compared the range of values found for K_l/K_c and for H_l/H_c (Fig. V). These values were obtained from

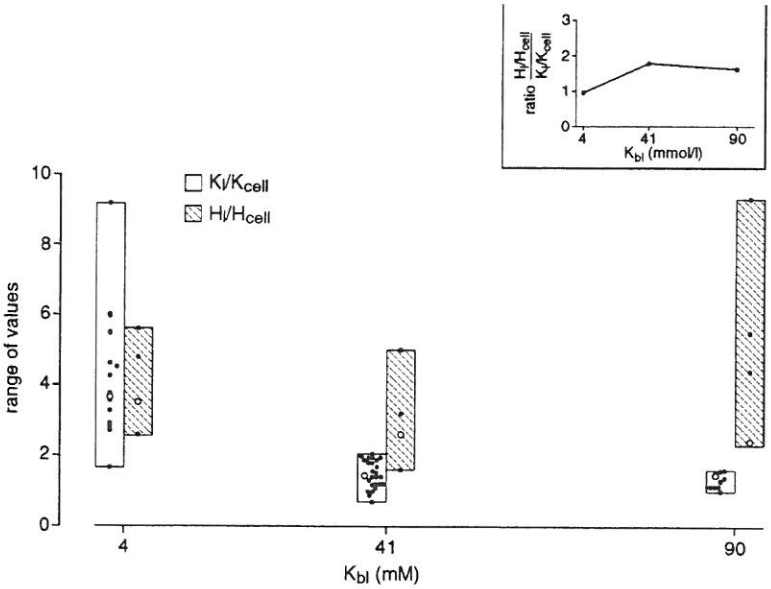

Fig V. Comparison of apical K^+ and H^+ concentration ratio's (H_l/H_c and K_l/K_c in different K_{bl} (in 57 mM Cl_{bl}). Inset: ratio H_l/H_c over K_l/K_c in different K_{bl}.
(o) ratio of mean values
(•) individual values of the ratio from "single tubule intracellular/luminal measurements".
Figure taken from Zhang *et al.* (Fig. 7, submitted).

either K^+- or H^+- (data taken from Zhang *et al.*, submitted) selective measurements where intracellular and luminal recordings were done consecutively on the same tubule. The ratios of the mean values for H_l/H_c and K_l/K_c are also given (i.e. the ratio of the mean luminal over the mean intracellular values from all experiments). All H_l/H_c values in 90 mM K_{bl} and most of them in 41 mM K_{bl} were larger than K_l/K_c. So the H^+ concentration gradient is large enough to drive K^+ extrusion. In 4 mM K_{bl}, the range of K_l/K_c and H_l/H_c overlap (except for one very high value of K_l/K_c), suggesting that in low K_{bl} the ratio H_l/H_c over K_l/K_c is near 1.0 and favouring the idea of an electroneutral K^+/H^+ exchange. The fact that H_l/H_c over K_l/K_c in 4 mM K_{bl} in the inset of Fig. V is 0.96 is due to one (out of 15) extremely high value found for K_l/K_c.

Also the inset in Fig. V shows that the ratio of the two concentration gradients increases in circumstances where K^+ secretion strongly accelerates, for instance when K_{bl} is raised from 5 to 51 mM. This was not the result of an increase in H_l/H_c as

would intuitively be expected. Zhang *et al.* (submitted) found that H_l/H_c has a tendency to rather decrease when secretion is stimulated by K_{bl}. At first sight this seemed to contradict the working hypothesis. The close dependence of K_c on K_{bl} however caused a reduction of K_l/K_c that was large enough to compensate for the decrease in H_l/H_c.

Paper 4: Both dinitrophenol and Ba^{2+} reduce KCl and fluid secretion in Malpighian tubules of *Formica*: The role of the apical H^+ and K^+ concentration gradient.

This paper provides more evidence for the hypothesis that the apical H^+ concentration gradient, built up by the apical H^+ pump is closely correlated with K^+ and fluid secretion in Malpighian tubules of *Formica*. Moreover further results support the idea that the K^+/H^+ antiporter can extrude K^+ to the lumen in exchange for H^+'s in an electroneutral way.

The effects of two agents, i. e. Ba^{2+} and 2,4-dinitrophenol (DNP), were studied on the apical H^+ and K^+ gradients in relation to their effect on fluid secretion.

The primary effect of Ba^{2+} is blockage of the K^+ channels in the basal membrane, slowing down net K^+ uptake by the cell. As a result the fluid secretion drops, and at the same time the apical electrical potential difference increases. This will hamper the electrogenic transfer of protons to the lumen.

DNP is well known as a protonophore that inhibits the ATP synthesis in the inner mitochondrial membrane; hence the apical H^+ pump will be depleted from its energy source. DNP may also exert its protonophore action at the cell membrane level where it may dissipate H^+ gradients. The addition of DNP resulted in a depolarization of V_{ap} (and V_{te}); and when applying DNP in the presence of Ba^{2+}, both V_{ap} and V_{bl} strongly depolarized. These effects suggested an inhibitory effect on an active hyperpolarizing component in the apical membrane. No changes in the passive electrical properties of both membranes were observed. At the given concentration (2.10^{-4} M), DNP did not change the electrical resistance of both membranes (see Dijkstra *et al.*, 1993), the sensitivity of V_{bl} and V_{ap} to a change in K_{bl} remained virtually unchanged, V_{bl} was still close to the equilibrium potential for K^+, and the transient changes of V_{bl} on varying K_{bl}, suggesting a change in K_c, were comparable wether DNP was present or absent.

It was demonstrated that both Ba^{2+} and DNP inhibited the realization of a cell-inward H^+ concentration gradient across the apical membrane.

Ba^{2+} increased the part of the electromotive force of the pump "lost" in building up an electrical potential difference across the apical membrane. DNP exerted its protonophore action at the basal cell membrane level. As result, the cell acidified. Due to the intracellular acidification, the gradient across the apical membrane against

which the pump is secreting H^+ ions would be diminushed and, as long as ATP is availabale, the pump would continue transferring H^+ ions at an increased rate, rapidly depleting the cell of ATP and eventually stopping the pump.
The apical K^+ concentration gradient did not change much on applying Ba^{2+} or DNP. Both conditions resulted in an unfavourable H_l/H_c over K_l/K_c ratio which was correlated with a reduced fluid secretion rate.
Only if electroneutrality of the K^+/H^+ antiporter was assumed, the calculated driving forces for the antiporter in the absence and presence of DNP were consistent with the expected values for secreting and non-secreting conditions.

Paper 5: Mechanisms of K^+ uptake across the basal membrane of Malpighian tubules of *Formica*: the effect of external ions and inhibitors.

In Malpighian tubules, basal K^+ transporting systems must exist to ensure a sufficiently large K^+ uptake to maintain fluid secretion and to explain the change in K_c when the K^+ concentration is varied in the surrounding medium. The possibility of K^+ uptake via K^+ channels down an electrochemical gradient has been clearly demonstrated (see paper 3). However the calculated electrochemical gradient for K^+ across the basal membrane was very small and less inward as bath K^+ decreased. Furthermore, in the presence of Ba^{2+}, when the basal electrical resistance was largely increased and the K^+ sensitivity of the basal membrane was lost, indicating a blocked state of the basal K^+ channels, the fluid secretion rate was reduced but not completely abolished. In this paper, we show that a rise of K_c on increasing K_{bl} still occurred in the presence of Ba^{2+}. Therefore, the existence of alternative pathways for K^+ transport was systematically investigated in different bath K^+ concentrations.
First of all, the existence and functioning of an active K^+ uptake system was investigated. To detect any Na^+/K^+ ATPase activity, Na^+ substitution experiments were performed and the effect of 10^{-3} M ouabain on fluid secretion rate and electrical potential differences was studied. Zhang *et al.* (submitted) suggested that the extrusion for protons at the basal side via a K^+/H^+ ATPase might be necessary in some conditions in Malpighian tubules of *Formica*. Therefore, the K^+/H^+ ATPase inhibitor Sch28080 (10^{-4} M) was tested on fluid secretion. Vanadate (10^{-4}, 10^{-3} M), a possible blocker of both active systems, was tested on fluid secretion, electrical potential differences and intracellular and luminal K^+ concentrations. However, we concluded that it tended to have an effect on the apical H^+ pump.
Summarizing the results, no conclusive evidence was found for the existence of active basal K^+ uptake systems in exchange for either Na^+ or H^+ ions. Apparently their role in maintaining an asymmetric ionic distribution across the basal membrane and a certain rate of fluid secretion is minimal.

Secondly, a reduction of the basal Cl^- concentration, replaced by $citrate^{3-}$ or SO_4^{2-}, had an inhibitory effect on the fluid secretion rate (Van Kerkhove *et al.*, 1989). Failing to observe a relative Cl^- conductance in the basal membrane, we have examined this epithelium for evidence of electroneutral coupled transport of K^+, Na^+, and Cl^-, i.e. via a K^+/Cl^- or $K^+/Na^+/2Cl^-$ cotransport system. In order to identify and distinguish between these hypothetical systems in different bath K^+ concentrations, different experimental approaches have been made: the sensitivity of the fluid secretion rate to bumetanide, its Na^+ dependence, and the potency of Br^- ions to inhibit or stimulate fluid secretion. The effects of bumetanide have been further investigated on electrical potential differences and on K_c.

Bumetanide, applied at a concentration of 10^{-4} M, did not influence fluid secretion in high K^+, Na^+-free conditions, but significantly diminished the fluid secretion rate in 51 mM K_{bl}. In a low K^+ high Na^+ medium (10 mM K_{bl}), a concentration of 10^{-5} M was already effective. Only in 10 mM K_{bl}, the fluid secretion rate was decreased when Na^+ was omitted from the bath solution. On replacing Cl^- by Br^-, the fluid secretion was stimulated in 51 mM K_{bl} and inhibited in 10 mM K_{bl}, suggesting the functioning of a K^+/Cl^- and $Na^+/K^+/2Cl^-$ cotransporter, respectively.

The effect of bumetanide (10^{-4} M) was electrically silent: no significant changes were observed on V_{te} and V_{bl}. The addition of bumetanide did not lower K_c. Moreover, the rise in K_c on increasing K_{bl} was not hampered on adding bumetanide to the bath solution.

Summarizing, in a high K^+, Na^+-free condition, a coupled entry mechanism of K^+ and Cl^- seems to be of little importance. In 51 mM K_{bl}, a substantial portion of K^+ entry can occur via a K^+/Cl^- cotransporter. In low K_{bl}, a $Na^+/K^+/2Cl^-$ cotransporter seems to take over.

It was concluded that different basal transport systems, i.e. K^+ channels, K^+/Cl^- and $K^+/Na^+/2Cl^-$ cotransporters, may function at different rates depending on the basal K^+ (and Na^+) concentration, and may determine thereby the amount of K^+ available to be transported across the apical membrane via the K^+/H^+ antiporter. The rise of K_c in response to an increased basal K^+ concentration can occur both via K^+ channels or via a K^+/Cl^- cotransport system.

GENERAL DISCUSSION AND CONCLUSIONS

The aim of this work was to clarify the transcellular route for K^+ transport in Malpighian tubules of the omnivorous species *Formica polyctena* . The properties of the basal and apical cell membranes have been investigated, and the regulatory mechanism by which the surrounding K^+ concentration can modulate the fluid secretion rate has been analysed.

When data were available, our findings were compared with those reported for Malpighian tubules of other insects species in similar (i.e. unstimulated, except when explicitely stated differently) conditions. These data came almost exclusively from three herbivorous insects, i.e. the locust *Locusta migratoria* L., the beetle *Onymacris plana* and the larva of the fly *Drosophila hydei*, and from three bloodsucking insects, i.e. the bug *Rhodnius prolixus* Stål., the mosquito *Aedes aegypti* and the tsetse fly *Glossina morsitans*. In herbivorous insects K^+ is the major transported cation. Bloodsucking insects can carry out rapid transport of fluid rich in Na^+ (for reviews see Phillips, 1981; Nicolson, 1993). However when not stimulated *Rhodnius* Malpighian tubules secrete a K^+-rich fluid containing only very low levels of Na^+ ions (Maddrell, 1980).

The high K^+ permeability of the basal membrane.

In the basal cell membrane of *Formica* Malpighian tubules, an appreciable K^+ permeability was present. The basal membrane potential difference (V_{bl}) was sensitive to the bath K^+ concentration (K_{bl}) but very little to the bath Na^+ and Cl^- concentration. These properties were comparable to those for Malpighian tubules of *Locusta*, *Onymacris* (with the exception of a moderate Cl^- permeability) and *Rhodnius* but distinctly different from those of *Aedes* and *Glossina* (see Table I). In *Formica*, the existence of basal K^+ channels was also suggested by the effects of Ba^{2+}: a strong reduction of the fluid secretion rate, a loss of the K^+ sensitivity of the basal membrane, and an increase of the basal membrane resistance. Furthermore, V_{bl} was almost equal to the equilibrium potential for K^+ (E_K^{bl}) in all bath K^+ and Cl^- concentrations tested. In tubules of *Onymacris*, the existence of basal K^+ channels was confirmed, indirectly by the effects of Ba^{2+}, and directly by a patch clamp study (Nicolson and Isaacson, 1987; Nicolson and Isaacson, 1990).

The intracellular K^+ concentration varies on changing the bath K^+ concentration.

The intracellular (K_c) and luminal (K_l) K^+ concentrations in Malpighian tubules of *Formica* fell within the range of cellular and luminal concentrations measured with K^+-selective electrodes found for Malpighian tubules of *Locusta* (K_c= 95 mM, K_l= 148 mM in 20 mM K_{bl}; Morgan and Mordue, 1983). In *Drosophila* Malpighian

Table I. Basal relative permeability to ions in Malpighian tubules of different insect species.

species	references	relative basal permeability to		
		K^+	Na^+	Cl^-
Formica polyctena	this study	high	low	low
Locusta migratoria L.	Baldrick *et al.* (1988) Fogg *et al.* (1989)	high	low	low
Onymacris plana	Nicolson and Isaacson (1987)	high	low	moderate
Rhodnius prolixus Stål	O'Donnell and Maddrell (1984)	high	low	low
Aedes aegypti	Sawyer and Beyenbach (1985)	moderate	moderate	low
Glossina morsitans	Isaacson and Nicolson (unpublished) see Nicolson (1993)	low	moderate	moderate

tubules, in 23 mM K_{bl}, K_c ranged from 57 mM in the proximal to 192 mM in the distal segment (Wessing *et al.*, 1987). In the rectal tubule complex of *Tenebrio molitor*, the high value for K_c (143 mM in 55 mM K_{bl}) was probably correlated with the very specialized structure of the tubule rectum complex where KCl is highly concentrated in the tubular lumen (up to 3000 mM) allowing a maximal amount of water to be reabsorbed from the rectal lumen (O'Donnell and Machin, 1991).

A remarkable feature of K_c in the Malpighian tubule cell of *Formica* was its fast and reversible response to changes in the bath K^+ concentration. The evidence that intracellular K^+ can vary between 29 and 123 mM in Malpighian tubules of *Formica* is in contrast with the general finding that in eukaryotic cells intracellular K^+ levels are relatively invariant and in the range of 120-140 mM (Yancey *et al.*, 1982).

A rise in K_c was also observed in Malpighian tubules of *Drosophila* on hormonal stimulation with 5-hydroxytryptamine (Wessing *et al.*, 1987). In contrast , in Malpighian tubules of *Tenebrio* (O'Donnell and Machin, 1991), and of *Locusta* (Baldrick *et al.*, 1988) and *Onymacris* (Nicolson and Isaacson, 1987) it was observed

(or inferred from V_{bl}) that K_c was not very sensitive to a change in K_{bl}. Luminal K^+-selective measurements in Malpighian tubules of *Formica* demonstrated that K_l varied as well, but to a smaller extent, on changing K_{bl}. Flame photometry of secreted droplets did not reveal a change in the luminal K^+ concentration of Malpighian tubules of *Rhodnius* (Maddrell, 1969) or *Glossina* (Gee, 1976) in a K_{bl} range from 0 to 150 mM; but in Malpighian tubules of *Musca* K_l increased on rising K_{bl} from 0 to 50 mM (Dalton and Windmill, 1980).

Intrinsic regulation of the K^+secretion by the surrounding K^+ concentration.

In Malpighian tubules of *Formica*, both the high K^+ conductance of the basal membrane, and the response of K_c to a change in the bath K^+ concentration played a significant role in the intrinsic regulation of K^+ secretion by the surrounding K^+ concentration: the higher the K^+ concentration in the medium, the higher the rate of transport, resulting in turn in a lowering of the surrounding K^+.

As a first regulatory mechanism, an increase in the bath K^+ concentration could stimulate fluid secretion by decreasing the height of the electrical voltage to be overcome by K^+ translocation across the apical membrane. This was possible because the resistance ratio of the apical over the basal membrane was high (around 40) and the basal membrane was dominated by a high K^+ conductance so that V_{bl} approached the E_K^{bl} on the one hand, and could impose its value on the apical membrane on the other. In Malpighian tubules of *Locusta*, a resistance ratio of the same order was expected to be present: both V_{bl} and V_{ap} responded to a change in bath K^+ concentration (Fogg *et al.*, 1989). In contrast, Malpighian tubules of *Aedes* had a ratio of 0.6 (Aneshansley *et al.*, 1988); the existence of a similar regulatory mechanism in this epithelium is therefore unlikely.

As a second regulatory mechanism, the bath K^+ concentration also determined the amount of cell K^+ available for extrusion to the lumen and hereby diminished the concentration step to be overcome for K^+.

The conclusions drawn above were consistent with the finding of a highly significant inverse relationship between the calculated apical electrochemical gradient for K^+ and the fluid secretion rate. The gradient itself was very closely correlated with K_{bl}.

The apical V-type H^+ ATPase.

In caterpillar (*Manduca sexta*) midgut, the so-called K^+-pump located in the apical membrane of insect transporting epithelia was shown to be a proton pump, belonging to the class of vacuolar type (V-type) ATPases, in parallel with a K^+/nH^+ antiporter (Wieczorek *et al.*, 1991). In the present study, evidence was presented that an electrogenic V-type H^+ ATPase was also involved in the active transport step at the apical membrane of Malpighian tubules of *Formica*.

The metabolic inhibitor monoiodo-acetic acid (MIA; 2.10^{-4} M) and two inhibitors of V-type proton ATPases, i.e. bafilomycin A_1 (Baf.A_1; 5.10^{-6} M) and N-ethyl

maleimide (NEM; 5.10^{-4} M) all inhibited fluid secretion rate significantly. Indirect evidence for the presence of a V-type H^+ ATPase came also from the inhibitory effect of Baf.A_1 on
fluid secretion rate in Malpighian tubules of *Drosophila*, *Onymacris* and *Glossina* , and *Aedes* (see Table II). A comparable effect on fluid secretion was reported for NEM on *Drosophila* Malpighian tubules (see Table II). In *Formica* tubules, in contrast to the pronounced effect on fluid secretion rate, MIA, NEM and Baf.A_1 had only mild effects on electrical potential differences and solely at the apical side. The effects of pump inhibition could be made more visible by blocking the K^+ conductance of the basal membrane and lowering the high resistance ratio of the apical over basal membrane by the application of Ba^{2+}. When acidifying the lumen and applying NEM and Baf.A1 in the lumen, evidence for an apical V-type H^+ pump was also provided in perfused *Formica* tubules (Dijkstra *et al.*, in preparation). As in *Formica* Malpighian tubules (in the absence of Ba^{2+}) Baf.A_1 depolarized the apical membrane potential difference (V_{ap}) and left V_{bl} unchanged in Malpighian tubules of *Onymacris* and *Glossina*. In contrast, both V_{ap} and V_{bl} depolarized on adding Baf.A_1 in *Aedes* Malpighian tubules (see Table II). Further evidence was provided by the immunocytochemical localization of a V-type H^+ ATPase in the apical microvilli of *Manduca* Malpighian tubules (Klein *et al.*, 1991; Russell *et al.*, 1992).

K^+ extrusion to the lumen is realized via the combination of an electrogenic H^+ pump and an electroneutral K^+/H^+ antiporter.

In Malpighian tubules of *Formica,* the apical H^+ pump has been demonstrated to be the prime mover in the active K^+ transfer to the lumen via an electroneutral K^+/H^+ antiporter.

Comparing the range of values found for K_l/K_c with the range of values found for the luminal (H_l) over intracellular (H_c) H^+ concentration, it was found that in each bath K^+ concentration the established cell-inward H^+ concentration gradient was large enough to drive apical K^+ extrusion to the lumen via an electroneutral exchange system.

By using Ba^{2+} and the protonophore 2, 4-dinitrophenol (DNP), evidence was presented that the apical proton concentration gradient, built up by the H^+ pump, was closely correlated with K^+ and fluid secretion. Both compounds inhibited the realization of the apical cell-inward H^+ concentration gradient: Ba^{2+} through increasing the part of the electromotive force of the pump "lost" in building up a large electrical potential difference across the apical membrane, and DNP by blocking and shunting the apical H^+ pump activity. As in *Formica* tubules, a depolarizing effect of DNP on the transepithelial potential difference (V_{te}) was observed in non-perfused and perfused Malpighian tubules of *Onymacris* and in perfused tubules of *Aedes* (see Table II). When injected luminally, similar effects of DNP on V_{te} were observed for the rectal tubule complex of *Tenebrio* (O'Donnell and Machin, 1991).

Table II. Effect of various drugs on fluid secretion rate (FS) and electrical potential differences (V_{bl}, V_{te}, V_{ap}) in Malpighian tubules of different insect species.

	Formica * *polyctena*	*Locusta migratoria* L.	*Onymacris plana*	*Drosophila hydei*	*Rhodnius prolixus* Stål	*Aedes aegypti*	*Glossina morsitans*
monoiodo-acetic acid							
FS	inhibition	-	-	-	-	-	-
V_{bl}	unchanged	-	-	-	-	-	-
V_{ap}	depolarization	-	-	-	-	-	-
N - ethyl maleimide							
FS	inhibition	-	-	inhibition (a)	-	-	-
V_{bl}	unchanged	-	-	-	-	-	-
V_{ap}	depolarization	-	-	-	-	-	-
bafilomycin A_1							
FS	inhibition	-	inhibition (b)	inhibition (a)	-	inhibition (c)	inhibition (b)
V_{bl}	unchanged	-	unchanged (b)	-	-	depolarization (c)	unchanged (b)
V_{ap}	depolarization	-	depolarization (b)	-	-	depolarization (c)	depolarization (b)
2,4 - dinitrophenol							
FS	inhibition	-	-	-	inhibition (f)	-	-
V_{bl}	small depolarization	-	V_{te} depolarization (d + e)	-	-	V_{te} depolarization (g + h)	-
V_{ap}	depolarization	-		-	-		-
ouabain							
FS	unchanged	strong inhibition (i)	-	weak inhibition (j)	stimulation (k)	inhibition (l)	unchanged (m)
V_{bl}	unchanged	slow depolarization (n)		depolarization (j)			unchanged (b)
V_{ap}	unchanged	slow depolarization (n)	V_{te} unchanged (d)	depolarization (j)	V_{te} increase (k)	V_{te} unchanged (l)	-
Sch 28080							
FS	unchanged	-	-	-	-	-	-
V_{bl}	-	-	-	-	-	-	-
V_{ap}	-	-	-	-	-	-	-
vanadate							
FS	inhibition	-	-	inhibition (a)	-	-	-
V_{bl}	unchanged	slow depolarization (n)	-	-	-	-	-
V_{ap}	depolarization	slow depolarization (n)	-	-	-	-	-
bumetanide/furosemide							
FS	inhibition	-	-	inhibition (j)	inhibition (o)	unchanged (inhibition + cAMP) (l)	-
V_{bl}	unchanged	unchanged (n)	V_{te} unchanged (d)	-	small depolarization (o)	slow depolarization (l)	small hyperpolarization (b)
V_{ap}	unchanged	slow increase (n)		-	transient hyperpolarization (o)	unchanged (l)	-

(* effects in the absence of Ba^{2+}, this study).

references : : (a) : Bertram *et al.*, 1991; (b) : see Nicolson, 1993; (c) : Pannabecker and Beyenbach, 1993; (d) : Isaacson *et al.*, 1989; (e) : Nicolson and Isaacson, 1987; (f) : Maddrell, 1969 (g) : Williams and Beyenbach, 1984; (h) : Pannabecker *et al.*, 1992.; (i) : Anstee *et al.*, 1979; (j) : Wessing *et al.*, 1987; (k) : Maddrell and Overton, 1988; (l) : Hegarty *et al.*, 1991 (m) : Gee, 1976; (n) : Baldrick *et al.*, 1988; (o) : O' Donnell and Maddrell, 1984

The decrease of the apical H^+ gradient by the action of Ba^{2+} and DNP resulted in an unfavourable H_l/H_c over K_l/K_c ratio wich was correlated with a reduced fluid secretion rate. Only if electroneutrality of the K^+/H^+ antiporter was assumed, the calculated driving forces for the antiporter in the absence and presence of DNP, were consistent with the expected values for secreting and non-secreting conditions, respectively. An inhibition of the fluid secretion upon administration of DNP was also observed in Malpighian tubules of *Calliphora* (Berridge, 1966), *Rhodnius* (see Table II) and *Musca* (Dalton and Windmill, 1980).

In Malpighian tubules of *Formica*, the intrinsic regulatory mechanism of transepithelial K^+ transport by the surrounding K^+ concentration consists of two effects on the apical membrane. (1) On increasing K_{bl}, V_{ap} decreased and the H^+ concentration gradient across the apical membrane decreased or remained unchanged (Zhang *et al.*, submitted). Both effects are expected to facilitate the turn-over rate of an electrogenic active H^+ pump. (2) On the other hand, the decrement in the apical H^+ concentration gradient was met by a more pronounced decrease in the apical K^+ concentration gradient. This resulted in a more favourable H_l/H_c over K_l/K_c ratio and hence in a facilitation of K^+ secretion via the K^+/H^+ antiporter.

Mechanisms for K^+ uptake across the basal membrane.

Basal K^+ transport systems had to exist to ensure a sufficiently large K^+ uptake to maintain fluid secretion, and to explain the adaptation of the intracellular K^+ concentration to the surrounding bath K^+ concentration in Malpighian tubules of *Formica*.

K^+ entry occurs passively through K^+ channels. The hyperpolarization of V_{bl} by Ba^{2+}, observed for each K_{bl}, seemed to favour the idea that passive K^+ movements were practically always in the inward direction. However, the calculated electrochemical gradient for K^+ across the basal membrane was very small and became less inward as the bath K^+ concentration decreased. A similar behaviour of the basal electrochemical K^+ gradient was found in *Tenebrio*: the calculated gradient was 3 mV inward in 120 mM K_{bl} and 3 mV outward in 55 mM K_{bl} (O'Donnell and Machin, 1991). Small outward K^+ gradients were found in Malpighian tubules of *Locusta* (in 20 mM K_{bl}, Morgan and Mordue, 1983) and *Drosophila* (in 23 mM K_{bl}, Wessing *et al.*, 1987).

In Malpighian tubules of *Formica*, no conclusive evidence was found for the presence of active K^+ uptake via a Na^+/K^+ ATPase or a K^+/H^+ ATPase. Only in low K_{bl}, the fluid secretion rate was decreased on omitting Na^+ from the bath solution. No ouabain effects were observed however in this K_{bl}. A comparison has been made with other studies (see Table II). Ouabain effects were reported for Malpighian tubules of *Locusta*, *Drosophila*, *Rhodnius* and *Aedes* . As in Malpighian tubules of *Formica*, no effect of ouabain could be demonstrated in Malpighian tubules of *Onymacris* and *Glossina*. Zhang *et al.* (submitted) suggested that the extrusion of protons at the basal side via a

K^+/H^+ ATPase may be needed to explain the lowering of the proton concentration level in both the lumen and the cell when increasing the bath K^+ concentration in Malpighian tubules of *Formica*. The fluid secretion rate was not affected by the K^+/H^+ ATPase inhibitor Sch28080 however. Unlike in Malpighian tubules of *Locusta* (see Table II), in Malpighian tubules of *Formica* there was a discrepancy between the significant effects of vanadate on fluid secretion and electrical parameters, and the lack of effect of ouabain. In Malpighian tubules of *Drosophila*, the much stronger effect of vanadate, compared to that of ouabain, was attributed to a broader action range of the compound. In our hands, the effects of vanadate on fluid secretion rate and electrophysiological parameters were comparable to, and followed a similar time course as the effects of the V-type ATPase inhibitors bafilomycin A_1 and NEM reported by Weltens *et al.* (1992) for the same preparation. We proposed an effect of vanadate on the apical H^+ pump in Malpighian tubules of *Formica*.

Secondary active K^+ uptake via a coupled entry of K^+ (,Na^+) and Cl^- seemed of little importance. In physiological conditions, i.e. 51 mM K_{bl} or lower, K^+ uptake could be realized via a K^+/Cl^- or $K^+/Na^+/2Cl^-$ cotransport system. In 51 mM K_{bl}, the functioning of a K^+/Cl^- cotransporter was suggested by the, rather low, sensitivity of the fluid secretion rate to bumetanide, and by the potency of Br^- to stimulate fluid secretion. In 5 and 10 mM K_{bl}, the higher sensitivity of the fluid secretion rate to bumetanide and the inhibitory effect of Cl^- substitution by Br^- on fluid secretion pointed to the functioning of a $Na^+/K^+/2Cl^-$ cotransporter. In the literature, inhibitory effects of bumetanide (or of another loop diuretic furosemide) on the fluid secretion rate have also been reported for Malpighian tubules of *Drosophila*, *Rhodnius* and *Aedes* (see Table II). Unlike in Malpighian tubules of *Formica* and *Onymacris*, significant electrical effects were reported for Malpighian tubules of *Locusta*, *Rhodnius*, *Aedes* and *Glossina*. In Malpighian tubules of *Formica*, the (small) Na^+ dependence of the fluid secretion rate observed in low bath K^+ concentration was attributed to an inhibitory effect on a $Na^+/K^+/2Cl^-$ cotransporter. In contrast, in Malpighian tubules of *Locusta*, effects of Na^+ omission, i.e. a depolarizing effect on V_{bl} and V_{ap}, were more in accordance with an inhibitory effect on a Na^+/K^+ ATPase (Baldrick *et al.*, 1988).

Transport model for transcellular K^+ transport.

The present work allowed to propose a model for transcellular K^+ transport in different bath K^+ concentrations in Malpighian tubules of *Formica*.

(see Fig. VI). At the apical side, K^+ extrusion into the lumen is realized via the combination of an electrogenic H^+ pump and an electroneutral K^+/H^+ antiporter.

The pathways for basal K^+ entry seem to be conductive and secundary active, and their relative importance varies depending on the bath K^+ (and Na^+) concentration. Active K^+ uptake systems, i.e. a Na^+/K^+ or K^+/H^+ ATPase, if present, do not seem to contribute significantly to transepithelial transport or to the maintenance of intracellular K^+ (and Na^+) levels. In high K^+, Na^+-free conditions, a coupled entry of K^+ and Cl^- seems to be of little importance. K^+ uptake probably occurs via basal K^+ channels, driven by a favourable electrochemical gradient. In more physiological conditions, i.e. 51 mM K_{bl}, when the basal electrochemical K^+ gradient seems to be less inward, K^+ entry via channels may become less important and a K^+/Cl^- cotransport system probably accounts for a large portion of K^+ uptake. In 5 or 10 mM K_{bl} (and high Na_{bl}) little K^+ is available for transepithelial transport; the basal electrochemical K^+ gradient is even smaller compared to 51 mM K_{bl} and possibly outward. The fluid secretion rate is low but still present. In this condition, a $Na^+/K^+/2Cl^-$ cotransporter may take over part of the basal K^+ uptake.

The different basal transport systems for K^+ entry may function at different rates depending on the basal K^+ (and Na^+) concentration and may thereby determine the amount of K^+ available to transport across the apical membrane via the K^+/H^+ antiporter. The rise of the cellular K^+ in response to an increased basal K^+ concentration can occur both via K^+ channels or via a coupled K^+/Cl^- entry mechanism.

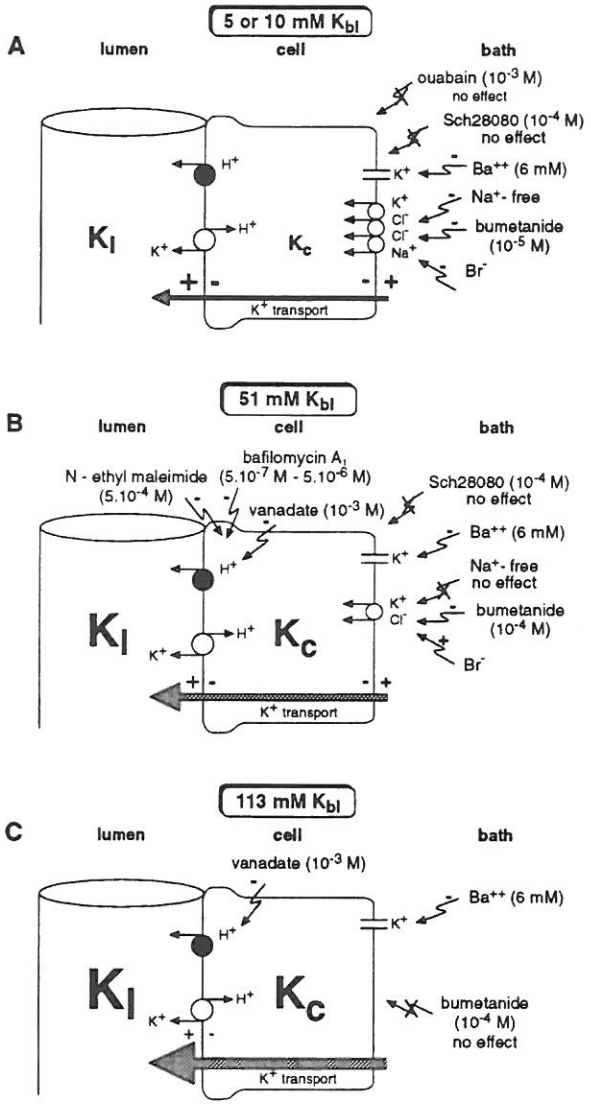

Fig. VI. Model for transcellular K^+ transport in Malpighian tubules of *Formica* in 5 or 10 (A), 51 (B) and 113 (C) mM K_{bl} (143 mM Cl_{bl}). It is shown that:

1) K^+ transport increases (larger arrows) in a higher K_{bl}
2) the apical and basal membrane potential differences decrease (smaller + and - signs in the figure) when K_{bl} increases
3) K_l and K_c increase (indicated by larger symbols in the figure) when K_{bl} increases
4) a vacuolar type H^+ pump is present in the apical membrane in parallel with a K^+/H^+ antiporter
5) depending on K_{bl}, K^+ channels, a K^+/Cl^- and/or a $Na^+/K^+/2Cl^-$ cotransporter are functioning at the basal side.

The effect of various inhibitors applied from the bath side on the different transport systems is shown.

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APPENDIX

PAPER 1

INTRINSIC REGULATION OF K^+ TRANSPORT IN MALPIGHIAN TUBULES OF (*FORMICA*) : ELECTROPHYSIOLOGICAL EVIDENCE.

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INTRINSIC REGULATION OF K^+ TRANSPORT IN MALPIGHIAN TUBULES (*FORMICA*): ELECTROPHYSIOLOGICAL EVIDENCE

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Abstract—Intracellular basolateral (V_{bl}) and, indirectly, apical membrane potentials (V_{ap}) have been measured in spontaneously secreting isolated Malpighian tubules of *Formica*. V_{bl} was sensitive to the bath K^+ concentration, $[K^+]_{bl}$, (42 mV/decade) and very little to the Na^+ and Cl^- concentration. V_{ap} was also sensitive to $[K^+]_{bl}$. The voltage changes in V_{ap} were 60–70% of those in V_{bl}, so the transepithelial potential (V_{te}) also changed: it increased with $[K^+]_{bl}$ (lumen became more positive). The overall result of an increase in bath K^+ concentration was a facilitation of KCl secretion: the apical electrical gradient to be overcome for K^+ extrusion was lowered and the transepithelial electrical gradient favouring Cl^- movement to the lumen was increased. Transient changes in V_{bl} on varying $[K^+]_{bl}$ also suggested dependence of the cellular K^+ concentration on $[K^+]_{bl}$. When plotting fluid secretion rate, i.e. K^+ transport, as a function of the calculated apical electrochemical gradient for K^+, a highly significant inverse relationship was found. The gradient itself was very closely correlated with $[K^+]_{bl}$. In another series of experiments tubules were luminally perfused with symmetrical solutions (51 mM K^+ 143 mM Cl^-) and resistances were measured after current injection. We report a transepithelial length-specific resistance of 23 ± 3 kΩcm, a tissue specific resistance of $182 \pm$ Ωcm^2 and a length constant of 402 ± 29 μm ($n = 38$). From intracellular measurements and luminal injection of current the voltage divider ratio could be obtained. This gave an estimate of the ratio of apical over basolateral resistance of 44 ± 8 ($n = 6$). Analysis of the electrical equivalent model, taking into account the high apical over basolateral resistance, explained why changes in the basolateral electromotive forces were reflected on the apical membrane. Taken together the results of this study were consistent with a role for the high basolateral conductance (relative to the apical conductance) in the intrinsic regulation of K^+ transport by bath $[K^+]$. A second mechanism of regulation could be the fact that $[K^+]_{bl}$ determines the amount of cellular K^+ available for transport.

Key Word Index: Malpighian tubules; K^+ transport; microelectrode study; luminal perfusion; resistance measurements

INTRODUCTION

Malpighian tubules of *Formica* actively secrete K^+ (Van Kerkhove *et al.*, 1989). This is in agreement with findings for tubules of many species (Phillips, 1981). The rate of K^+ transport was sensitive to the bath K^+ and Cl^- concentration. A 10-fold increase in the bath K^+ concentration for instance caused up to a five-fold increase in the rate of transport (Van Kerkhove *et al.*, 1989). Intrinsic regulation of the K^+ transport must be present: the higher the K^+ concentration in the medium, the higher the rate of transport, resulting in turn in a lowering of the surrounding K^+. In view of the K^+ concentrations found in the haemolymph of the adult stage of *Formica* species [< 5–70 mM (see Van Kerkhove *et al.*, 1989)] it seemed interesting to further investigate the effects of an increase of the surrounding K^+ concentration.

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As K^+ transport from the cell interior across the apical membrane was active we expected that an increase in the pumping rate would be facilitated by a decrease in the electrochemical gradient against which K^+ had to be moved across this barrier. The apical side might therefore be the site of action of the intrinsic regulation. In the present study basolateral (V_{bl}) and (indirectly) apical (V_{ap}) membrane potential differences were measured. It was found that an increase in bath K^+ concentration did have an effect on both V_{bl} and V_{ap}. V_{bl} depolarized and this was accompanied by a decrease in V_{ap}. So bath K^+ influenced the apical barrier and when plotting fluid secretion as a function of V_{ap} in the different K^+ concentrations an inverse relationship was found.

To date, analysis of the interaction between the basolateral and the apical membrane in a Malpighian tubule epithelium is rare and information about relative and/or absolute electrical resistances of the different barriers is extremely limited. Only for *Onymacris* (Isaacson *et al.*, 1989) and *Aedes aegypti* data are available (Williams and Beyenbach, 1984; Aneshansley *et al.*, 1988). The present paper reports data of electrical parameters in *Formica* tubule epithelium. The ratio of apical over basolateral membrane resistance was estimated in luminally perfused tubules and found to be high. Using this finding in the analysis of the electrical equivalent circuit of the Malpighian tubule epithelium, helped to explain the impact of bath $[K^+]$ on the apical membrane potential.

Furthermore an estimate could be made of the cellular K^+ available for secretion in different K^+ concentrations. The relation of this parameter with the rate of K^+ secretion was examined and discussed.

MATERIALS AND METHODS

Animals

The species studied was *Formica polyctena* (*Hymenoptera, Insecta*). Worker ants were collected from natural nests located at the periphery of woods and kept in artificial nests at a temperature of 20°C and a relative humidity of 30–40%. The animals were fed with sugar and water, and occasionally with insects.

Artificial salines

The composition of the bathing solutions is summarized in Table 1. The low Cl^-, Hepes buffered Ringers (57 mM, the anion gap filled with organic acid) mimick the low Cl^- concentration in the haemolymph *in vivo* (Van Kerkhove *et al.*, 1989). The effect of a high Cl^- concentration was studied in Ringers where Cl^- was the only anion present (143 mM). Solutions were freshly prepared each week, filtered through 0.22 μm Millipore filters and kept at 2°C until use. pH (7.2) remained stable for at least 1 week under these conditions.

Set up for the measurement of basolateral (V_{bl}) and transepithelial (V_{te}) potential difference

A single Malpighian tubule (2–3 mm) was dissected out and set up as described earlier (Van Kerkove *et al.*, 1989). Briefly, a small bathing droplet (50 μl) was covered with paraffin oil and continuously perfused (150 μl/min). Each end of the tubule was fixed in a holding pipette. A microelectrode was then advanced with a hydraulic micromanipulator (Narishige, Japan) and a cell impaled from the basolateral side. A sudden negative voltage deflection (V_{bl}) was seen. When advancing the electrode further, the tip reached the lumen and the transepithelial (V_{te}) was measured.

Table 1. Composition of experimental solutions

		57 mM Cl^-		143 mM Cl^-	
		A (K^+ free)	B (Na^+ free)	C (K^+ free)	D (Na^+ free)
NaCl	mM	27	/	113	/
KCl	mM	—	27	/	113
$CaCl_2$	mM	2	2	2	2
$Mg\ Cl_2$	mM	13	13	13	13
Na_3 citrate	mM	28.7	/	/	/
K_3 citrate	mM	—	28.7	/	/
Alanine	mM	2.8	2.8	2.8	2.8
Trehalose	g/l	4	4	4	4
Maltose	g/l	4	4	4	4
Glucose	g/l	27.7	27.7	15	15
Hepes	mM	12.1	12.1	12.1	12.1

pH was adjusted to 7.20 by adding ~3.5 mmol/l NaOH (solutions A and C) or KOH (solutions B and D). Osmolality of all solutions was kept at 375 mOsm by adjusting the glucose concentration.
Different K^+ concentrations were obtained by mixing solutions A and B or C and D.

The tubules were either totally immersed in the bathing solution, or part of the tubule was pulled out of the bath into the oil and nicked, so that secretory fluid could leave the tubule. This prevented hydrostatic pressure to be built up. In a series of control experiments no statistically significant difference was found in either V_{bl} ($P > 0.1$, $n = 6$ and 4) or V_{te} ($P > 0.1$, $n = 9$ and 7) using either method, in a high K (113 mM) and Cl (143 mM) solution, where fluid secretion was fast. Results obtained with both methods were therefore pooled.

Microelectrode characteristics, cell impalements and effect of microelectrode fillings on intracellular (V_{bl}) and transepithelial (V_{te}) potential measurements

Microelectrodes were pulled from borosilicate filament glass (o.d. 1.3 mm, i.d. 0.7 mm) to a tip diameter smaller than 0.5 μm. Microelectrodes were filled with either KCl (0.1, 1 or 3 M) or Na acetate (1 or 3 M with 10 mM KCl) and had resistances ranging from about 20 to 300 MΩ depending on the filling solutions. The microelectrode was connected to the B channel of a WPI Duo 773 electrometer via a Ag/AgCl wire. A coarse, low resistance (1 MΩ) 3 M KCl filled electrode was placed in the bath solution and connected to earth.

Figure 4(A) shows a typical experiment: on impaling a cell a fast negative deflection was seen and no further gradual increase in the measured potential was observed, so the basolateral membrane seemed to seal around the electrode immediately. Also, the potential deflection remained stable for several minutes. When examining the frequency distribution of a large number of impalements (Fig. 1) the histogram had a near symmetrical shape. After withdrawing the electrode the potential reading should return to zero within 2–3 mV.

In a preliminary series of experiments the effect of different microelectrode fillings on potential measurements was investigated: it was shown by Isenberg (1979) that KCl tended to leak out of 3 M KCl filled microelectrodes. This would change the cell KCl content (in our case also the luminal K and Cl concentrations, when measuring V_{te}) and consequently influence the cell's membrane potential (V_M) and/or V_{te}. When comparing V_{bl} and V_{te} measured in 51 mM K^+, 143 mM Cl^- Ringer solution with electrodes filled with either a high (3 M) or low (0.1 M) KCl concentration no statistically significant difference was seen: V_{bl} was $-19\,mV \pm 3$ (5) and $-18\,mV \pm 1$ (5), V_{te} was $+33\,mV \pm 7$ (6) and $+32\,mV \pm 7$ (10), respectively. So the tips of the electrodes seemed to be sufficiently small not to induce major KCl leaks. Microelectrodes filled with 3 M Na acetate (and 10 mM KCl), gave similar results: $-19\,mV \pm 1$ (4) for V_{bl} and $+40\,mV \pm 7$ (11) for V_{te}. Therefore all results were pooled.

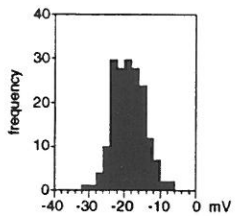

Fig. 1. Frequency distribution of V_{bl}, measured in 51 mM K^+ 57 mM Cl^- in spontaneously secreting tubules. Frequency was given for 2 mV intervals. Mean value was -19 ± 1 mV ($n = 179$).

Resistance measurements

Set-up. A Malpighian tubule was dissected as described above in Ringer containing 5 mM K^+ and the middle segment of the tubule was cut out and transferred to the chamber (total volume of 2.5 ml) of the perfusion system with a Pasteur pipette. The average length of the perfused tubules was 637 ± 60 μm ($n = 38$). The intraluminal perfusion technique used (see Fig. 2) has been extensively described by Greger and co-workers (Greger, 1981; Greger and Hampel, 1981; Greger and Schlatter, 1983). The lumen perfusion rate was high (around 20 nl/min), so that the lumen was well dilated. The perfusion rate was far above the secretion rate. Therefore the composition of the luminal solution was not influenced by secretion. The bath perfusion rate could be varied by gravity between 10 and 60 ml per min. This flow rate ensured effective changes in bath composition within 10 s.

Transepithelial potential difference (V_{te}) was measured at both ends of the perfused segment using a symmetrical arrangement of Ag/AgCl half cells. The half cells connected the perfusion and collection solutions on the one hand and the bath solution on the other to a high impedance electrometer (Keithley, model 602) via an Agar bridge.

Measurement of the transepithelial resistance (R_{te}). R_{te} was determined by constant current pulses (amplitude: 30–90 nA, duration: 1 s). The pulses were injected via a Ag/AgCl wire in one of the channels of the perfusion pipette at a frequency of 6/min. The current loop was closed by a separate Ringer–Agar bridge, Ag/AgCl half cell in the bath and by a stimulation isolation unit, so that the current was off ground. This arrangement prevented electrical coupling artifacts between the current injection and the voltage recording channel.

The parameters that were measured were: (1) the volume resistivity ρ of the luminal perfusion solution

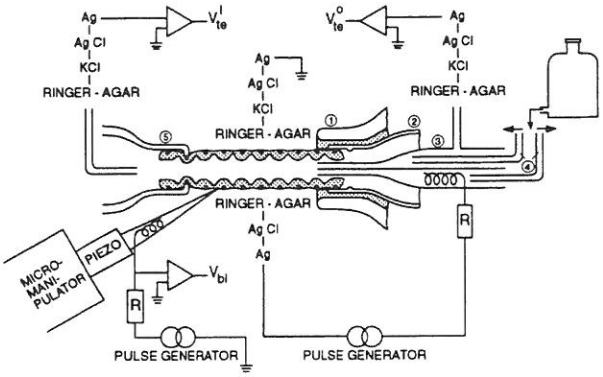

Fig. 2. Diagram of experimental set-up of a luminally perfused tubule [adapted from Greger and Schlatter (1983)]. The perfusion side includes four pipettes: (1) an outer Sylgard pipette with a tip diameter of 0.5 mm; (2) a perfusion holding pipette with a constriction of a diameter ranging from 23 to 32 μm; (3) a dual channel perfusion pipette, manufactured from theta-shaped borosilicate glass with a tip diameter of about 8 μm; (4) fluid exchange pipette inserted in each of the two channels. This arrangement allows the luminal solution to be exchanged very promptly, if needed. The collection side consists of a tubule holding pipette (5) with the tip shaped into a liplike collar which fitted tightly over the tubule to prevent electrical leaks as much as possible. Transepithelial potential was measured at the perfusion (V_{te}^{o}) and collection site (V_{te}^{l}). The basolateral potential V_{bl} was measured with a conventional microelectrode. Current pulses for resistance measurements were sent through the second perfusion barrel and were collected via a separate reference electrode.

(65.6 Ωcm for the 51 mM K^+ 143 mM Cl^- Ringer solution, measured with a WTW conductivity meter LBR, Weilheim, FGR); (2) the current induced voltage deflections at the perfusion (ΔV_{te}^{o}) and collection end (ΔV_{te}^{L}) of the tubule; (3) R_{inp} (Ω), the input resistance, equal to $\Delta V_{te}^{o}/I_0$, where I_0 was the injected current (A) at the perfusion side; (4) L (cm), the length of the stretch of tubule under study, from the perfusion to the collection pipette; (5) the inner radius of the tubule, r_{opt} (cm), measured by an ocular micrometer.

Method A. Using the first four parameters only, an estimate of the length constant, λ(cm), and of the length specific resistance R_{te}^{l} (Ωcm) can be derived. Isaacson *et al.* (1989) and Aneshansley *et al.* (1988) used cable equations as described by Helman *et al.* (1971) to do so for Malpighian tubules of *Onymacris* and *Aedes*, respectively. The tissue-specific resistance R_{te}^{l} (Ωcm²) and the radius of the tubule can also be calculated from these data (r_{elec}) and compared with the optical radius (Boulpaep and Giebisch, 1978).

Method B. If r_{opt} is easy to measure, λ, the length specific R_{te}^{l} (Ωcm) and the tissue specific resistance, R_{te}^{l} (Ωcm²), can be estimated from parameters (1), (3), (4) and (5), ΔV_{te}^{L} is not needed then [see Appendix in Greger (1981) and equation (13) in Boulpaep and Giebisch, 1978)].

Measurement of the voltage divider ratio. After impaling a cell from the basolateral side, an estimate of the ratio of apical over basolateral resistance (R_{ap}^{tot}/R_{bl}) could be obtained. R_{ap}^{tot} was the lumped electrical resistance of the apical membrane, consisting of two postulated resistances in parallel, i.e. a pump resistance, R_p, and a diffusional resistance, R_{ap} (see Fig. 3). The voltage divider ratio was calculated from the current induced voltage deflections across the basolateral membrane and across the entire epithelium at the site of the impalement. The latter value was obtained from the appropriate cable equations [equations (13) and (14) from Sackin and Boulpaep (1981)]. To this end the measured transepithelial voltage deflections (ΔV_{te}^{o}), the total tubule length (L) and the distance between the impalement and current injection sites (x) were inserted in the appropriate equations.

Theoretical considerations

Electrical equivalent circuit. In Fig. 3 an electrical equivalent model is shown. Several authors have made an analysis of such a model (e.g. Boulpaep and Sackin, 1979a; Frömter, 1972). At least three barriers

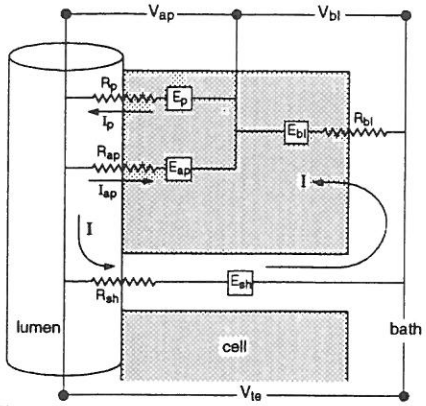

Fig. 3. Electrical equivalent circuit of a Malpighian tubule.

have to be considered: (1) the apical membrane, (2) the basal and lateral membrane and (3) the paracellular barrier. If the paracellular pathway has a certain conductance and if e.m.f.'s [i.e. electromotive forces: diffusion potentials and/or electrogenic pump(s)] are present, a circular current will flow and the actual electrical potentials across the barriers may be quite different from the e.m.f.'s present. In our model the e.m.f.'s at the basolateral and shunt barrier were thought to consist of the diffusion potentials E_{bl} and E_{sh}, respectively. At the level of the apical membrane a diffusion potential and an electrogenic pump in parallel were postulated. Taking into account the steep relationship between K^+ transport and V_{ap} (see Results section), the electrogenic pump was most probably not a constant current pump, but could be represented by a pump electromotive force E_p, sending a current I_p through a pump resistance R_p. Diffusion currents, I and I_{ap}, flow through the diffusional resistances R_{bl}, R_{ap} and R_{sh}.

Defining:

$$E_{bl} = E_{cell} - E_{bath} \quad \text{and} \quad V_{bl} = V_{cell} - V_{bath},$$

$$E_{ap} = E_{cell} - E_{lumen} \quad \text{and} \quad V_{ap} = V_{cell} - V_{lumen},$$

$$E_{sh} = E_{lumen} - E_{bath} \quad \text{and} \quad V_{te} = V_{lumen} - V_{bath},$$

expressions for V_{bl}, V_{ap} and V_{te} can be derived as a function of E, R, and I_p, by applying Kirchoff's first and second law.

$$I = I_{ap} + I_p, \tag{1}$$

$$I = (E_{bl} - E_{ap} - E_{sh})/(R_{bl} + R_{ap} + R_{sh}) + (I_p . R_{ap})/(R_{bl} + R_{ap} + R_{sh}), \tag{2}$$

$$V_{bl} = E_{bl} - I . R_{bl}, \tag{3}$$

$$V_{ap} = E_{ap} + I_{ap} . R_{ap} = E_{ap} + (I - I_p) . R_{ap}, \tag{4}$$

$$V_{te} = E_{sh} + I . R_{sh}. \tag{5}$$

Combining equations (1) and (2) with (3)–(5):

$$V_{bl} = [1/(R_{ap} + R_{bl} + R_{sh})] . [E_{bl} . (R_{sh} + R_{ap}) + (E_{ap} + E_{sh}) . R_{bl} - I_p . R_{ap} . R_{bl}], \tag{6}$$

$$V_{ap} = [1/(R_{ap} + R_{bl} + R_{sh})] . [E_{ap} . (R_{bl} + R_{sh}) + (E_{bl} - E_{sh}) . R_{ap} - I_p . R_{ap} . (R_{bl} + R_{sh})], \tag{7}$$

$$V_{te} = [1/(R_{ap} + R_{bl} + R_{sh})] . [E_{sh} . (R_{ap} + R_{bl}) + (E_{bl} - E_{ap}) . R_{sh} + I_p . R_{ap} . R_{sh}]. \tag{8}$$

It should also be noted that

$$R_{ap}^{tot} = (R_{ap} . R_p)/(R_{ap} + R_p) \tag{9}$$

$$R_{te} = [R_{sh} . (R_{bl} + R_{ap}^{tot})]/[R_{ap}^{tot} + R_{bl} + R_{sh}]. \tag{10}$$

Computation of the equivalent short-circuit current (I_{scc}). In flat sheet epithelia I_{scc} can be measured directly. If the active transport is electrogenic and the epithelium is bathed in symmetrical solutions, I_{scc} should be equal to the net transfer of the actively transported ion(s). In tubules perfused with symmetrical solutions in the lumen and the bath ($E_{sh} = 0$) I_{scc} can be calculated as the ratio of the open-circuit transepithelial potential (V_{te}) over the transepithelial resistance (R_{te}) (Sackin and Boulpaep, 1981).

Statistics

Results were presented as mean values ± SE.

Statistical computations were obtained using Statview II (Abacus Concepts Inc., Berkeley, CA, U.S.A., 1987).

RESULTS

Effect of different bathing solutions on the basolateral membrane potential (V_{bl})

In Fig. 4(A) a typical experiment is shown. V_{bl} was clearly sensitive to the bath K^+ concentration, $[K^+]_{bl}$. It depolarized to around zero in a high K^+ concentration (113 mM) and hyperpolarized to −60 mV in 5 mM K^+. Some tubules showed an overshoot when hyperpolarizing in 5 mM K^+ and stabilized at a less negative value. Upon readmitting a higher K^+ concentration the membrane then showed an undershoot, the cell potential reaching a stable value at a more negative level [see Fig. 4(B) and Table 2)]. So these transient changes didn't seem to be an effect of a leak around the microelectrode, but rather of a change in intracellular K^+ concentration (see Discussion). The reversibility of the effect of K^+ on V_{bl} in paired experiments is shown in Table 3.

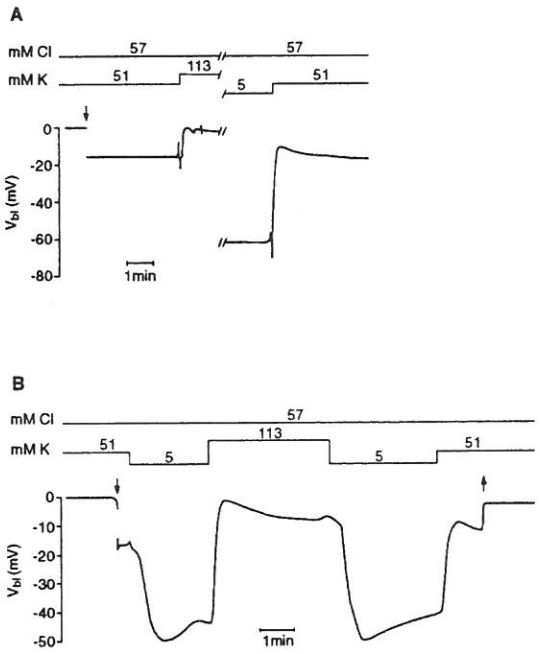

Fig. 4. Effect of different K^+ concentrations on V_{bl} of a spontaneously secreting tubule. Arrows indicate impalement (↓) or withdrawal of the electrode (↑). Transient changes in V_{bl} on changing $[K^+]_{bl}$ were more (B) or less (A) pronounced.

Fifty-seven mM was the control concentration for Cl^-, resembling that in the haemolymph (see Van Kerkhove *et al.*, 1989). Increasing the Cl^- concentration from 57 to 143 mM in a constant chemical K^+ concentration unexpectedly caused a depolarization in paired experiments (different solutions applied to the same cell) in the presence of 113 or 51 mM K^+. It caused either a depolarization or a hyperpolarization in 5 mM K^+. A possible explanation for the depolarization might be a difference in extracellular K^+ activity in the two Cl^- concentrations (Cl^- replaced by citrate in 57 mM Cl^- Ringer). This was verified with a K^+ selective electrode (unpublished results) which showed a drop to 80% of the extracellular K^+ activity in 57 mM Cl^- with respect to 143 mM Cl^- Ringer. The results (V_{bl}) of 11 paired experiments (six different solutions applied to the same cell, with 5, 51 or 113 mM K^+ and 57 or 143 mM Cl^-) were plotted as a function of the logarithm of the relative K^+ concentration (Fig. 5). It was found that the values could be fitted by a straight line with a slope of 42 mV/decade ($r = 0.999$, $P < 0.001$).

The effect of a change in Cl^- concentration on V_{bl} was further investigated by replacing all Cl^- by SO_4^{2-}, an anion that very probably does not permeate the membrane. If cell Cl^- left the cell through a conductive pathway across the basolateral membrane, one would expect at least a transient depolarization. Instead a hyperpolarization was seen. Again the change in potential could be explained by a change in K^+ activity: for an equal chemical K^+

Table 2. Transient changes in V_{bl} when changing $[K^+]_{bl}$ (paired experiments) $[Cl^-]_{bl} = 57$ mM

	113mM	5mM		113 mM	
$[K^+]_{bl}$	Steady state	Transient	Steady state	Transient	Steady state
V_{bl} ($n = 11$)	-3 ± 1 mV	-50 ± 2 mV	-42 ± 3 mV		
V_{bl} ($n = 9$)			-43 ± 2 mV	$+1 \pm 1$ mV	-4 ± 1 mV

Mean values ± SE.

Table 3. Reversibility of the effect of $[K^+]_{bl}$ on V_{bl} (paired experiments)
$[Cl^-]_{bl} = 57$ mM

$[K^+]_{bl}$	5 mM	113 mM	5 mM	113 mM
V_{bl} ($n = 11$)	-46 ± 3 mV	-2 ± 1 mV	-42 ± 3 mV	
V_{bl} ($n = 4$)		-2 ± 2 mV	-42 ± 3 mV	$+3 \pm 1$ mV

Mean values $\pm$ SE.

concentration, the K^+ activity in the SO_4^{2-} Ringer, as measured with a K^+ selective electrode, was only 70% of that in the Cl^- Ringer. This was expected to cause a hyperpolarization of 9 mV across a primarily K^+ selective membrane. The actual hyperpolarization of 8 ± 0.4 mV ($n = 6$) was very close.

Effect of different bathing solutions on the apical membrane potential (V_{ap})

V_{ap} was not measured directly, but was derived from the measurements of V_{bl} and V_{te}. Figure 6 summarizes the results of a large number of experiments (mostly unpaired, i.e. V_{bl} and V_{te} measured on different tubules). From the inset in Fig. 6 it is clear that there was a close correlation between V_{ap} and V_{bl}. This means that there was electrical cross talk between the two membranes. The linear regression was statistically significant ($r = 0.992$, $P < 0.01$). It had a slope of 0.63 ± 0.04 and an intercept with the V_{ap} axis of -42 ± 1.4 mV. These findings were confirmed in paired experiments: V_{bl} was measured in three different K^+ concentrations (either in 57 mM Cl^-, $n = 9$, or in 143 mM Cl^-, $n = 6$); the electrode was then pushed through into the lumen of the tubule and the effect of the three K^+ concentrations on V_{te} was measured. Again a significant correlation was found between V_{bl} and V_{ap} ($r = 0.901$, $P < 0.01$): the slope of the regression was 0.67 ± 0.16 and the intercept with the V_{ap} axis was -42 ± 5.0 mV. In a low $[K^+]_{bl}$ V_{bl} and V_{ap} were highly polarized and V_{te} had a low positive value. An increase in $[K^+]_{bl}$ depolarized

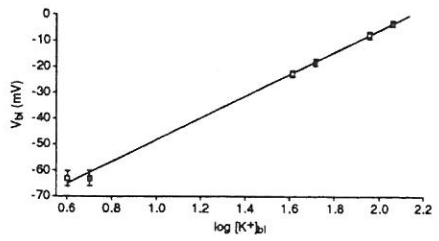

Fig. 5. Mean values $\pm$ SE for V_{bl} as a function of $[K^+]_{bl}$. Paired experiments ($n = 11$). Open symbols (□), measurements in 57 mM Cl^- (citrate), solid symbols (■) in 143 mM Cl^-. The K^+ activity in the presence of 57 mM Cl^- (anion gap filled with citrate) was only 80% of that in 143 mM Cl^- for the same chemical concentration. K^+ concentrations on the abscissa were shifted accordingly, to allow for this relative difference in activity. The slope of the curve was 42 mV/decade ($r = 0.999$, $P \ll 0.001$).

V_{bl} and V_{ap}, but the change in V_{ap} was only 60–70% of the change in V_{bl} (see inset of Fig. 6), so V_{te} hyperpolarized. In a high $[K^+]_{bl}$, when V_{bl} was near 0 mV, V_{ap} was around -42 mV and V_{te} was $+42$ mV, respectively. The overall result of increasing $[K^+]_{bl}$ was: a decrease in the apical potential gradient to be overcome by K^+ and, at the same time, an increase of the passive electrical gradient for Cl^- across the epithelium. This should facilitate the transepithelial movement of KCl.

Correlation between V_{ap} and fluid secretion rate

In a previous study (Van Kerkhove *et al.*, 1989) we found that Malpighian tubules primarily secreted KCl and fluid secretion could be used as a measure for net K^+ transfer across the epithelium. When plotting the relative fluid secretion rate [values taken from Table 3 (Van Kerkhove *et al.*, 1989)] as a function of V_{ap} (present study) we found a highly significant inverse relationship (Fig. 7).

Total transepithelial resistance (R_{te}) and relative apical (R_{ap}^{rel}) over basolateral (R_{bl}) membrane resistance

Resistance measurements were performed with luminally perfused tubules. Symmetrical solutions were used for intraluminal and bath perfusion (51 mM K^+ 143 mM Cl^-). A microelectrode in the cell measured V_{bl}. At the same time V_{te} was monitored. Constant current pulses were applied. Total transepithelial resistance and the ratio of apical over basolateral resistance could then be calculated.

Figure 8 shows a typical experiment. The basolateral membrane potential amounted to a high and stable value (-23 ± 1 mV, $n = 29$), indicating that the cells were healthy and could withstand the deformation due to the intraluminal perfusion pressure.

Total transepithelial transverse resistance (R_{te}). This can be expressed in Ωcm ($R_{te}^{\perp}$), i.e. the resistance of the tubule multiplied by the experimental length. As this value will depend on the luminal radius of the tubule under study, it can also be expressed in Ωcm^2 (resistance times the surface of the transporting epithelium), if we want to compare it with the tissue-specific resistance $R_{te}^{\perp}$ in other epithelia. In order to calculate the tubule's tissue-specific resistances one can use either the optically measured luminal radius and the current-induced voltage deflection at the

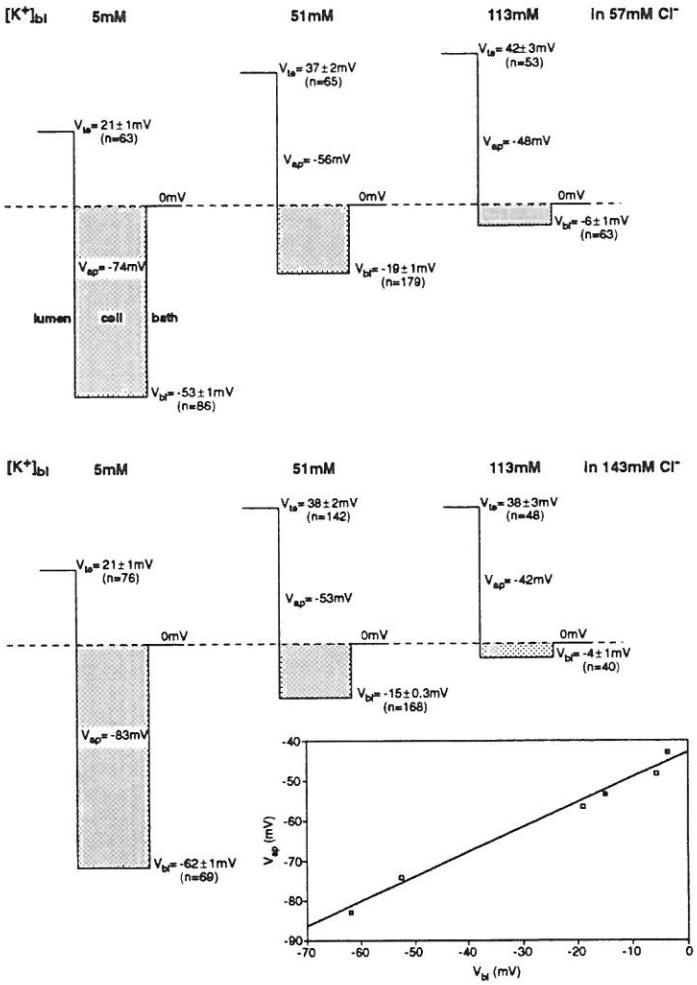

Fig. 6. Summary of V_{bl} and V_{te} measurements (unpaired experiments) in six different solutions. Inset shows V_{ap} (= mean value of V_{bl} minus mean value of V_{te}) as a function of V_{bl}. Open symbols (□), values obtained in 57 mM Cl^-, solid symbols (■), values in 143 mM Cl^-.

perfusion site (Method B) or the voltage deflections at the perfusion and the collection site and compute the radius from the data (Method A, see Materials and Methods section). The optical radius was measured with an eyepiece micrometer. The measurement was quite accurate: the cells were transparent and the lumen was dilated by the luminal perfusion pressure, so the boundary of the luminal membrane could easily be detected. For 38 measurements (in 22 tubules) the optical radius and the length constant of the perfused tubules were 12.5 ± 0.5 and 402 ± 29 μm, respectively. This gave a value of 23.2 ± 2.8 kΩcm for the length specific and 182 ± 21 Ωcm^2 for the tissue-specific resistance.

The electrical radius on the other hand, calculated from the measured transepithelial voltage changes and the resistivity of the perfusate (see Greger, 1981), was systematically lower (by about 40%) than the optical radius (giving a mean value for R^t_{te} of 41 ± 5 Ωcm^2, $n = 34$). Also the length constant,

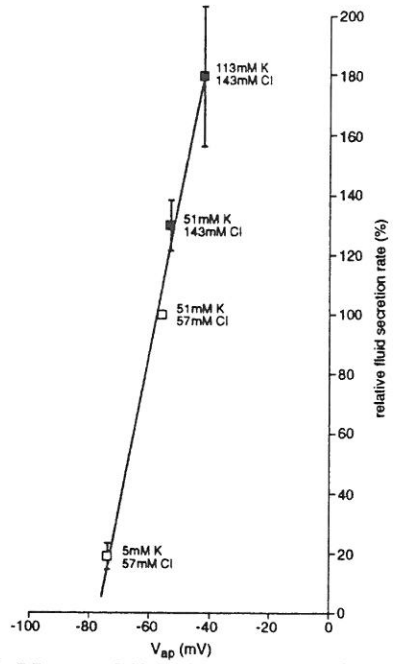

Fig. 7. Percentage fluid secretion rate [values taken from Van Kerkhove *et al.* (1989)] as a function of V_{ap} (present study, see Fig. 6). Correlation was significant: $r = 0.988$, $P < 0.01$.

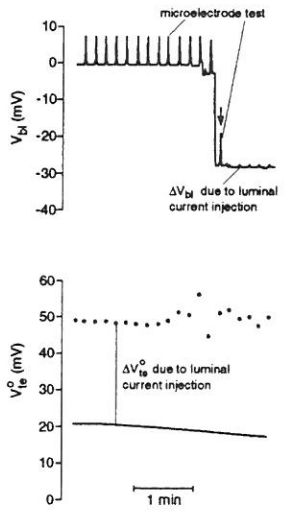

Fig. 8. Original tracing of transepithelial (V^0_{te}) and basolateral (V_{bl}) potential measurement in an isolated perfused Malpighian tubule. Pulses on the V_{bl} trace before impalement are due to current injection into the microelectrode, in order to measure the input resistance of the electrode. This pulse is switched off (↓) after a successful impalement. The pulses (ΔV_{bl}) seen afterwards are caused by luminal current injection. Clearly the ΔV_{bl} are much smaller than the ΔV^0_{te}, indicating that the basolateral membrane resistance is much smaller than the apical one.

calculated by this method varied with the length of the tubule. As we have no indication of morphological inhomogeneity along the length of the tubule, these results were most likely artefactual and due to an electrically leaky collection site. This caused an underestimate of the voltage deflection at this site and thus a short λ and an apparently low tissue specific resistance.

Relative apical over basolateral resistance. This was obtained from a series of six experiments. It was shown that the resistance of the apical membrane was about 40 times higher than that of the basolateral membrane: $R^{tot}_{ap}/R_{bl} = 44 \pm 8$ ($n = 6$). The resistance of the basolateral membrane must be low, because these measurements were possible only when the injected current was increased to about 100 nA and if the impalement site was relatively close to the current injection site (see Fig. 2). In our experiments the ratio of this distance (x) over the length constant (λ) was 0.67 ± 0.15 ($n = 6$; range 0.36–1.23).

Computation of the equivalent short-circuit current (I_{scc})

As K^+ transport was most probably electrogenic, the net K^+ flux should be equal to the I_{scc}. In 51 mM K^+ 143 mM Cl^- Ringer the net K^+ flux per cm tubule was calculated to be 1 μA/cm [from Van Kerkhove *et al.* (1989), assuming the effectively secreting length of the tubule to be 1 mm]. The I_{scc} calculated from V_{te} and the length specific R^L_{te} (Ωcm) was 1.2 ± 0.1 μA/cm ($n = 38$), which was very close.

When the tissue specific resistance R^t_{te}, (Ωcm^2) was used, I_{scc} was 175 ± 25 $\mu A/cm^2$ ($n = 38$). The radius of the lumen of a perfused and of a spontaneously secreting tubule was of the same order of magnitude: in 51 mM K^+, 143 mM Cl^- Ringer the tubules secreted fluid at a relatively high rate and the lumen was distended. Using the radius found for the perfused tubule, the net K^+ flux was calculated to be 127 $\mu A/cm^2$. This was somewhat lower, but still in good agreement with I_{scc}.

DISCUSSION

High apical over basolateral resistance

In order to understand the behaviour of the basolateral and the apical membrane potentials as a function of the bath K^+ concentration, it is necessary to comment first on the finding that the apical membrane resistance was substantially higher than the basolateral one.

First of all we want to make some comments on the measurement itself. R_{ap}^{tot}/R_{bl} was derived from the ratio of the voltage deflections across the apical and the basolateral membrane (voltage divider ratio) after current injection. This is legitimate only if the current density across the basolateral membrane is equal to the current density across the apical membrane. Two factors may give either an under- or an overestimation of the resistance ratio. An underestimation will occur if the lateral space significantly contributes to the total paracellular resistance (Boulpaep and Sackin, 1979b). In epithelia with narrow lateral spaces this resistance may be important. In the present tissue the height of the cells was about 10 μm (unpublished observation). This implies that the lateral space pathway was not very long and therefore would not be a major factor in the paracellular resistance. According to the calculations of Weber and Frömter (1988), the influence of the lateral spaces on the tubular current propagation was practically negligible, when the space width was about 0.2 μm, a lower value would lead to an underestimation of the apical membrane resistance. On the other hand it was shown by Cook and Frömter (1985) that, if the voltage divider ratio is measured close to the luminal current source, an overestimation of the ratio of apical to basal membrane resistance results. The reason is that part of the injected current is lost to the neighbouring cells through the intercellular junctions. A similar observation has been reported by several authors for renal tubules (Greger and Schlatter, 1983; Hoshi *et al.*, 1981) and rectal gland tubules of spiny dogfish (Greger and Schlatter, 1984). The error was small if the luminal core resistance was low compared to the cell core resistance, i.e. if the cell cable had a length constant far shorter than the lumen cable. We have no way to verify this in the present tissue. Thus, where possible, cell impalements were performed at a reasonable distance from the site of current injection ($x/\lambda \approx 0.7$). In our experiments the voltage divider ratio was not dependent on the impalement distance, although we could not completely exclude that the ratio values overestimated R_{ap}^{tot}/R_{bl}. Nevertheless, even if small quantitative errors were present, the experimental findings clearly indicated that the resistance of the apical membrane was much larger than the basolateral one. And, if a passive conductance was present in the apical membrane, it was probably very low.

These findings permitted some simplifications to be made, when analysing the electrical equivalent model of the epithelium (see Fig. 3 and theoretical considerations in the Materials and Methods section). For a Malpighian tubule of *Formica*, knowing that $R_{ap}^{tot} \gg R_{bl}$, which implied that $R_{ap} \gg R_{bl}$, equations (6)–(8), could be simplified to:

$$V_{bl} \approx E_{bl}, \tag{11}$$

$$V_{ap} \approx f'_{sh} \cdot E_{ap} + f_{ap} \cdot (E_{bl} - E_{sh}) - f'_{sh} \cdot V_{pump}, \tag{12}$$

$$V_{te} \approx f_{ap} \cdot E_{sh} + f_{sh} \cdot (E_{bl} - E_{ap}) + f_{sh} \cdot V_{pump}, \tag{13}$$

where f_{ap}, f_{sh} and f'_{sh} express the relative resistances, R_{ap}, R_{sh} and $R_{sh} + R_{bl}$, respectively, of the different barriers. They will determine the impact of the e.m.f.'s on the actual potential differences measured.

$$f_{ap} = R_{ap}/(R_{ap} + R_{sh}),$$

$$f'_{sh} = (R_{bl} + R_{sh})/(R_{ap} + R_{sh}),$$

$$f_{sh} = R_{sh}/(R_{ap} + R_{sh}),$$

$$V_{pump} = I_p \cdot R_{ap}.$$

It followed that V_{bl} almost equaled the e.m.f. (E_{bl}), present across the basolateral membrane. V_{ap} and V_{te} however were sensitive to the e.m.f.'s present in the other barriers: the higher the resistance of a barrier the greater the impact of the e.m.f.'s of the other barriers. This meant that it was possible to study the electrical properties of the basolateral membrane directly, by changing the bath ion concentrations for instance. Changing E_{bl} on the other hand had an impact on both V_{ap} and V_{te}.

The voltage divider ratio in Malpighian tubules in *Formica* (around 40) was high, when compared to other epithelia. Values varying between 1–4 were much more common [e.g. rabbit and *Ambystoma* renal proximal tubule (Bello-Reuss, 1986); *Necturus* proximal tubule (Sackin, 1986); *Amphiuma* collecting tubule (Hunter *et al.*, 1987); *Necturus* urinary bladder, Thomas *et al.* (1983); rabbit cortical thick ascending limb (Greger and Schlatter, 1983); *Calliphora* salivary gland (Berridge *et al.*, 1975); locust rectum (Hanrahan and Phillips, 1984)]. Only in the unstimulated rectal gland in shark [$R_{ap}/R_{bl} = 31$ (Greger *et al.*, 1984)], in the rabbit inner stripe of the outer medullary collecting duct [$R_{ap}/R_{bl} = 99$ (Koeppen, 1985) and rat pancreatic duct [$R_{ap}/R_{bl} = 16$ (Novak and Greger, 1991)] for instance comparably high values were found. The reason for this is not clear yet, but may have to do with the transport mechanisms and their regulation. In a Malpighian tubule of *Formica* for instance the high basolateral over apical conductivity allowed an intrinsic control of the rate of transport of K^+ by bath [K^+] (see below). Further study will show whether the basolateral conductance (and voltage divider ratio) changes when bath K^+ concentration is altered.

We expect that in Malpighian tubules of *Locusta* a voltage divider ratio of the same order of magnitude is present: V_{bl} (and V_{ap}) in these tubules primarily responded to a change in bath K^+ concentration, but factors that affected V_{ap} had little or no effect on V_{bl} (Fogg *et al.*, 1989; Baldrick *et al.*, 1988). The only other species where voltage divider ratio has been measured in Malpighian tubules is *Aedes aegypti*. These tubules had a ratio of 0.6 (Aneshansley *et al.*, 1988). In this preparation the Na^+ conductance and its regulation played a more important role in transport of salt and water (Sawyer and Beyenbach, 1985) and total basolateral conductance might therefore be lower and closer to the apical conductance and/or the apical conductance might be higher than that of *Formica* tubules.

Basolateral membrane potential: dependence on K^+, Na^+ and Cl^-

As shown above, due to the high conductance of the basolateral membrane relative to the apical membrane, it was possible to study the e.m.f. at the basolateral membrane directly, without having to take into account interference by e.m.f.'s of the other barriers on the actually measured membrane potential.

It was clear from the results that the conductance of this membrane for Na^+ (g_{Na+}) or Cl^- (g_{Cl-}) was not very high. When testing the effect of decreasing the bath K^+ concentration on the potential all K^+ was replaced by Na^+. This caused a hyperpolarization, meaning that g_{K+} was much larger than g_{Na+}. Also replacing Cl^- by other anions (SO_4^{2-} or citrate^{3-}) had little effect on the membrane potential that could not be explained by a relative shift in extracellular K^+ activity by the Cl^- substitution.

A K^+ permeability has been found in the basolateral membrane of all Malpighian tubule cells studied up to now [*Rhodnius* (O'Donnell and Maddrell, 1984); *Onymacris* (Nicolson and Isaacson, 1987); *Locusta* (Baldrick *et al.*, 1988; Fogg *et al.*, 1989); *A. aegypti* (Sawyer and Beyenbach, 1985)]. In the blood-sucking yellow fever mosquito a Na^+ permeability was also present and it increased on hormonal stimulation, facilitating Na^+ entry in the cells and resulting in preferential Na^+ over K^+ transport. This occurred after a blood meal, that contained an appreciable NaCl load (Sawyer and Beyenbach, 1985).

Nevertheless the membrane did not seem to behave as a perfect K^+ electrode, when plotting V_{bl} as a function of $\log[K^+]_{bl}$ (see Fig. 5). The slope was only 42 mV/decade. One of the suppositions that was made when expecting a slope of 58 mV/decade when making such a plot, was that the intracellular K^+ concentration stayed constant in the different $[K^+]_{bl}$ used. The presence, in some cells, of a transient overshoot in the depolarization or hyperpolarization (on increasing or decreasing $[K^+]_{bl}$, respectively) seemed to contradict this. In a low K^+ concentration in the bath these cells might have a relatively low intracellular K^+ concentration. On increasing $[K^+]_{bl}$ the membrane depolarized to a low negative value corresponding to the $[K^+]$ gradient present. If during the next few minutes the cell K^+ content rose, the membrane potential would consequently repolarize and stabilize at a level corresponding to the new concentration gradient. A slope of less than 58 mV/decade in the plot of V_{bl} against $[K^+]$bath would result from this. The fact that V_{bl} sometimes hyperpolarized in 5 mM K^+ when increasing the Cl^- concentration from 57 to 143 mM (when we expect a depolarization due to a relative increase in $[K^+]_{bl}$) could be the result of either a Cl^- conductance, becoming more important in the low $[K^+]_{bl}$, or a stimulation of a Cl^- dependent K^+ uptake by the cell in this low K^+ concentration. An increase in cell K^+ seemed plausible: preliminary results with K^+ selective double-barrelled microelectrodes corroborated this interpretation (Leyssens *et al.*, 1991): V_{bl} closely followed E^{bl}_{K+} within a few millivolts in all solutions and $[K^+]_{cell}$ varied with $[K^+]_{bl}$.

This dependence of cell K^+ concentration on $[K^+]_{bl}$ has not been seen in salivary gland of *Calliphora* for instance by Berridge and Schlue (1978), who measured intracellular K^+ directly with K^+ sensitive microelectrodes. In these cells the K^+ concentration was rather stable and decreased only if the bath K^+ concentration was lower than 2 mM. Baldrick *et al.* (1988) also indirectly came to the conclusion that $[K^+]_{cell}$ in Malpighian tubule cells of *Locusta* did not vary appreciably with the concentration in the bath (1–128 mM). The authors assumed a low Na^+ and a negligible Cl^- conductance. When they plotted $e^{EF/RT}$ as a function of $[K^+]_{bl}$ the data fitted a perfectly straight line, indicating that the intracellular K^+ concentration had a constant value of 164 mM. Plotting the data for *Formica* Malpighian tubule cells in this way (not shown) did not produce a straight curve and when trying to estimate the cellular K concentration from the slope(s) of the curve unlikely high values were found (200–300 mM).

Relationship between $[K^+]_{bl}$, the apical electrochemical gradient for K^+ and the rate of transcellular K^+ transport

From the previous considerations it was clear: (1) that the basolateral K^+ concentration contributed in determining the height of the electrical step to be overcome by K^+ transport across the apical

membrane; and (2) at the same time $[K^+]_{bl}$ determined the amount of K^+ available to the active mechanisms present in the apical membrane through its influence on $[K^+]_{cell}$. Knowing the K^+ concentration in the lumen to be 195 mM (Van Kerkhove *et al.*, 1989) and calculating $[K^+]_{cell}$ (assuming that V_{bl} was close to $E^{bl}_{K^+}$), we could obtain an estimate of the apical electrochemical gradient for K^+, i.e. $V_{ap} - E^{ap}_{K^+}$ (all potentials expressed with respect to the lumen). Figure 9(A) illustrates that the apical electrochemical gradient for K^+ was strongly correlated with $[K^+]_{bl}$

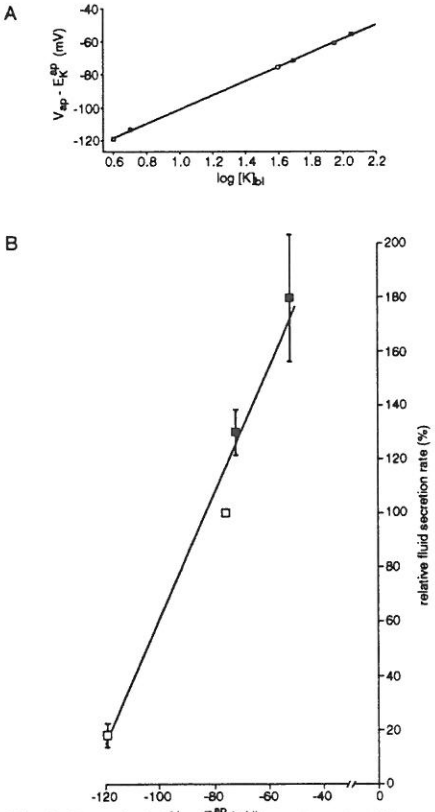

Fig. 9. (A) Apical electrochemical gradient for K^+ as a function of log $[K^+]_{bl}$. Open symbols (□) values in 57 mM Cl^-, solid symbols (■) in 143 in M Cl^-. K^+ concentrations in 57 mM Cl^- were shifted on the abscissa, as in Fig. 5. Values for V_{ap} were taken from Fig. 6. $E^{ap}_{K^+}$ was calculated from $[K^+]_{cell}$, derived from V_{bl}, and from the luminal K^+ concentration found by Van Kerkhove *et al.* (1989). Correlation was significant: $r = 1.00$, $P \ll 0.001$. Slope was 42 mV/decade. (B) Percentage fluid secretion rate [values taken from Van Kerkhove *et al.* (1989)] as a function of the apical electrochemical gradient for K^+, taken from (A). Correlation was significant: $r = 0.987$, $P < 0.02$. Slope was 2.3%/mV.

and that the secretion rate was steeply dependent on it [Fig. 9(B)]. When plotted in this way no direct effect of the basal Cl^- concentration on K^+ transport was found: the data obtained in 57 and 143 mM Cl^- lay on exactly the same curve.

The strong dependence of K^+ transport on the apical electrical potential (Fig. 6) suggested the presence of an electrogenic pump in this membrane. This will have to be further investigated: the electrical and the concentration component of the gradient have to be varied independently, i.e. by other means than a change in $[K^+]_{bl}$ and the impact of both on the transport rate has to be compared. It was already possible however to have an idea of the reversal potential (E_r) of such an electrogenic pump from Fig. 9(B). As K^+ transport corresponded well to the equivalent short circuit current, Fig. 9(B) was equivalent to a current voltage curve. The point of intersection with the abscissa, which gave the value of the electrochemical gradient in mV, when transport stopped, might correspond to the E_r of an electrogenic pump, when no concentration gradient was present across the barrier (Chapman and Johnson, 1978). The value of about −130 mV was close to the one found for the H^+-ATPase in the apical membrane of frog skin for instance (Ehrenfeld *et al.*, 1985). We could also calculate the free energy necessary to extrude K^+ across the apical membrane from it: $\Delta G^{ap}_{K^+} = F.E_r = 13$ kcal/mol. If ATP hydrolysis was the exergonic reaction to which K^+ transport was coupled, it should have a molar free energy of at least 13 kcal. As we have no data about the efficiency of the pump or about the molar free energy of ATP hydrolysis in a Malpighian tubule cell it is impossible for the moment to have an idea of the number of K^+ ions transferred across the membrane per ATP molecule.

R_t and the equivalent short circuit current

Table 4 summarizes the transepithelial length and tissue specific resistances, reported for Malpighian tubules of three species studied up to now.

The values were of a comparable order of magnitude and similar to those found for other insect epithelia: 150 Ωcm² (Wood and Moreton, 1978), 253

Table 4. Transepithelial resistance

Species	R^l_{te} kΩcm	R^t_{te} Ωcm²	Reference
A. aegypti	21	130	Williams and Beyenbach (1984)
A. aegypti	13	186	Aneshansley *et al.* (1988)
O. plana	8	72*	Isaacson *et al.* (1989)
F. polyctena	23	182	Present study

*Calculated from the author's data for R^l_{te} and electrical inner diameter [Table 2 (Isaacson *et al.*, 1989)].

and 177 Ωcm² [locust rectum: Hanrahan and Phillips (1984) and (1985), respectively]. The data for the tissue specific resistance in tubules were subject to caution however. In *Onymacris* for instance (Isaacson *et al.*, 1989) visualization of the lumen was difficult, while the tubule was heavily pigmented. Also the luminal cross section was irregular and contractions occurred. The authors found no correlation between the optically measured outer diameter and the inner diameter, calculated from the cable equations. They therefore preferred to report the length specific data, (calculated with Method A, see Materials and Methods section) that were much less sensitive to changes in the inner diameter. For *Aedes* Method A was used to obtain R^{L}_{te} and either 20 μm (Williams and Beyenbach, 1984) or 45 μm (Aneshansley *et al.*, 1988) was chosen as the relevant inner diameter to obtain a value for R^{L}_{te}. In our hands preference was given to method B, using r_{opt}: in *Formica* the luminal diameter was easy to measure optically. Also the requirement that the length constant should not change with the length of the stretch of tubule under study was fulfilled only if Method B was used in the calculations. And the I_{scc} (175 μA/cm²) was in better agreement with the K^+ transport [127 μA/cm², calculated from Van Kerkhove *et al.* (1989)]. An I_{scc} of 700 μA/cm², was found if the value of 41 Ωcm² was used, based on the electrical radius (Method A).

In order to decide on the basis of R^{L}_{te} whether the Malpighian tubule of *Formica* belonged to the category of leaky or tight epithelia, the relative magnitude of the sum of cell membrane resistances over shunt resistance should be determined. According to Guggino *et al.* (1982) in a leaky epithelium the resistance to flow of ions is less through the paracellular pathway than through the cellular pathway, irrespective of the absolute magnitude of the resistance of a given pathway. In order to quantitatively evaluate the ratio of the cell resistance ($R^{tot}_{ap} + R_{bl}$) over the shunt resistance (R_{sh}) it would have been necessary to perform three independent measurements, given the three independent variables. However from the high ratio of R^{tot}_{ap}/R_{bl} (≈40) and the absolute value of the transepithelial resistance (≈180 Ωcm²), it was possible to give a qualitative estimate of the leakiness of this tubule. Since the transepithelial resistance is the net result of two resistances in parallel, it can only vary between 50 and 100% of the lowest resistance [see equation (10)]. If the present tissue was tight and the flow of ions mainly followed the cellular pathway, the maximal possible value for the apical membrane resistance would be 180 Ωcm², whereas the basolateral membrane resistance would amount to only 4–5 Ωcm² or less. This seems low. Therefore we think that the total transepithelial resistance was an estimate of the paracellular rather than the cellular resistance. Moreover, its absolute value compared well with that reported for *Necturus* proximal tubule [260 and 265 Ωcm², for the transepithelial and the shunt resistance, respectively (Guggino *et al.*, 1982)]. With these values the latter was defined as a leaky epithelium.

A model for KCl transport and the ionic basis for the circular current: a working hypothesis

Figure 10 shows a model for K^+ transport through a Malpighian tubule of *Formica*, based on the data obtained up to now. It also allows us to plan for further research.

The basolateral membrane. A large K^+ conductance is present in the basolateral membrane. Whether the K^+ electrochemical gradient is inward and large enough to explain at least part of the net uptake of K^+ through this membrane has to be investigated with K^+ sensitive microelectrodes. A Na/K ATPase and cation/anion symporter(s) are also postulated in the basolateral membrane. The presence of these systems will have to be confirmed. In any case, if $[K^+]_{cell}$ changes with $[K^+]_{bl}$, it has to replace (or be replaced by) another cation, most likely Na^+. In a low bath K^+ concentration, mechanisms have to be present explaining net Na uptake by the cell: one possibility is switching from a K/Cl symporter (using an inward Cl concentration gradient) in the higher K^+ concentrations to a Na/K/2Cl symporter in the high Na^+ low K^+ solutions. Also if a minimal Na^+ conductance is present in the membrane the increased electrochemical gradient for Na^+ in the latter circumstances, combined with a reduced Na/K ATPase activity, can explain an increased Na^+ over K^+ uptake by the cells. A high $[K^+]_{bl}$ on the other hand might stimulate a Na/K pump, replacing Na^+ by K^+. Preliminary results showed however that ouabain had no effect on V_{bl} or on secretion (De Decker, unpublished results), like in many other insects (Berridge, 1968; Maddrell, 1969; Pilcher, 1970).

The apical membrane. The presence of an electrogenic cation pump in the apical membrane is very likely. Whether this is a H^+-ATPase, combined with a K/H antiporter, as suggested by the findings of Wieczorek *et al.* (1986) has to be investigated with H^+ sensitive microelectrodes.

Pathway for chloride. No evidence is available at present to decide what the relative importance is of the cellular over the paracellular pathway for Cl^- transfer. The high apical over basolateral membrane resistance in *Formica* Malpighian tubules suggests that, if a Cl^- conductance is present in the apical membrane, it must be smaller than that for K^+ in the

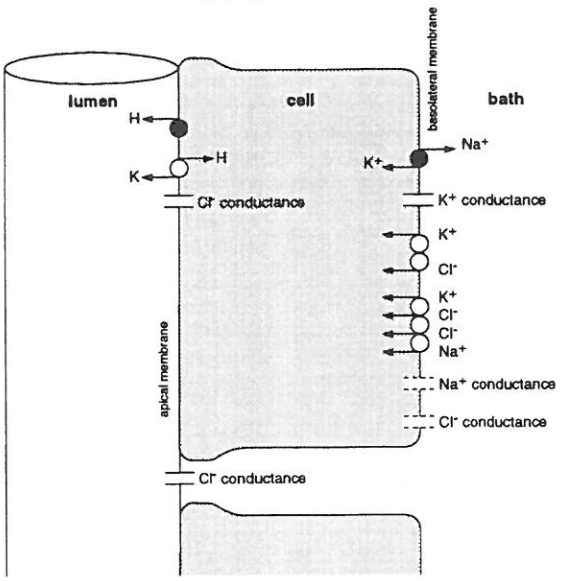

Fig. 10. Model for KCl transport through a Malpighian tubule cell of *Formica*. Solid symbols (●) represent active mechanisms, coupling an exergonic chemical reaction (e.g. ATP hydrolysis) to uphill ion transfer. Open symbols (○) represent secondary active systems using the downhill gradient substance or ion to move another ion(s) uphill. Channels or conductive pathways were indicated by openings in the barriers.

basolateral membrane. As in other epithelia, such a Cl^- conductance, in our case probably having a low basal value, may be a site for hormonal regulation. In any case if Cl^- uptake does occur through a cation/anion symporter at the basolateral side and the cell remains in steady state the same quantity of Cl^- ions has to leave the cells. So part of the net KCl transport may occur transcellularly. The importance of the shunt vs the cellular pathway for Cl^- transport will have to be investigated in luminally perfused tubules, where the composition of the luminal fluid can be controlled.

Circular current. As the shunt conductance is not zero, a circular current (I, see Fig. 3) will flow. This current could be carried by Cl^- through the shunt: a cation conductance is not very likely as this would cause a large backflux of K^+. The current I through the membranes may be an inward K^+ current at the basolateral side. It must then be equal to a positive outward current across the apical membrane = $I_p + I_{sp}$, where I_p is the positive pump current and I_{sp} may be a conductive Cl^- efflux from the cell into the lumen. If a negative ion is moving outward, I_{sp} is negative and the total positive current across the apical membrane is smaller than I_p. Consequently I_p is greater than I across the basolateral membrane. The pump current would then be larger than the conductive K^+ entrance. If the cell is in steady state in a particular bathing solution, K^+ entrance and exit must be equal. The extra K^+ (and Cl^-) uptake, necessary to compensate for the exit, could occur via electroneutral K/Cl symporter systems. In view of the high ratio R_{sp}^{tot}/R_M however, the ionic conductance of the apical membrane is probably low and most of the Cl^- current is expected to pass through the shunt, at least in control conditions.

Possible basis for electrical cross-talk between apical and basolateral membrane

In a symmetrical cell with an electrogenic pump and ionic conductance(s) (g_m) present in parallel in the membrane, the polarizing current produced by the pump will create a deviation from the diffusion potential for the permeable ions, generating an electrochemical gradient for these ions. If the conductance g_m of the membrane is high, the pump current I_p will easily be counterbalanced by a passive ionic current and the impact on the actual membrane potential value will be small and equal to I_p/g_m.

In an asymmetrical cell the apical and the basolateral part of the cell membrane have different properties. In our model, due to the high apical over

basolateral resistance, we think that the apical membrane contains an electrogenic cation pump, producing an outward current, and little or no ionic conductance, while the basolateral membrane is dominated by a high K^+ conductance. Passive ionic current can occur through this membrane. Electroneutrality demands that any outward current should be balanced by an inward current of equal size. However, if the inward and outward current come from and go to a different compartment, respectively (in this case the bath and the lumen), the current loop has to be closed by a current of equal size from one compartment to the other via the shunt. In this case the impact of the pump current on the membrane potential (apical and basolateral) will depend on the passive conductances of the shunt and the basolateral membrane: if these conductances are high the pump current will easily be shortcircuited by K^+ coming into the cell via the basolateral membrane and Cl^- following via the shunt. In a high $[K^+]_{bl}$ (i.e. close to $[K^+]_{bath}$ and $E_{K^+} \approx 0$) for instance, K^+ being pumped out of the cell will easily be counterbalanced by K^+ coming in through the basolateral K^+ channels and the effect on the basolateral potential will be minimal. Part of the apical pump current will be used to build a positive potential in the lumen to attract Cl^- through the shunt. In a low $[K]_M$ the new diffusion potential across the basolateral membrane will polarize the membrane and the inward K^+ current will slow down (less K^+ is available to start with), more of the pump current will now be used to further polarize the apical membrane. This will in itself slow down the electrogenic pump until outward current becomes equal to the (smaller) inward current again and until the (lowered) transepithelial potential gradient meets the (decreased) demand for Cl^- ions.

CONCLUSION

In a previous study (Van Kerkhove *et al.*, 1989) it was found that fluid and K^+ secretion were dependent on the K^+ concentration in the bath. We now showed that the bath K^+ concentration can regulate fluid secretion by decreasing the height of the electrical and concentration steps to be overcome by K^+ transport across the apical barrier. This is possible because the resistance ratio of the apical over the basolateral membrane is high and the basolateral membrane is dominated by a high K^+ conductance so that V_M approaches E_{K^+} on the one hand, and can impose its value on the apical membrane on the other. In view of the range of concentrations found in the haemolymph of the adult stage of *Formica* (<5–70 mM) the mechanism may be operative in the animal.

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Errata

p. 432 (last line) :

When advancing the electrode further, the tip reached the lumen and the transepithelial **potential difference** (V_{te}) was measured.

p. 432 (legend of Table I) :

Osmolality of all solutions was kept at **375 mosm/kg H_2O**.

p. 442 :

We could also calculate the free energy necessary to extrude K^+ across the apical membrane from it : $\Delta G_{K^+}^{ap} = K. E_r = 13$ **kJ**/mol. If ATP hydrolysis was the exergonic reaction to which K^+ transport was coupled, it should have a molar free energy of at least 13 **kJ**.

PAPER 2

UNMASKING OF THE APICAL ELECTROGENIC H^+ PUMP IN ISOLATED MALPIGHIAN TUBULES (*FORMICA POLYCTENA*) BY THE USE OF BARIUM.

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Unmasking of the Apical Electrogenic H Pump in Isolated Malpighian Tubules *(Formica polyctena)* by the Use of Barium

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Key Words
Malpighian tubules
H pump
V type ATPase
Barium
Electrophysiology

Abstract
In the present study evidence is given for the presence of an electrogenic, vacuolar type ATPase (V type ATPase) in the apical membrane of malpighian tubules of *Formica*. Barium (6 m*M*), the metabolic inhibitor monoiodo-acetic acid (MIA; $5 \cdot 10^{-4}$ *M*) and two inhibitors of V type proton ATPases, i.e. bafilomycin A1 (Baf-A1; $5 \cdot 10^{-6}$ *M*) and N-ethyl maleimide (NEM; $5 \cdot 10^{-4}$ *M*), all inhibited fluid secretion significantly ($p < 0.05$). This is in agreement with the hypothesis that K enters passively via K channels in the basolateral membrane and that a V type ATPase is involved in the active transport step at the apical membrane. Also MIA, NEM and Baf-A1 slightly depolarized the apical membrane potential, V_{ap}, by 16, 17 and 30 mV, respectively, whereas they had virtually no effect on the basolateral membrane potential (V_{bl}). The mild effect on V_{ap}, in contrast with the pronounced effect on fluid secretion, can be explained by the high apical over basolateral membrane resistance: the voltage divider ratio, VDR, was 47 ± 9 (n = 6). As a consequence the basolateral membrane will impose its value on the other barriers. VDR was decreased to 1.4 ± 0.2 (n = 19) by Ba. Ba also caused a strong and reversible hyperpolarization of both V_{bl} and V_{ap} (from −16 ± 1 to −84 ± 4 (n = 8) and from −51 ± 4 to −96 ± 6 mV, respectively). As expected MIA, NEM and Baf-A1 now had a much more pronounced depolarizing effect, i.e. they drastically reduced the Ba-induced hyperpolarization of both V_{ap} and V_{bl}. The reduction in V_{ap} was 67, 67 and 54 mV, respectively. V_{bl} depolarized from −73 ± 4 to −15 ± 4 mV (n = 7), from −72 ± 10 to 13 ± 2 mV (n = 5) and from −78 ± 3 to −30 ± 5 mV (n = 12) in the presence of Ba and MIA, NEM or Baf-A1, respectively. From cable analysis and total transepithelial resistance in the absence and presence of barium it was also possible to make an estimate of the resistances across the different barriers: total basolateral resistance = 10 Ω·cm², total apical resistance = 475 Ω·cm², total shunt resistance = 228 Ω·cm². It was concluded that in malpighian tubules of *Formica* an H pump of the V type is present in the apical membrane. As suggested in other epithelia this pump can be the prime mover in active K transport: the proton concentration gradient built up across the apical membrane can drive a K/H exchanger.

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Introduction

The secretion rate of KCl-rich primary urine by the isolated malpighian tubules of *Formica* is, as in many insects [1], strongly dependent upon the K^+ concentration in the bathing Ringer solution. From measurements of the transepithelial potential (V_{te}) and the luminal fluid composition in different bathing K^+ concentrations it was clear that K^+ is transported against a considerable transepithelial electrochemical gradient [2]. Measurements of the intracellular potential (V_{bl}) revealed that the basolateral membrane acts as an almost perfect K^+ electrode [3]. The primary active step in K^+ transport is located at the apical cell membrane. Active K^+ transport occurs in a lot of insect epithelia [4]. The transport system that is responsible for the transport of cations across the apical membrane was designated electrogenic K^+ pump or 'common cation pump' since other alkali metal ions were transported too [5]. The presence of such a pump was suggested in most transport models for malpighian tubule cells [1, 6–9].

From vesicle studies on the apical membrane of the goblet cells of the midgut of *Manduca sexta* the cation pump has been identified as a primary active proton pump that belongs to the vacuolar type ATPases [10–14]. Net K^+ secretion is assumed to be achieved by a K^+/H^+ exchanger, the necessary proton gradient being built up by the active H pump. On the basis of the use of blockers Bertram [15] provided indirect evidence for this mechanism in the apical membrane of malpighian tubule cells of larval *Drosophila hydei*.

The aim of the present study is to provide evidence for the existence of such a V type electrogenic proton pump in the apical membrane of malpighian tubule of *Formica*. Resistance measurements in symmetrically perfused tubules revealed a very high voltage divider ratio ($R_{ap}{}^t/R_{bl}$ = 47 in control solutions; $R_{ap}{}^t$ = total apical resistance, R_{bl} is total basolateral resistance). In these conditions the electromotive force of the apical membrane (E_{ap}) is masked and the effect of stimulating or inhibiting the apical electrogenic pump causes only mild electrical effects and solely at the apical side [3]. In the present study the voltage divider ratio is lowered by adding barium to the basolateral side. This causes a considerable increase in the basolateral resistance and therefore changes in E_{ap} will now be visible in V_{ap} and V_{bl}.

The effects of the metabolic inhibitor monoiodo-acetic acid (MIA) and of the two (typical) V type ATPase inhibitors N-ethyl maleimide (NEM; [16]) and bafilomycin A1 (Baf-A1; [17]) are tested on secretion rate in control Ringer. To characterize the apical pump the effect of the same drugs on the intracellular and transepithelial potentials is investigated, both in the presence and absence of barium. The results strongly suggest that in malpighian tubules of *Formica* an electrogenic proton pump of the V type is involved in the active transport of K^+ through the cells. Part of these results have been published as an abstract [18].

Materials and Methods

Animals

Formica polyctena (forest ant; *Hymenoptera, Insecta*) were collected from natural nests at the periphery of woods and kept in artificial nests until use. Room temperature was about 20 °C and humidity was around 40%. The ants were fed sugar and water ad libitum.

Artificial Salines

The composition of the control solution is summarized in table 1. Solutions were freshly prepared each week, filtered through 0.22 μm Millipore filters and kept at 2 °C until use. pH remained stable for at least 1 week under these conditions. Justification for the pres-

ence of the different components in the physiological solution was discussed previously [2]. Different K^+ concentrations were obtained by substituting K^+ for Na^+ or vice versa. A barium-containing solution was prepared by adding 6 m*M* $BaCl_2$ to the control solution. This increased the chloride concentration with 8% and the osmolality by about 4%. The osmolality was adjusted by an appropriate decrease in the glucose concentration. The effect of the slightly higher chloride concentration was not tested separately since it was not expected to have much impact on potential differences and secretion rate.

Test Substances

Substances were tested at the following concentrations: barium ($BaCl_2$; Janssen Chimica): $6 \cdot 10^{-3}$ *M*, MIA (Merck): $2 \cdot 10^{-4}$ *M*, NEM (Sigma): $5 \cdot 10^{-4}$ *M*. Bafilomycin-A1 (Baf-A1) was a gift from Dr. K. Altendorf (Universität Osnabrück, FRG). The concentration of this substance was determined by spectrophotometry [17]. It was noted that the electrical response to the same concentration of Baf-A1 disappears when the drug ages. This is most probably due to a decreased activity per concentration unit. Since the secretion experiments were done at a later moment, a concentration of $5 \cdot 10^{-6}$ *M* Baf-A1 was used to test its effect on secretion rate, while only $5 \cdot 10^{-7}$ *M* was needed to investigate its effect on electrical potentials. NEM and bafilomycin are not soluble in water. Therefore these substances were first dissolved in DMSO (dimethyl sulfoxide: Janssen Chimica). The final concentration of DMSO in the control solution was 1‰ or less. The effect of 1‰ DMSO was tested in control experiments: no significant effects were seen on secretion rate ($n = 5$), V_{bl} ($n = 5$) or V_{te} ($n = 3$). The same amount of DMSO was added to the control solution to obtain the same osmolality in control and test solutions.

Dissection and Experimental Set Up for Measurement of Fluid Secretion

This technique was described in detail elsewhere [2]. Briefly, the abdomen of the animal was cut open and the gut with the attached malpighian tubules (12–18) was removed. One tubule was isolated by cutting it off as closely to the gut as possible with small scissors. The isolated malpighian tubule (1–2 mm long) was transferred, on the stage of an inverted microscope (Zeiss IMC 401), to a continuously and rapidly perfused bathing droplet (50–100 µl), covered with paraffin oil to avoid evaporation. The cut end of the tubule was sucked into the tip of a holding pipette, provided with a constriction to obstruct the open end of the tubule. This pipette was moved to pull the tubule out of the droplet into the oil for about one third of its length. In this part a hole was made with the tip of a broken pipette. The luminal fluid is forced out of this hole by the secretion pressure. The appearing droplets were collected every 10 min. They were blown out of the hydrophobic collection pipette under paraffin oil and by measuring the diameter of the perfectly spherical droplet with an eyepiece, the secretion rate could be calculated.

Secretion rate in control conditions in different tubules shows a large variability and moreover it decreases in time [2]. Therefore each tubule served as its own control and the experimental protocol consisted of 3 collection periods in control solution (51 m*M* K, 143 m*M* Cl) followed by 4 experimental periods. The secretion rate was expressed as a percentage of the last period in control solution. Reversibility of effects of experimental conditions was evaluated by 3–4 washout periods.

The time effect was ruled out by the use of the following ratio:

$$\frac{\text{Experimental secretion percentage of collection period x}}{\text{Control percentage of collection period x}}$$

The control percentage for each collection period was obtained in a series of experiments ($n = 9$) where only the control solution was admitted during 110 min. Results of secretion rate were expressed as this ratio.

Table 1. Composition of the control solution (51 m*M* K 143 m*M* Cl)

Component	Concentration, m*M*
KCl	51
NaCl	62
$CaCl_2$	2
$MgCl_2$	13
Alanine	2.8
Trehalose	10.6
Maltose	11.7
Glucose	139
Hepes	12.1

pH was adjusted to 7.20, osmolality of the solution was 375 mosm.

Electrical Potential Measurements

The method was described in detail previously [3]. Intracellular (V_{bl}) and transepithelial (V_{te}) measurements were performed with 100 m*M* filled KCl microelectrodes (Borosilicate filament glass, Hilgenberg, FRG; OD 1.5 mm, ID 1 mm; tip diameter < 0.5 µm; R about 300 MΩ; for discussion on the filling solution: [3]), connected to a high impedance electrometer (WPI KS-700) via an Ag/AgCl wire. The reference electrode was a low resistance (1 MΩ) 3 *M* KCl/agar (4%) bridge, mounted in the bathing droplet and connected to the earth via an Ag/AgCl wire. The tubule was placed in the perfused bathing droplet and to facilitate impalement the tubule was immobilized by sucking each end into a holding pipette. Intracellular potentials were measured when the tip of the electrode (mounted on a Narashige hydraulic manipulator) punctured the basolateral membrane. The measurement was accepted if a sudden negative potential deflection occurred and was stable for at least a few minutes, and if the electrode potential differed maximally 3 mV from the baseline after withdrawal. Transepithelial potential was measured by advancing the microelectrode through the cell layer into the lumen of the tubule. The apical membrane potential (V_{ap}) was calculated as the difference between the measured transepithelial and basolateral potentials.

Electrical Resistance Measurements

This technique has been described in detail by Greger [19], Greger and Hampel [20] and Greger and Schlatter [21]. The middle segment of an isolated malpighian tubule was transferred to the perfusion system and mounted as described in reference [3]. The tubule was symmetrically perfused with control solution except when barium was used: it was only added to the bathing solution. Constant current pulses (30 nA; 100 ms; every 10 s) were supplied via an Ag/AgCl wire in one of the perfusion barrels. The current circuit was closed by a separate reference electrode (Ag/AgCl wire) in the bath. Total resistance of the epithelium (R_{te}, $\Omega \cdot cm^2$) and the voltage divider ratio (VDR; R_{ap}^t/R_{bl} with R = resistance, t = total, ap = apical and bl = basolateral) were estimated using the appropriate cable equations [22]. The experimental values used for calculations were ΔV_{te} at the perfusion pipette, ΔV_{bl} at a measured distance from the perfusion pipette and the optically measured radius of the lumen of the perfused tubule.

Theoretical Considerations

Epithelial tissues can be compared to electrical circuits [23]. The following equations were deduced from the electrical equivalent circuit for malpighian tubule of *Formica* [fig. 3 in 3]. Briefly, the circuit consists of three barriers: (1) the basolateral membrane: consists of a diffusional resistance R_{bl} and an electromotive force (EMF) E_{bl} ($\cong E_K$); (2) the apical membrane (in series with the basolateral membrane): consists of a diffusional resistance (R_{ap}^d) and EMF (E_{ap}), in parallel with a pump resistance (R_{pump}) and EMF (E_{pump}). The total resistance of this membrane is indicated by $R_{ap}^t = (R_{ap}^d \cdot R_{pump})/(R_{ap}^d + R_{pump})$; (3) the paracellular pathway: in parallel with the cellular pathway, consisting of EMF E_{sh} and diffusional resistance R_{sh}.

$$V_{bl} = \frac{E_{bl}(R_{sh} + R_{ap}^d) + R_{bl}(E_{sh} + E_{ap}) - I_p R_{ap}^d R_b}{R_{sh} + R_{bl} + R_{ap}^d} \quad (1)$$

$$V_{te} = \frac{E_{sh}(R_{ap}^d + R_{bl}) + R_{sh}(E_{bl} - E_{ap}) + I_p R_{ap}^d R_{sh}}{R_{sh} + R_{bl} + R_{ap}^d} \quad (2)$$

$$V_{ap} = \frac{E_{ap}(R_{sh} + R_{bl}) + R_{ap}^d(E_{bl} - E_{sh}) - I_p R_{ap}^d(R_{bl} + R_{sh})}{R_{sh} + R_{bl} + R_{ap}^d} \quad (3)$$

R = resistance (Ω); E = electromotive force (mV); $V_{bl} = V_{cell} - V_{bath}$; $V_{te} = V_{lumen} - V_{bath}$; $V_{ap} = V_{cell} - V_{lumen}$.

The resistance quotient f is defined as:

$$f = (R_{bl} + R_{sh})/(R_{ap}^t + R_{bl} + R_{sh}) \quad (4)$$

i.e. an estimate of the impact of E_{ap} on V_{ap}. This is a maximal estimate, while R_{ap}^d (eq.3) will be equal to or more likely greater than R_{ap}^t. In this study we could only calculate R_{ap}^t. It should be noted that if $R_{bl} \ll R_{ap}^t$, a fortiori $R_{bl} \ll R_{ap}^d$.

Since VDR (R_{ap}^t/R_{bl}) and R_{te} were determined in a paired way, in the presence and absence of barium, it was possible to estimate the individual resistance values (R_{ap}^t, R_{bl}, R_{sh} and R_{cell}) on condition that R_{ap}^t and R_{sh} are not influenced by the barium treatment [24].

Taking the simplest equivalent circuit for the epithelium:

$$1/R_{te} = 1/R_{sh} + 1/(R_{ap}^t + R_{bl}) = 1/R_{sh} + 1/R_{ap}^t (1 + VDR^{-1}) \quad (5)$$

Since it is assumed that R_{sh} and R_{ap}^t do not change under barium treatment, the following can be derived:

$$1/R_{te} - 1/R_{ap}^t (1 + VDR^{-1}) = 1/R_{te'} - 1/R_{ap}^t (1 + VDR'^{-1}) \quad (6)$$

where (') indicates the situation with barium.

R_{ap}^t is the only unknown value in eq.5. From the calculated VDR and VDR' it is then possible to deter-

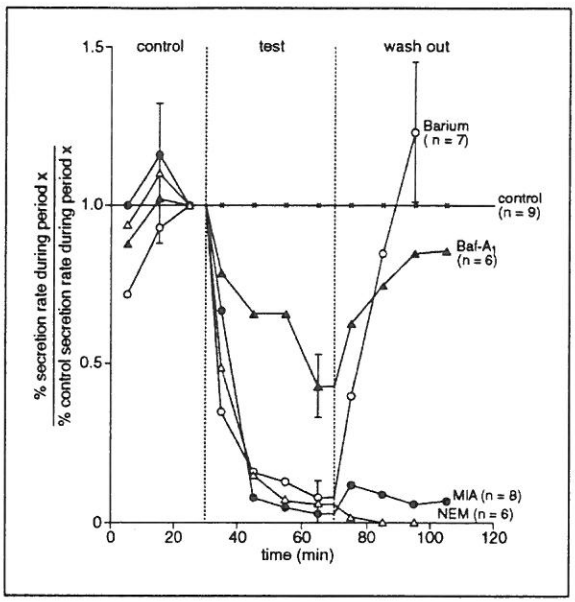

Fig. 1. The effect of 6 m*M* barium (n = 7), $6 \cdot 10^{-3}$ *M* MIA (n = 8), $2 \cdot 10^{-4}$ *M* NEM (n = 6) and $5 \cdot 10^{-6}$ *M* Baf-A1 (n = 6) on the fluid secretion rate of isolated malpighian tubules. To overcome the variability in the absolute values for secretion rate in different tubules, the absolute value of each secretion period within an individual experiment was expressed as a percentage of the value measured during the third collection period. The secretion rate was also determined in a series of experiments during which the tubules were kept in control solution during the total duration of the experiment (110 min). To rule out the time effect secretion rates were then expressed as the ratio of the test value (in %) of one period over the control value (in %) of the same collection period. For each tested substance the ratio of test over control value was significantly smaller than 1 ($p < 0.05$) during the last test period (60–70 min). The ratio was not significantly different from 1 during the last washout period after barium treatment (80–90 min).

mine R_{bl} and R_{bl}'. Subsequently R_{ab} can be determined from eq.5.

$$R_{cell} = R_{ap}^{t} + R_{bl} \quad (7)$$

where R_{cell} is the total cellular resistance ($\Omega \cdot cm^2$).

Statistics

Results are presented as mean values ± SE (n = number of tubules). Statistical significance of paired or unpaired differences was evaluated by the (two-tailed) Student's t test.

Results

Effect of Barium

The effect of 6 m*M* barium on secretion rate is shown in figure 1. Secretion is inhibited to about 8% of the control value by the end of the test period, and the effect is completely reversible within the washing out period. Figure 2 shows a typical potential profile in a symmetrically perfused tubule in control

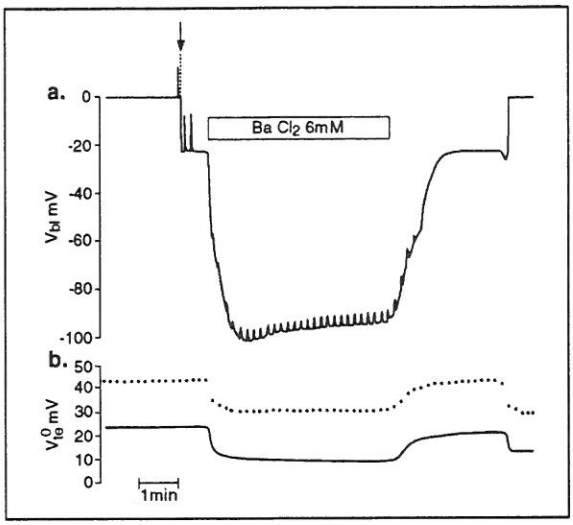

Fig. 2. Original trace of an electrical measurement of the basolateral (**a**) and transepithelial potential (**b**) in a perfused malpighian tubule, showing the response to luminal current injection (30 nA during 100 ms every 10 s) in the absence and presence of 6 m*M* barium in the bathing solution. ΔV_{bl} in response to these current injections is visible as the smaller peaks (**a**) and is only observed when barium is present. ΔV_{te} in response to luminal current injections (**b**) is indicated by the distance between the dotted line and the lower trace. The penetration of the basolateral membrane by a conventional microelectrode is indicated by the arrow. The resistance of the microelectrode was determined just before and after impalement (visible as the larger voltage deflections on V_{bl}, 3 ×, at the beginning of the experiment).

solution: adding 6 m*M* barium causes a sudden hyperpolarization of the basolateral membrane and a decrease in V_{te}. In the presence of barium basolateral voltage deflections in response to the luminal current injections appear, indicating an increase in VDR, and therefore suggesting an increase in R_{bl}. Figure 3 summarizes the effect of barium on the intracellular potentials in unperfused tubules. V_{bl} (n = 8) and V_{te} (n = 21) were not measured in paired experiments. The hyperpolarization of the apical membrane potential, calculated from the mean V_{te} and V_{bl}, was confirmed in 3 paired experiments (V_{ap} was −51 ± 4 mV and −96 ± 6 mV in the absence and presence of barium, respectively).

The basolateral hyperpolarization caused by barium is reversible: V_{bl} in control conditions is not significantly different before and after adding barium (n = 5). The decrease in V_{te} from 42 ± 5 before to 29 ± 3 mV (n = 10) after washing out barium (applied for 10 min) could simply be due to a decrease in V_{te} in time observed in many tubules (time effect). Because of the large variability amongst tubules in the magnitude of this time effect we cannot conclude whether the effect of barium on the apical potential is completely revers-

ible, but it is at least partially. Table 2 summarizes the effect of barium on R_{te} for a series of paired experiments (n = 21). The effect of barium on VDR was not measured in a paired way, but the results clearly show significant differences between the values in the presence or absence of barium. The significant increase in R_{te} from 155 to 178 $\Omega \cdot cm^2$ is mainly due to an increase in the basolateral membrane resistance, as is indicated by the drop in VDR from 47 to 1.4. When the absolute values of the resistances are calculated, an increase in R_{bl} is seen from 10 to 339 $\Omega \cdot cm^2$, and in R_{cell} from 485 to 814 $\Omega \cdot cm^2$. It must be noted that in these circumstances $R_{ap}{}^t$ and R_{sh} are assumed to remain constant and have an estimated value of 475 $\Omega \cdot cm^2$ and 228 $\Omega \cdot cm^2$, respectively. It was previously shown in paired experiments that V_{bl} behaves as a K^+ electrode in control solutions [3]. Figure 4 shows an example of an intracellular measurement in an unperfused tubule that clearly illustrates the reduced response to changes in $[K^+]_{bath}$ in the presence of barium. This loss of K^+ sensitivity of the basolateral membrane is summarized in figure 5. For each K^+ concentration the effect of barium was tested in a paired way (n = 4 in 5 m*M* K^+, n = 9 in 51 m*M* K^+ and n = 6 in 113 m*M* K^+). All paired values were significantly different from one another ($p < 0.005$). Figure 4 also illustrates the sustained hyperpolarization of V_{bl} in different K^+ concentrations as long as barium is present. This demonstrates that the hyperpolarization is not spontaneously lost for at least 15 min.

Effect of Metabolic Inhibition

Active transport should be sensitive to metabolic inhibition. In a first series of experiments the effect of a blocker of anaerobic ATP production, MIA, was tested on fluid secretion. Figure 1 clearly shows that the transport is inhibited by MIA. Fluid secretion rate drops to very low values (about 5%), but

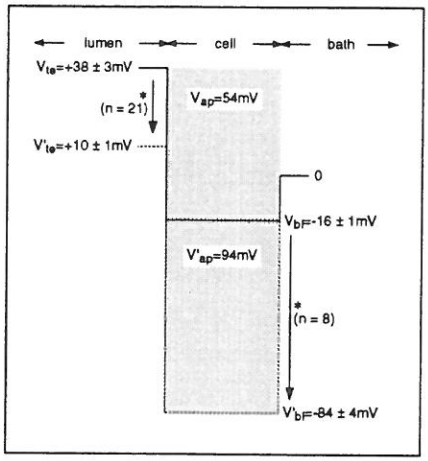

Fig. 3. Effect of adding 6 m*M* barium to the basolateral medium on the transepithelial potential profile in unperfused tubules. Full lines indicate the control situation (V_{bl}, V_{te}), dotted lines indicate the situation in the presence of barium (V'_{bl}, V'_{te}). V_x and V'_x were measured in a paired way, V_{te} and V_{bl} were not measured in the same series of experiments. V_{ap} and V'_{ap} were calculated from the mean values. An asterisk indicates significant differences between paired values ($p < 0.005$).

Table 2. Effect of 6 m*M* barium on VDR and on R_{te}

	Without barium	With 6 m*M* barium
VDR	47 ± 9 (6)	1.4 ± 0.2 (19)
R_{te}, $\Omega \cdot cm^2$	155 ± 18 (21)	178 ± 18 (21)

Values in the presence and absence of barium are significantly different ($p < 0.005$) from each other (VDR was determined in nonpaired experiments, R_{te} was measured in paired experiments).

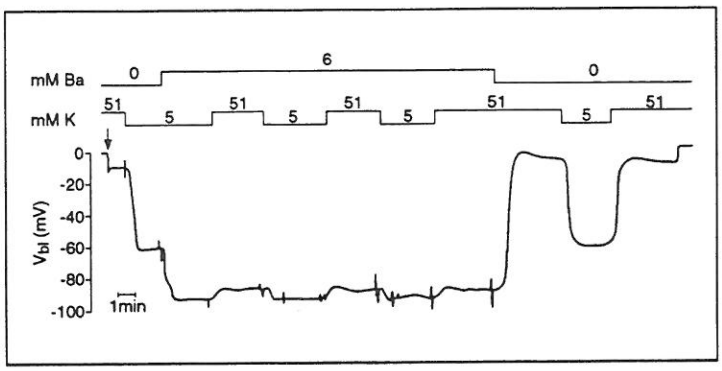

Fig. 4. Original trace of a measurement of the response of the basolateral membrane potential to different bathing K^+ concentrations in the presence and absence of 6 m*M* barium. The arrow indicates the penetration of the membrane with a conventional microelectrode.

does not recover within the 40 min washout period. Besides the effect of MIA on secretion rate its influence on the electrical potential differences was investigated. The active step for cation transport resides in the apical membrane. If this step is electrogenic we expect that MIA mainly affects the apical membrane potential. In control conditions VDR is high and the absolute value of R_{bl} is low. From equation 1 ($R_{bl} < R_{ap}$) we can deduce that V_{bl} is in these conditions mainly dependent upon E_{bl}. The expression of changes in the electrical characteristics of the apical membrane on V_{ap} will be small (33%). This can be derived from equation 4: $f = 0.33$. In the presence of barium the effects of changes in the apical electrical characteristics become more pronounced on V_{ap} ($f = 0.54$) and will also be reflected on V_{bl} (equation 1: R_{bl} increases 30–40 times). The results summarized in figure 6a corroborate this hypothesis. In a first series of experiments the basolateral and concomitant transepithelial potentials were measured, and the apical membrane potential was calculated, before and after adding MIA in the absence of barium. Both V_{ap} and V_{te} depolarize after adding MIA, but V_{bl} is not affected. In another series of experiments the procedure was repeated in the presence of barium. The apical membrane depolarization is now much more pronounced (from −91 to −24 mV) and also V_{bl} is markedly reduced. A typical experiment is shown in figure 7a.

Effect of V Type ATPase Blocking Agents

In order to gain further insight into the nature of the active mechanism that is present in the apical membrane, the effect of two V type ATPase blockers was studied on fluid secretion (fig. 1; NEM, $5 \cdot 10^{-4}$ *M* and Baf-A1, $5 \cdot 10^{-6}$ *M*). Both drugs inhibited the fluid secretion significantly although some differences in their effects can be seen: NEM reduced the fluid secretion to very low values (to about 7%) and the secretion did not recover within the 30 min washing out period.

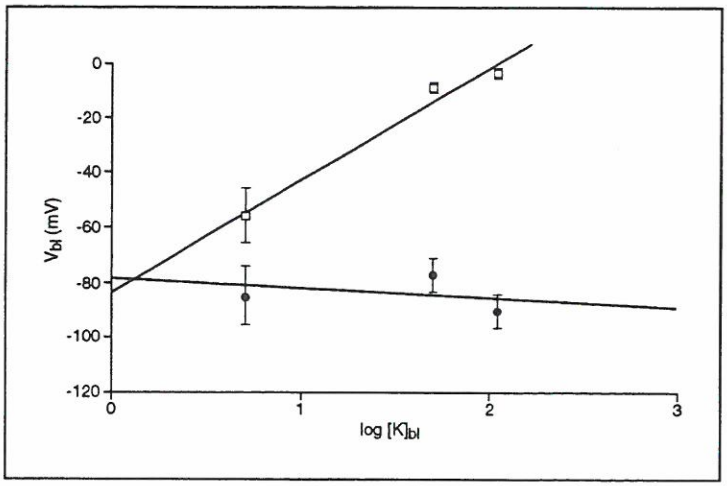

Fig. 5. The basolateral membrane potential (V_{bl}) of unperfused malpighian tubules versus log $[K^+]_{bl}$ in the presence (•) and absence (□) of 6 m*M* barium in the basolateral medium. The barium effect was tested in a paired way but each $[K^+]_{bl}$ was tested in a different series of experiments.

Baf-A1 lowered the secretion rate to about 45% and its effect was more reversible (86% recovery within 40 min). The effect of both substances was also tested on the potential profile in the presence and absence of barium. For each drug two series of experiments were performed: one to evaluate the drug effect in control solution and a second one to investigate its effect in the presence of barium. Results are summarized in figure 6b,c. As has been shown for MIA, in the absence of barium the effects are small and only visible at the apical side, while in the presence of barium a large depolarization of both membrane potentials is seen. Typical V_{bl} measurements in the presence of barium and drugs are shown in figure 7b,c. The effect of NEM on V_{bl} seems to be biphasic: a first decrease to about -53 ± 10 mV and a second decrease to about -12 ± 2.4 mV ($n = 5$). The mean effect of Baf-A1 is smaller than the NEM effect (fig. 6c), but in individual experiments the values can decrease to the same amount as in NEM treatment as is the case in the example shown in figure 7c.

Discussion

Unperfused isolated malpighian tubules of *Formica* produce a KCl-rich primary urine: K^+ and Cl^- concentrations of about 200 m*M* were measured in the lumen [2]. A working hypothesis to explain the transport of K^+ and Cl^- has been described recently [3]. In the present study we focus on the primary active transport system in the apical membrane (fig. 8).

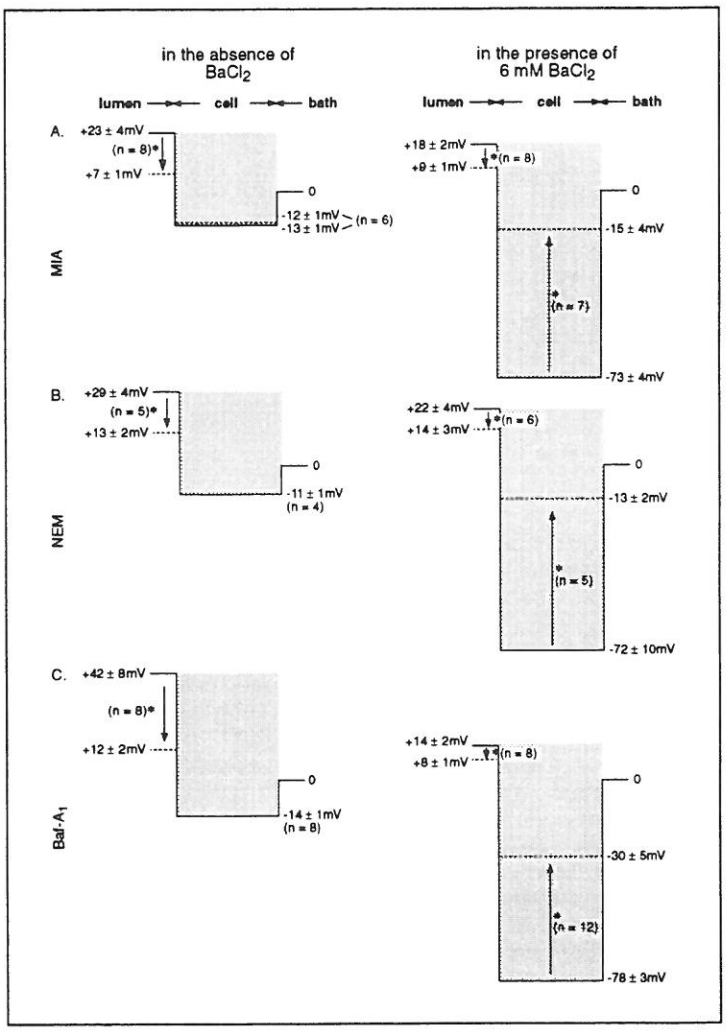

Fig. 6. Effects of different drugs (indicated by arrows) on the normal transepithelial potential profile (full lines) in the absence (left) and presence (right) of 6 m*M* barium in the basolateral medium. (a) Effect of $2 \cdot 10^{-4}$ *M* MIA. (b) Effect of $5 \cdot 10^{-4}$ *M* NEM. (c) Effect of $5 \cdot 10^{-7}$ *M* Baf-A1. The effect of the drugs per se was tested in a paired way, but the effects of the drugs in the absence or the presence of barium were investigated on a separate series of tubules. An asterisk indicates significant differences between paired values ($p < 0.005$).

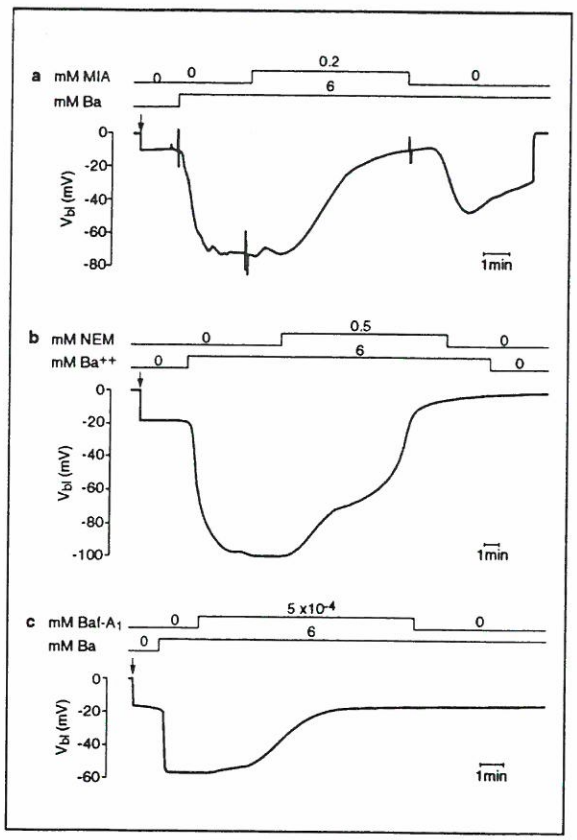

Fig. 7. Typical examples of measurements of the intracellular potential (V_{bl}) to illustrate the effect of different drugs on the hyperpolarization caused by barium. (**a**) Effect of $2 \cdot 10^{-4}$ *M* MIA. (**b**) Effect of $5 \cdot 10^{-4}$ *M* NEM. (**c**) Effect of $5 \cdot 10^{-7}$ *M* Baf-A1. Arrows indicate the penetration of the basolateral membrane by conventional microelectrodes.

Barium Increases the Basolateral Membrane Resistance

The basolateral membrane of the *Formica* malpighian tubule cell closely reflects E_K [3], indicating that K^+ channels contribute almost exclusively to the conductivity of this membrane. Barium is known to selectively block K^+ channels in a variety of tissues [25]. In the presence of barium R_{te} increases from 155 to 178 $\Omega \cdot cm^2$ while VDR drops from 47 to about 1.4. These results strongly indicate that barium causes a rise in R_{bl} (estimated increase from 10 to 339 $\Omega \cdot cm^2$) because the main conductive pathway is blocked. This is confirmed by the loss in K^+ sensitivity of V_{bl} under barium treatment (fig. 5). It is concluded that barium is an effective blocker of the K^+ channels in the basolateral membrane of the malpighian tubule cells of *Formica*. The estimated basolateral resistance in control condi-

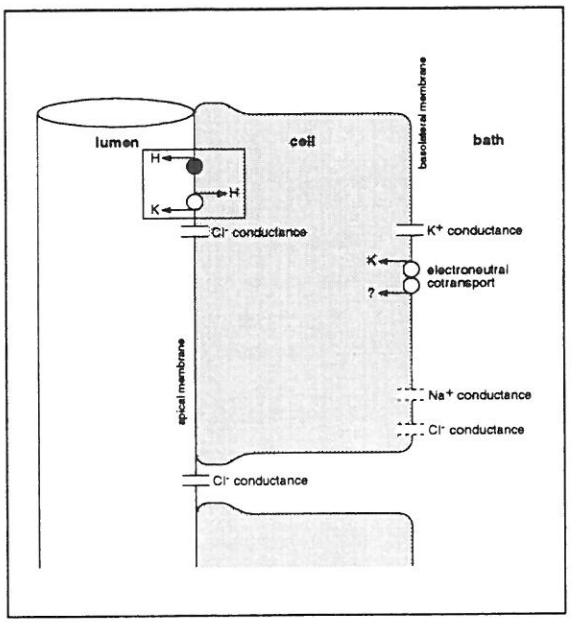

Fig. 8. Model for K^+ and Cl^- transport from bath to lumen in the malpighian tubules of *Formica*. The primary active pump at the apical side pumps protons into the lumen. The proton gradient built up by this system allows efflux of K^+ across the apical membrane via a K^+/H^+ exchanger. K^+ entry at the basolateral membrane is passive via K^+ channels. In this membrance other permeabilities are possible and a cotransport system can be present. The anions diffuse mainly (passive transport) via the shunt but chloride channels in the cell membranes are not ruled out.

tions is very low (10 $\Omega \cdot cm^2$), indicating that the K^+ permeability is very high indeed. Although the resistance increases significantly under barium treatment, the new value of 339 $\Omega \cdot cm^2$ is still not extremely high. Therefore it can not be excluded that other conductances are present in the basolateral membrane. As suggested in the model (fig. 8) an Na^+ or Cl^- permeability could be taken into account.

Apical Electrical Events Are Visible under Barium Treatment

From previous work [2, 3] it is clear that the active transport system is localized in the apical membrane. Electrical events at this membrane are masked however by the basolateral membrane, due to its high conductance. When reducing this conductance with barium, the voltage divider ratio drops from 47 to 1.4. One then expects the electromotive forces of the apical membrane to become more visible in the measured potential values. This is indicated on the one hand by the resistance quotient f: its value increases from 0.334 in control conditions to 0.544 in barium-containing solution. Looking at equation 3 it is clear then that the term ($E_{ap} \cdot f$) becomes more important in the measured apical membrane potential. On the other hand it is clear from equation 1 that an increased R_{bl} implicates a stronger reflection of E_{ap} on V_{bl} via the term $R_{bl}(E_{sh} + E_{ap})/(R_{ap}^t + R_{bl} + R_{sh})$. The most marked effect of adding barium to the basolateral medium is the strong hyperpolarization of both membrane potentials (fig. 3). This is most probably due to electrical events

in the apical membrane that become visible in this condition. This interpretation is corroborated by the results shown in figure 6. When applying MIA, NEM or Baf-A1 in the absence of barium only the apical potential depolarizes (by 17, 16 and 30 mV, respectively). This effect is systematic but not very large although the drugs block the apical transport system effectively: secretion rate is highly reduced (fig. 1). In the presence of barium the depolarization of the apical membrane is much more pronounced (67, 67 and 54 mV, respectively) and the effect is also reflected on the basolateral membrane.

Ionic Mechanisms Underlying the Hyperpolarization in Barium Containing Solution

In the cells of *Formica* malpighian tubules K^+ ions are assumed to be transported actively into the tubule lumen: K^+ enters the cell via the basolateral K^+ channels and is secreted into the lumen by an active pump mechanism. The accompanying Cl^- ion moves possibly mainly through the paracellular pathway (fig. 8). In view of this hypothesis the most likely explanation for the strong bilateral effect of barium is the electrical coupling between the apical and basolateral K^+ movement. At the apical side K^+ is removed from the cytoplasm, thereby creating a more negative intracellular potential and a more positive intraluminal potential. Under control conditions these potentials are counterbalanced by a fast inward movement of K^+ across the highly conductive K^+ channels in the basolateral membrane and by paracellular movement of Cl^-. When barium is added, the basolateral K^+ conductance is reduced and therefore a much larger negative intracellular potential is built up because the counterbalancing K^+ movement is much slower. The membrane potentials will increase until either the basolateral membrane potential is large enough to allow K to move through the low conductive channels (secretion is not completely abolished in this case) or until the apical membrane potential reaches the maximal value that can be built up by the electrogenic transport (in which case the secretion actually stops). It should be stressed that this description is only true if anions are mainly moving through the shunt resistance and if the apical transport mechanism is electrogenic.

A strong bilateral hyperpolarization in barium containing solution is also seen in the midgut of *Manduca sexta* (V_H hyperpolarizes from −40 to −70 mV [25]). The authors suggest that depletion of chloride from the cytoplasm is responsible for the hyperpolarization. This is only true if chloride is transported transcellularly via apical and basolateral chloride channels and if both membranes show the chloride equilibrium potential when barium is added. But also in this preparation an electrogenic cation secreting transport mechanism is at the origin of the potential difference.

Although the anion pathway in the malpighian tubule of *Formica* is not completely understood yet, there seem to be more arguments in favor of the paracellular anion route. We therefore believe that the electrical coupling theory might be more suited to explain the results.

Active Transport Is Responsible for the Hyperpolarization in the Presence of Barium

The hyperpolarization caused by the admission of barium disappears when the active transport is ruled out by metabolic inhibition (fig. 6a). The same phenomenon is seen in *M. sexta* midgut [26, 27], where hypoxia abolishes the hyperpolarization that has been built up in barium containing solution. These results strongly indicate the involvement of the active transport in the hyperpolarization. It

can be concluded that this transport mechanism is electrogenic.

Nature of the Apical Active Transport System

It is now well established that in the midgut of *M. sexta* the so-called 'common cation pump' in the apical membrane is actually a proton pump, showing the characteristics of the classical V type ATPases [10–14]. This proton pump can function as the driving force for K^+ extrusion since it maintains a proton gradient, favorable for proton influx via a K^+/H^+ exchanger. Bertram [28] suggested that an identical system is responsible for the transport of cations in the malpighian tubules of *D. hydei* and recently described the sensitivity of the pump to typical V type blockers [15], confirming these predictions.

Since the presence of this system in the malpighian tubules of *Formica* was considered, the effect of typical V type ATPase inhibitors like bafilomycin and NEM was investigated [16, 17]. Bafilomycins inhibit the proton V type ATPases very specifically and at low concentrations [17]. The concentration of Baf-A1 used in the present study is rather high compared to those reported by Bowman, but it is comparable to the one used in the malpighian tubules of *D. hydei* [15]. As the authors suggest the need for a rather high concentration is probably due to the fact that intact epithelia are used instead of isolated membrane fractions. Anyway, the concentrations used in this study (up to $5 \cdot 10^{-6}$ *M*) are far below the concentration needed for effects on other types of ATPases [17]. The inhibition of the fluid secretion by $5 \cdot 10^{-6}$ *M* Baf-A1 therefore strongly indicates that the apical pump belongs to the V type ATPases. The reduction of the barium hyperpolarization after adding $5 \cdot 10^{-7}$ *M* Baf-A1 confirms the electrogenicity of this H pump. It was noted that the activity per concentration unit of Baf-A1 decreases in time. This became obvious by the fact that $5 \cdot 10^{-7}$ *M* Baf-A1 had no longer an effect on the membrane potentials at the moment we planned secretion experiments. A 10 times higher concentration did have an effect on the electrical potential, so this concentration was used to investigate the effect on the fluid secretion. The concentration of NEM used in the present study (500 μM) is much higher than the one used in malpighian tubule of *D. hydei* (10–100 μM), and is in fact above the limit of specificity [15, 17]. A lower concentration (100 μM) did not have any effect on the measured parameters, but no concentrations in between have been tested. Like MIA the effect of this high concentration of NEM is rather drastic and irreversible. This could mean that these substances are toxic to the cells. It should be noted however that in the absence of barium the basolateral membrane potential does not change and remains stable for several minutes after adding the drugs. Therefore it is concluded that the cell metabolism is not completely blocked as expected in toxic situations. In the fluid secretion experiments shown by Bertram [15] no washout period followed the experimental period. It is therefore not possible to know whether the effects of NEM and Baf-A1 were reversible in their preparation. The sensitivity of the fluid secretion to nitrate is in agreement with the presence of a V type proton pump: in previous experiments all chloride was substituted by nitrate which resulted in a complete stop of the fluid secretion [2]. It is known that nitrate is toxic to the V type ATPase [11, 17].

Conclusions

We conclude that the barium-induced hyperpolarization is due to an active and electrogenic transport mechanism in the apical membrane. The sensitivity of this pump to Baf-A1 strongly suggests that we are dealing with a proton pump of the V type ATPase. To explain the net movement of K^+ the presence of a K^+/H^+ exchanger is considered and will be investigated by intracellular and intraluminal pH measurements [29].

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Errata

p. 53 (legend Table 1) :

pH was adjusted to 7.20, osmolality of the solution was **375 mosm/kgH_2O.**

p. 104 (last line but one) :

$R_{ap}{}^t$ is the only unknown value in eq. **6**

p. 105 (legend of Fig. 1) :

The effect of 6 mM barium (n = 7), **2.10^{-4} M** MIA (n = 8), **5.10^{-4} M** NEM (n = 6) and 5.10^{-6} M Baf - A1 (n = 6) on the fluid secretion rate of isolated malpighian tubules.

PAPER 3

MEASUREMENT OF INTRACELLULAR AND LUMINAL K^+ CONCENTRATIONS IN A MALPIGHIAN TUBULE (*FORMICA*). ESTIMATE OF BASAL AND LUMINAL ELECTROCHEMICAL K^+ GRADIENTS.

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MEASUREMENT OF INTRACELLULAR AND LUMINAL K^+ CONCENTRATIONS IN A MALPIGHIAN TUBULE (*FORMICA*). ESTIMATE OF BASAL AND LUMINAL ELECTROCHEMICAL K^+ GRADIENTS.

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Key words : Malpighian tubules, K^+ transport, ion-selective microelectrodes, transmembrane gradients

Running title : cell and luminal K^+ in Malpighian tubule

ABSTRACT

Cellular and luminal K^+ concentrations were measured in different bath K^+ concentrations using double-barrelled K^+-selective electrodes. The electrochemical gradient for K^+ across the basal and the apical cell membrane was estimated. The experiments were performed in two Cl^- concentrations. In control Ringer (41 mM K^+ and 57 mM Cl^-) cell K^+ was 82 mM and luminal K^+ was 119 mM. On lowering bath K^+ to 4 mM or increasing it to 90 mM cell K^+ and to a lesser extent luminal K^+ followed: cell K^+ changed to 29 and 90 mM and luminal K^+ to 106 and 132 mM, respectively. In a 143 mM Cl^- Ringer solution cellular and luminal K^+ concentration showed a similar change with the basal K^+ concentration. The basal membrane potential difference was almost equal to the equilibrium potential for K^+ in all bath K^+ and Cl^- concentrations tested and the estimated electrochemical gradient for K^+ across the basal membrane was very small (inward, zero, or even outward in low bath K^+). It tended to become more inward as the bath K^+ increased. The methods for estimating such a small gradient and the idea of passive K^+ uptake through K^+ channels across this barrier was critically evaluated. The large transepithelial electrochemical gradient against which K^+ was transported was primarily situated in the apical membrane. This gradient decreased as the bath K^+ was elevated (and fluid secretion accelerated): an increase in the bath K^+ reduced both the electrical and concentration step to be overcome for K^+ secretion across the apical membrane. A double role for the bath K^+ in the regulation of K^+ secretion is proposed.

INTRODUCTION

Malpighian tubules of forest ants (*Formica polyctena*) secrete fluid and concentrate K^+ and Cl^- in the lumen. Total luminal K^+ and Cl^- concentrations were obtained from X-ray analysis of secreted droplets. It was found that K^+ was always transported across the epithelium against its electrochemical gradient (Van Kerkhove *et al.*, 1989).

In this study, we focus on electrochemical K^+ gradients across the cell membranes. Experiments were performed with double-barrelled K^+-selective electrodes in order to obtain the luminal and intracellular K^+ concentrations together with the electrical gradients across the apical and basal membrane.

The high permeability for K^+ of the basal membrane in Malpighian tubules of *Formica* suggests the possibility of passive K^+ entry via conductive K^+ channels (see also Leyssens *et al.*, 1992). In this study, the existence of a favourable electrochemical gradient for K^+ entry across the basal membrane is discussed.

The K^+ extrusion from the cell to the lumen was found to be active. It is assumed to be established by a K^+/H^+ exchanger. To realize the necessary cell-inward H^+ concentration gradient, the presence of an electrogenic H^+ pump, similar to the H^+ ATPase described in *Manduca sexta* midgut cells (Wieczorek *et al.*, 1989), was suggested in Malpighian tubules of *Formica* by Weltens *et al.* (1992) and in a preliminary study an apical cell-inward H^+ concentration gradient was reported by Zhang *et al.* (1992). Furthermore, the fluid secretion rate is strongly dependent on the bath K^+ concentration (Van Kerkhove *et al.*, 1989). In this study we demonstrate that the basal K^+ concentration determines the intracellular K^+ concentration, as suggested in a previous study (Leyssens *et al.*, 1992) when transient changes of the basal membrane potential on varying the basal K^+ concentration were first observed. This change in intracellular K^+ moduling the apical K^+ concentration gradient may be an important factor in the intrinsic regulation of apical K^+ transport by K^+.

Part of these results have been published as an abstract (Leyssens *et al.*, 1991).

MATERIALS AND METHODS

1.Preparation and experimental set-up.

Worker ants of the species *Formica polyctena* were collected from natural nests at the periphery of woods. They were kept in artificial nests at a constant temperature (20 °C) and fed with sugar and water.

The procedure followed for the dissection of one Malpighian tubule has already been described by Van Kerkhove *et al.* (1989). Briefly, after decapitation, the abdomen of the ant was opened and part of the gastrointestinal tract with the Malpighian tubules attached was removed. One Malpighian tubule was cut as close to the gut as possible and transferred to the experimental set-up on the stage of an inverted microscope (Nikon, Diaphot).

The experimental set-up for the ion-sensitive measurements was as follows. Shortly, an isolated spontaneously secreting Malpighian tubule with a length of about 1 mm was immobilized by two holding pipettes in a small droplet of Ringer solution (50 µl) on the bottom of a petri-dish. The droplet was covered with paraffin oil to avoid evaporation. Using high resistance ion-selective electrodes (50-100 GΩ), it was technically not possible to measure potential differences while secretory fluid was allowed to leave the tubule in the paraffin oil due to the noise induced. The bathing droplet was perfused continuously at a rate of 150 µl/min. A cell or the lumen was impaled with a double-barrelled electrode. The K^+-selective and reference barrel were connected to the channels of a high impedance electrometer (Duo 773, WPI) via Ag/AgCl half-cells and potentials were recorded on a Linseis pen recorder. A low resistance 3 M KCl electrode was placed in the bathing droplet and connected to earth. Cells were punctured with a double-barrelled K^+-selective electrode in order to determine the basal membrane potential difference (V_{bl}) and the intracellular K^+ activity (Kc). Measurements of the transepithelial potential difference (V_{te}) and the luminal K^+ activity (K_l) were performed by advancing the electrode through the cell layer into the lumen of the tubule; V_{bl} and V_{te} were expressed with reference to the bath side. The apical potential difference (V_{ap}) was expressed with reference to the lumen and the absolute value was calculated as the difference between the transepithelial and basal membrane potential [$V_{ap} = -(V_{te} - V_{bl})$]. V_{te} and V_{bl} were measured in experiments where intracellular and luminal recordings were made consecutively in the same tubule (termed "single tubule V_{bl} and V_{ap} measurements"). Experiments were paired if bath solutions were changed during continuous measurement of one parameter. Impalements were accepted if 1) a sudden voltage deflection of the reference barrel was recorded, 2) the potential of the reference and ion-sensitive barrel remained stable for at least a few minutes in a certain bath solution and if 3) the reference and ion-selective barrel potential differed 3 mV at the most from the baseline on withdrawal. Furthermore, on calibrating the ion-selective barrel before and after the experiment, the readings had to coincide within a range of 3 mV.

2. Construction and calibration of double barrelled K^+-selective electrodes.

The double barrelled microelectrodes were drawn on a horizontal puller with an air jet cooling system (Model-77, Sutter Instrument U.S.A.). Paired filament containing glass capillaries with unequal diameter were used (OD_1=1.5 mm, ID_1=0.87 mm; OD_2=0.75 mm, ID_2=0.35 mm; Hilgenberg, Malsfeld, Germany). A tip diameter of approximately 1 µm was

obtained. The inside of the larger barrel was silanized (hexamethyldisilazane; Janssen Chimica), the smaller barrel remained hydrophylic. A short column of the K^+-selective liquid ion exchanger, Corning 477317, was introduced into the tip of the larger barrel, i.e. the K^+-selective barrel, and the rest of this barrel was backfilled with 1 M KCl. The other barrel, i.e. the reference barrel was filled with either 1 M Na acetate + 10 mM KCl or with 100 mM KCl. The filling solution of the reference barrel may interfere with the accuracy of the estimate of the membrane potential difference by inducing a change in liquid junction potential (LJP) and/or a tip potential (V_{tip}) on impaling a cell (see Vaughan Jones and Aickin, 1987). Table 1 summarizes the effects of an increase in K^+ (replacing Na^+) or a decrease in Cl^- on LJP and V_{tip} of conventional microelectrodes filled with either KCl (3M or 0.1 M) or Na acetate (3M +10 mM KCl). Electrodes filled with 0.1 M KCl were the least sensitive to changes in ion composition around the tip of the electrode. Unfortunately, the use of this filling solution for the reference barrel in double barrelled electrodes was limited: the very high resistances (up to 1 GΩ) often resulted in unstable recordings that had to be discarded. As a higher KCl concentration in the reference barrel might cause leakage of KCl (Isenberg, 1979; Berridge and Schlue, 1978) and interfere with the K^+ measurements, most experiments shown were performed using 1 M Na acetate + 10 mM KCl. This can of course give rise to systematic errors when impaling a cell. Although the properties of the reference barrel were not identical to those of the convential microelectrodes (higher resistances, 1 instead of 3 M Na acetate + 10 mM KCl as filling solution), the overall effect of a decrease of Cl^- concentration combined with an increase in K^+ concentration and concomitant decrease in Na^+ concentration, simulating the expected changes in ionic concentrations on entering the cell, would be a more negative value for the LJP and V_{tip} (see Table 1). In the lumen on the other hand, both K^+ and Cl^- concentrations are expected to be high: in Malpighian tubules of *Locusta* for instance the luminal K^+ and Cl^- concentration was about 140 mM and 200 mM, respectively, the luminal Na^+ concentration was 20 mM (Morgan and Mordue, 1983). In the ant the chemical KCl concentration was around 200 mM, and the Na concentration was low (Van Kerkhove *et al.*, 1989). So the impact on the reference barrel is expected to be less important, K^+ and Cl^- effects cancelling each other out.

In order to improve the responsiveness of the electrode to K^+ and to reduce the resistance, the filled double-barrelled electrode was beveled in a rotating polishing alumina solution (No.3 AB Gama Polishing Alumina, Buehler, U.S.A.). Thereby, a compromise had to be made between the sharpness of the tip (to allow stable cell impalement), and the improvement of the K^+ sensitivity (the slope of the calibration curve is much closer to the expected Nernstian slope when the electrode tip was broken for instance; see also Tripathi *et al.*, 1985) and the reduction of the resistance of the electrode. After beveling, the calibration curves had slopes of approximately 50 mV, and the ion-sensitive and the reference barrel had tip resistances ranging from 50 to 100 GΩ and from 0.1 to 1 GΩ, respectively.

Table 1. Effects of changes in the bath Cl^-, K^+, and Na^+ concentration on liquid junction and tip potentials of microelectrodes filled with different solutions.

bath solution		from 143 to 57 mM Cl_{bl} [a]		from 4 to 90 mM K_{bl} [b]	
filling solution	n	Δ LJP(mV)	Δ V_{tip} (mV)	Δ LJP(mV)	Δ V_{tip}(mV)
3 M Na acetate + 10 mM KCl	7	- 4,2 ± 1,4* (P < 0.05)	- 2,5 ± 1,1* (P < 0.05)	- 2,0 ± 1,0* (P < 0.05)	- 0.2 ± 0.3
3 M KCl	6	- 1,6 ± 0,8	- 3,3 ± 0,6* (p < 0.01)	0.0 ± 0,4	0.2 ± 0.3
100 mM KCl	10	0.8 ± 0.7	0.1 ± 0.2	0.2 ± 0.1* (P < 0.001)	0.2 ± 0.4

The effect of a change in bath ion concentrations was tested by using different Ringer solutions (see Table 2).
The change in liquid junction potential (Δ LJP) is equal to the electrode response, after breaking the tip; the tip potential (V_{tip}) is the electrode response with the tip intact minus the response, in the same solution, after the tip was broken.
Changes in LJP and V_{tip} significantly different from zero are indicated (*).
[a] Relative K^+ concentration in 143 and 57 mM Cl_{bl} was 51 and 41 mM (see also Materials and Methods, Section 3)
[b] Cl^- concentration was 57 mM, K^+ was replacing Na^+.

The recorded K^+-sensitive signal (i.e. the potential difference signal between the ion-sensitive and the reference barrel or E_{ISE}-E_{ref}) was calibrated in a bathing medium, containing either 57 mM or 143 mM Cl^- Ringer solution (see "Methods: section 3, Solutions"), in which the chemical K^+ concentration varied from 5 to 113 mM, using Na^+ as a substitute. The ion-sensitive electrodes were accepted for use when a stable potential signal was obtained for both the ion-sensitive and the reference barrel, when the K^+ sensitivity of the reference barrel was less than 1 mV/decade and when the calibration curve could be fitted by a straight line with a slope of at least 48 mV/decade. The electrodes were calibrated in terms of concentration: the measured intracellular or luminal "K^+ signal" was always expressed as the chemical K^+ concentration that would be needed in a 143 mM Cl^- Ringer solution to result in the same reading of the K^+-selective barrel. This method assumes that activity coefficients are the same in cellular fluids, luminal fluids and the calibration solutions (see also pp.89-91 in Thomas, 1978).

3. Solutions.

The chemical composition of the bathing solutions is summarized in Table 2. In the 57 mM Cl^- Ringer solutions, the anion gap is filled with the organic acid $citrate^{3-}$. Since high concentrations of $citrate^{3+}$ chelate Ca^{2+}, in the latter solution a reduction of the Ca^{2+} activity can be expected (Pitman, 1979; Kenyon and Gibbons, 1977). However, in control experiments Ca^{2+} omission of Ca^{2+} from the bath solution did not affect fluid secretion or electrical potential differences (De Decker, personal communication). In the $citrate^{3-}$ containing solution the ionic strength is different and as a result the activity coefficient for K^+ is lower than that measured in a 143 mM Cl^- solution with the same chemical K^+ concentration. A single barrelled K^+-selective electrode, calibrated in the 143 mM Cl^- Ringer solutions, was used to measure the K^+ signal in the 57 mM Cl^- Ringer solutions and showed a drop to 80% of this signal for the same chemical K^+ concentration (see also Leyssens *et al.*, 1992). These relative K^+ concentration values were used to calculate cellular and luminal electrochemical K^+ gradients in the 57 mM Cl^- Ringers. It must be noted that the slope of the calibration curve was similar whether the calibration was done in 57 or in 143 mM Cl^-. So the $citrate^{3-}$ containing solution didn't interfere with the selectivity of the K^+ resin, it only reduced the activity coefficient of the solution.

Table 2. Composition of the experimental solutions.

		A high K^+ 57 Cl	B high K^+ 143 Cl	C K^+ free 57 Cl	D K^+ free 143 Cl
NaCl	mM	-	-	27	113
KCl	mM	27	113	-	-
$CaCl_2$	mM	2	2	2	2
$MgCl_2$	mM	13	13	13	13
Na_3 citrate	mM	-	-	28.7	-
K_3 citrate	mM	28.7	-	-	-
Alanine	mM	2.8	2.8	2.8	2.8
Trehalose	g/l	4	4	4	4
Maltose	g/l	4	4	4	4
Glucose	g/l	27.7	15	27.7	15
Hepes	mM	12.1	12.1	12.1	12.1

pH was adjusted to 7.20 by adding approximately 3.5 mM NaOH (solutions C and D) or KOH (solutions A and B). Osmolality (Mean ± SE) of all solutions was kept at 375 mosm/kg H_2O by adjusting the glucose concentration. Different K^+ concentrations were obtained by mixing solutions A and C or B and D.

The replacement of Cl^- by $citrate^{3-}$ will also result in a reduction of the Cl^- activity: we expect that the Cl^- signal in this solution will also drop to about 80% (i.e. 46 instead of 57 mM) of the signal that would be obtained in a solution with the same chemical Cl^- concentration but an ionic strength comparable to that of the 143 mM Cl^- Ringer. Since it was not measured

directly with a Cl^--sensitive electrode, the Cl^- activity is simply expressed as a chemical concentration.

Solutions were freshly prepared each week, filtered through 0.22 µm Millipore filters and stored at 2 °C until use. The pH was adjusted with NaOH or with KOH (in the 113 mM K^+, Na^+-free solutions) to a value of 7.2.

4.Statistics.

Results are presented as mean values ± SE, n=number of cells (intracellular measurements) or n=number of tubules (luminal measurements). Results were statistically analyzed (regression analysis, two-tailed Student's t-test) using Statview II (Abacus Concepts, Inc., Berkeley, C.A., 1987). Unless explicitely stated, the Student's t-test was performed in a paired way.

RESULTS

1. The basal membrane potential difference and the intracellular K^+ concentration in the presence of different bath K^+ concentrations.

The effect of a change in the basal K^+ concentration (K_{bl}) was tested in a high (143mM) and in a low (57mM) Cl^- bathing solution.

Fig. 1 shows two examples of recordings in the 57 mM Cl^- bathing solution. The upper trace shows V_{bl} as measured with the reference barrel. The lower trace gives the potential difference between the ion-sensitive and the reference barrel, translated into K^+ concentrations. The sensitivity of the basal membrane potential difference (V_{bl}) and the intracellular K^+ concentration (K_c) to changes in K_{bl} is demonstrated.

After puncturing the cell in 41 mM K_{bl} (Fig. 1A), a V_{bl} of -18 mV was recorded. When K_{bl} was lowered to 4 mM, V_{bl} hyperpolarized and K_c gradually decreased. An increase in K_{bl} to 90 mM resulted in a depolarization of V_{bl} and a gradual rise in K_c. Small overshoots of V_{bl} were observed on changing K_{bl}. In some cells the response of K_c (closely followed by V_{bl}) to a change in K_{bl} was even much more pronounced as can be seen in Fig. 1B.

The difference in response between cells with a large or a small change in K_c (and concomitantly V_{bl}) was not marked enough however to distinguish two cell types: in all cells tested K_c changed with K_{bl} to some extent and the shifts of K_c ranged from small to more pronounced: a decrease of K_{bl} from 41 to 4 mM for instance could result in a drop of K_c varying from 30 % to 75 %. Similar observations were made in 143 mM Cl_{bl} for values of K_{bl} ranging from 5 to 113 mM. Also the effect of a change in K_{bl} on V_{bl} and K_c was fully reversible.

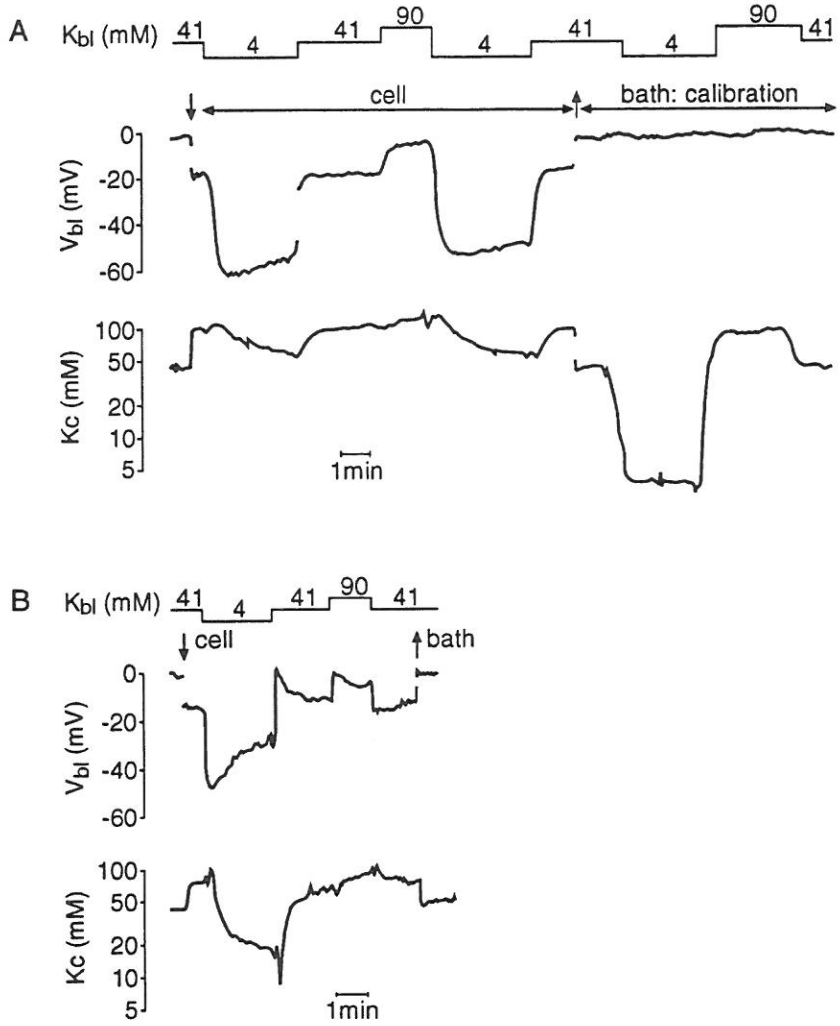

Fig. 1. Effect of different K^+ concentrations of the bath solution (K_{bl}) on the basal membrane potential (V_{bl}) and the intracellular K^+ concentration (K_c) of a spontaneously secreting tubule in a 57 mM Cl_{bl} Ringer solution. Arrows indicate impalement or withdrawal of the electrode. Transient changes in V_{bl} on changing K_{bl} are less (A) or more (B) pronounced. At the end of the experiment, the sensitivity of the double-barrelled K^+-selective electrode to changes in K_{bl} is shown (A).

The results of all V_{bl} and K_c measurements are summarized in Table 3. The steady state values are given. Both in 57 and 143 mM Cl_{bl}, the sensitivity of V_{bl} to changes in K_{bl} is evident. A shift of K_c towards higher concentration levels when K_{bl} increases is also obvious. The increase of K_c with K_{bl} was confirmed in two subsets of the experiments that had been performed in a paired way: in the presence of 57 mM Cl_{bl}, K_c increased

significantly ($P<0.01$) from 28±2 mM to 76±3 mM (n=32) and from 77±3 mM to 90±2 mM (n=26) for a change in K_{bl} from 4 to 41 mM and from 41 to 90 mM, respectively.
The results of the present study confirm the hypothesis suggested in a previous paper that K_c seems to change with K_{bl} (Leyssens *et al.*, 1992). It was based on the observation of transient changes in V_{bl} occurring on changing K_{bl}.
The observations were further analysed. If the steady state value of V_{bl} was plotted as a function of the logarithm of K_{bl}, it was found that the values could be fitted by a straight line with a slope of 29 mV/decade ($r=0.928$, $P<0.001$) in 57 mM Cl_{bl} and 33 mV/decade ($r=0.868$, $P<0.001$) in 143 mM Cl_{bl}. Apparently the basal membrane does not behave as a perfect K^+ electrode. Compared to a previous study (Leyssens *et al.*, 1992) and due to variability among the tubules, the slope deviated even further from the ideal Nerstian slope. A change of 58 mV per decade is expected if the membrane is primarily permeable to K^+ and if K_c remains constant in different K_{bl}.

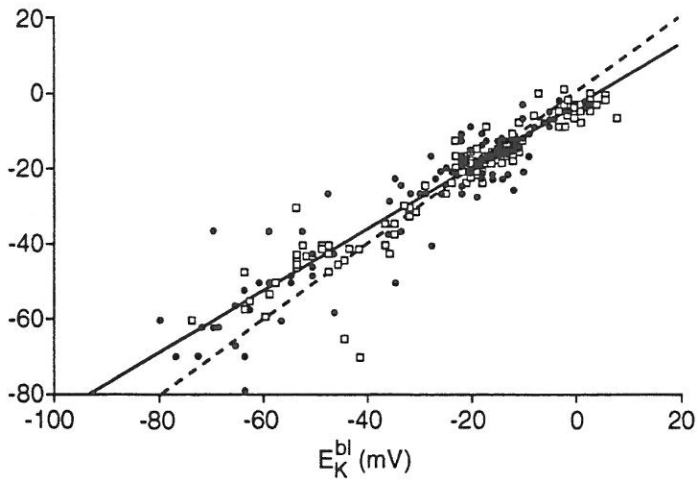

Fig. 2. V_{bl} as a function of the calculated equilibrium potential for K^+ (E_K^{bl}). V_{bl} and E_K^{bl} values obtained from all K_{bl} and Cl_{bl} conditions were pooled. Different symbols are used for the values, obtained in 57 mM (•) and 143 mM (▫) Cl_{bl}. The slope has a value of 0.822 ($r=0.941$, $P<0.001$, $n=242$). The dotted line is the line of identity.

This is clearly not the case (see Table 3). In order to investigate the K^+ sensitivity of the basal membrane potential difference further, V_{bl} was plotted as a function of the calculated equilibrium potential for K^+ (E_K^{bl}). Plotting V_{bl} against E_K^{bl} in 57 and 143 mM Cl_{bl} separately resulted in curves which were not distinguishable (slopes were 0.815, $r=0.95$, $P<0.001$, $n=135$; and 0.833, $r=0.93$, $P<0.001$, $n=107$, in 57 and 143 mM Cl^- respectively). Therefore V_{bl} and E_K^{bl} values obtained from all K_{bl} and Cl_{bl} conditions tested were pooled (Fig. 2).

Table 3. Basal membrane potential (V_{bl}) and intracellular K^+ concentration (K_c) as measured with a double-barrelled K^+- selective electrode. Mean value ± SE, (range).

Cl_{bl} (mM)	57			143		
K_{bl} (mM)	4	41	90	5	51	113
V_{bl} (mV)	- 44 ± 2 (-70, -29)	- 16 ± 1 * (-26, -7)	- 3 ± 1 *° (-8,+ 2)	- 48 ± 3 (-79,-24)	- 16 ± 1 § (-27,- 6)	- 3 ± 1 §α (- 6,– 1)
K_c (mM)	29 ± 2 (13, 75)	82 ± 2 * (59, 128)	90 ± 2 *° (67, 121)	51 ± 5 (12, 121)	103 ± 4 § (59, 200)	123 ± 8 §α (99, 153)
V_{bl}-E_K^{bl} (mV) a	+ 4 ± 1 ∞	+ 1 ± 0,5	- 4 ± 1 ∞	+ 5 ± 2 ∞	+ 1 ± 1	- 1 ± 1
n b	39	70	26	35	63	9

a : Basal electrochemical gradient for K^+ with $E_K^{bl} = 58 \log \frac{K_{bl}}{K_c}$, b : n = number of cells

* : significantly different from 4 mM K_{bl} ($P<0.01$), ° : significantly different from 41 mM K_{bl} ($P<0.01$), § : significantly different from 5 mM K_{bl} ($P<0.01$), α : significantly different from 51 mM K_{bl} ($P<0.05$) (unpaired student's t-test); ∞ : significantly different from zero ($P<0.01$)

A straight line could be fitted with a slope of 0.822 ($r=0.941$, $P<0.001$, $n=242$). The line of identity, representing perfect equilibrium between the electrical and the chemical gradient, is drawn as well. It is very close to the experimental line. Whether a small basal electrochemical K^+gradient is present will be discussed more extensively in section 3 of the "Results" for the different K_{bl} tested.

2. The transepithelial potential difference and the luminal K^+ concentration in the presence of different bath K^+ concentrations.

The effect of K_{bl} on the transepithelial potential difference (V_{te}) and the luminal K^+ concentration (K_l) was tested in the same bathing solutions as those used for the intracellular measurements.

Fig. 3 shows recordings of luminal measurements in 57 mM K_{bl}. The sensitivity of V_{te} to changes in K_{bl} is obvious. V_{te} hyperpolarizes (lumen more positive) when K_{bl} is increased or depolarizes when the bath K^+ concentration is lowered.

In contrast to the results obtained by X-ray analysis of secreted droplets (Van Kerkhove *et al.*, 1989), K_l did not always maintain a constant value in different K_{bl}. Fig. 3 shows an example of the response of K_l on a change in K_{bl}. An increase in K_{bl} to 90 mM did not cause an appreciable rise of K_l, but K_l dropped to a lower value in 4 mM K_{bl} (Fig. 3). The effect was reversible. The change in K_l was variable in this series of experiments , but in general K_l always responded to some extent to a change in K_{bl}. In 143 mM Cl_{bl}, the effect of K_{bl} on K_l was comparable.

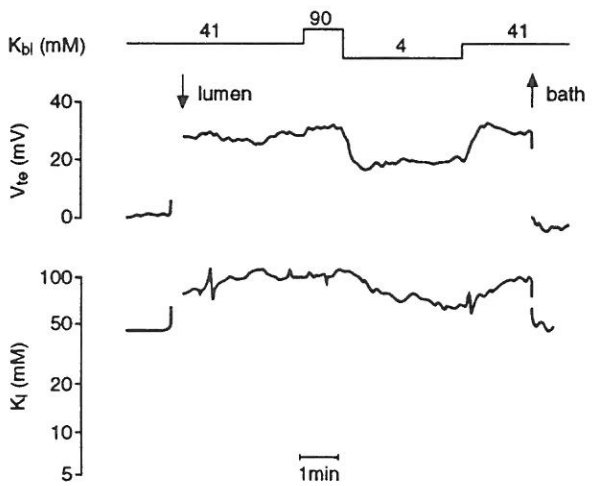

Fig. 3. Effect of a change in K_{bl} on the transepithelial potential (V_{te}) and the luminal K^+ concentration (K_l) of a spontaneously secreting tubule in a 57 mM Cl^- Ringer solution. Arrows indicate luminal impalement or withdrawal of the electrode.

The results of all luminal measurements are summarized in Table 4. There is a tendency of V_{te} to shift towards more positive potentials when K_{bl} increases. A tendency of K_l to shift towards higher concentration levels in response to an increase of K_{bl} can also be observed. This was confirmed in two subsets of the experiments that had been performed in a paired way: in 57 mM Cl_{bl}, V_{te} increased significantly from 18±2 mV to 33±2 mV ($P<0.001$) and K_l from 107±8 mM to 119 ±7 mM ($P<0.02$), when K_{bl} increased from 4 to 41 mM (n=25). For an increase of K_{bl} from 41 to 90 mM (n=20), the increase of V_{te} from 32±2 mV to 35±2 mV ($P<0.001$) was accompanied by an increase of K_l from 127±8 mM to 132±8 mM ($P<0.05$).

From Table 4, it is also clear that K^+ is transported actively from the bath towards the lumen against a large electrochemical gradient (V_{te} - E_K^{te}). In a previous paper, Van Kerkhove *et al.* (1989) came to the same conclusion using the chemical K^+ concentration of the secreted droplets obtained from X-ray analysis. Furthermore, V_{te} - E_K^{te} decreases as K_{bl} increases. For a given K_{bl}, no significant correlation was found between K_l and V_{te}. Thus the paracellular pathway does not seem to play any role in determining the transepithelial K^+ gradient. This is to be expected, while even a small K^+ conductance in the shunt would result in an appreciable backleak of K^+ along the established electrochemical gradient.

Table 4. Transepithelial potential difference (V_{te}) and luminal K^+ concentration (K_l) as measured with a double - barrelled K^+-selective electrode. Mean value ± SE, (range).

Cl_{bl} (mM)	57			143		
K_{bl} (mM)	4	41	90	5	51	113
V_{te} (mV)	18 ± 2 (4, 36)	31 ± 2 * (10, 54)	35 ± 3 * (12, 62)	19 ± 2 (6, 39)	30 ± 3 § (13, 58)	26 ± 6 (13, 51)
K_l (mM)	106 ± 7 (36, 208)	119 ± 6 (65, 208)	132 ± 8 * (88, 208)	92 ± 9 (22, 180)	128 ± 7 § (54, 190)	134 ± 7 § (102, 151)
V_{te}-E_K^{te} (mV) [a]	+99 ± 3	+57 ± 3 *	+44 ± 4 *°	+89 ± 4	+51 ± 3 §	+30 ± 6 §α
n [b]	30	30	20	18	30	6

a : Transepithelial electrochemical gradient for K^+ with $E_K^{te} = 58 \log \frac{K_{bl}}{K_l}$, b : n = number of tubules; * : significantly different from 4 mM K_{bl} ($P< 0.05$), § : significantly different from 5 mM K_{bl} ($P< 0.05$); ° : significantly different from 41 mM K_{bl} ($P< 0.01$), α : significantly different from 51 mM K_{bl} ($P< 0.01$) (unpaired student's t-test).

3. The basal electrochemical K^+ gradient.

From resistance measurements in perfused tubules, we know that the basal membrane conductance is of the order of 0.1 S/cm^2 (Weltens *et al.*, 1992). This low membrane conductance is mostly due to K^+ and a small basal inward electrochemical gradient could account for a net uptake of K^+ sufficient to sustain secretion (see "Discussion"). However, the assessment of a small inward electrochemical gradient is extremely difficult. Therefore two different approaches were used: the basal K^+ gradient was calculated as the difference between V_{bl} and $E_K{}^{bl}$ and it was estimated directly from the ion-selective barrel on impalement (E_{ISE}).

In Table 3, the calculated electrochemical K^+ gradient (V_{bl}-$E_K{}^{bl}$) is given for all intracellular measurements, i.e. including those cells for which a direct measure from the ion-selective barrel could not be made. In the low K^+ concentrations a significant outwardly directed K^+ gradient was found, in the other solutions the mean gradient was not significantly different from zero or inward (in 90 mM K_{bl} 57 mM Cl_{bl}). In all solutions tested, except in 4 mM K_{bl} 57 mM Cl_{bl} (where the K^+ gradient was zero or outward) when looking at the individual experiments the three possibilities were present, a fraction of the cells had no gradient, a fraction had an inwardly directed gradient, other cells had an outward K^+ gradient. The different types of cells were often found in one and the same tubule. There was a tendency however for the fraction of cells with no or an inward gradient to increase with K_{bl} (Fig. 4).

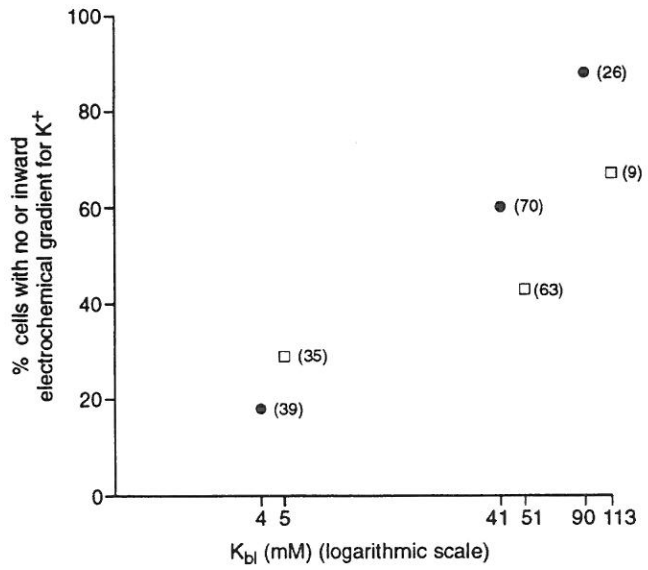

Fig. 4. Number of cells (in %) with no or an inward electrochemical (V_{bl}-$E_K{}^{bl}$) in different K_{bl}; (•) in 57 mM Cl_{bl}, (⊏) in 143 mM Cl_{bl} ; (n)=total number of cells tested in each condition.

In 57 mM Cl_{bl}, the effect of K_{bl} on V_{bl}-E_K^{bl} was also statistically tested in a subset of experiments where the effect of a change in K_{bl} was tested in a paired way (see Fig. 5): V_{bl}-E_K^{bl} changed from outward to zero and from zero to inward, when K_{bl} increased from 4 to 41 mM and from 41 to 90 mM, respectively. This tendency (to a more inwardly directed gradient in high K_{bl}) explains the slight deviation from unity of the slope of the experimental curve in Fig. 2.

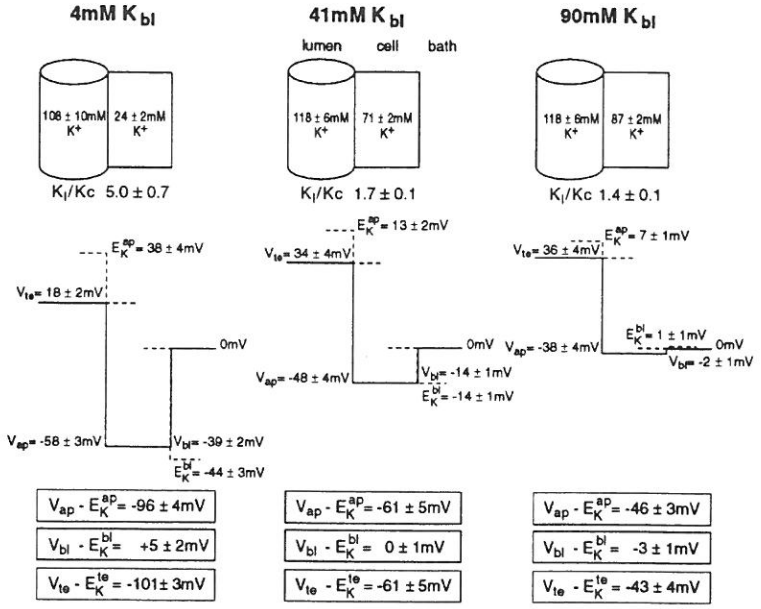

Fig. 5. Summary of intracellular and luminal K^+-selective measurements in 57 mM K_{bl} (n=9). Mean values ± SE are given. "single tubule V_{bl} and V_{ap} measurements": cellular and luminal measurements were made, consecutively, in one and the same tubule. Furthermore, in each tubule, the three different K_{bl} were tested. The apical membrane potential difference (V_{ap}) was expressed with reference to the lumen. All the calculated electrochemical gradients (except V_{bl}-E_K^{bl} in 41 mM K_{bl} were significantly different ($P<0.05$) from zero. All the electrophysiological parameters in 41 mM K_{bl} were significantly different from 4 mM K_{bl}, and all the parameters in 90 mM K_{bl} (except K_l, not significantly different from 41 mM K_{bl}) were different from 4 and 41 mM K_{bl}, at the 5 % level at least.

Table 5 summarizes the results for a subset of cells that had been impaled in the respective bath solutions and for which both methods could be compared in one and the same cell. The values slightly differ from Table 3 as they concern a smaller number of measurements. In this series of experiments the mean value for the calculated K^+ gradient was not significantly different from zero or outward (Table 5, row A). Important was however that in this subset of experiments the calculated gradient could be compared with the electrochemical K^+ gradient estimated directly from the potential change registered by the ion-sensitive barrel

(E_{ISE}) on impaling the cell (Table 5, row B). The gradient found by the ion-sensitive barrel was always significantly more negative, i.e. less outward (or more inward), than the calculated (V_{bl}-E_K^{bl}) gradient.

Table 5. Basal electrochemical gradient for K^+ for a series of cells impaled with a double-barrelled electrodein different solutions. Mean value ± SE.

Cl_{bl} (mM)	57		143		
K_{bl} (mM)	4	41	5	37	51
A. V_{bl}-E_K^{bl} (mV)	+ 8 ± 1 $^{\infty}$	+ 4 ± 1	+ 2 ± 1	+ 1 ± 1	+ 2 ± 1 $^{\infty}$
B. E_{ISE} (mV)	+ 1 ± 1 $^{\bullet}$	- 1 ± 0.3 $^{\bullet\infty}$	- 2 ± 0.5 $^{\bullet\infty}$	- 2 ± 1 $^{\bullet}$	- 3 ± 0.4 $^{\bullet\infty}$
n	10	45	8	9	35

A : calculated basal electrochemical gradient for K^+ with $E_{Kbl} = 58 \log (K_{bl}/K_c)$,
B : basal electrochemical gradient read from the ion-sensitive barrel on impalement
• : significantly different from A (paired student's t-test) ($P<0.05$)
∞ : value significantly different from zero ($P<0.05$)

4. <u>The apical electrochemical K^+ gradient.</u>

From the results in Table 3 and 4 it can already be inferred that V_{ap} [= - (V_{te} - V_{bl})] will depolarize when K_{bl} is increased (see also Leyssens *et al.*, 1992). Another important observation was that the ratio K_l/K_c seemed to be smaller in a higher K_{bl}. This was confirmed in "single tubule V_{ap} and V_{bl} measurements": after an intracellular recording the double-barrelled K^+-selective electrode was advanced through the cell layer into the lumen of the tubule. V_{ap} is expressed with reference to the lumen. The results are summarized in Table 6. In 9 experiments in 57 mM Cl_{bl}, the 3 different K_{bl} were tested on one and the same tubule, measuring K_c and K_l (see Fig. 5). It is clear that both V_{ap} and K_l/K_c decrease significantly when K_{bl} is raised. Consequently, the higher K_{bl}, the lower the gradient (V_{ap} - E_K^{ap}) against which K^+ has to be extruded into the lumen. This should correlate with a faster K^+ secretion. Fluid secretion can be used as a measure for net K^+ transfer across the epithelium (Van Kerkhove *et al.*,1989). When plotting the relative fluid secretion rate in

57 mM Cl_{bl} (original values, n=24 for each K_{bl}, Zhang, unpublished results) against the averaged values of V_{ap}-E_K^{ap} (from Table 6),as expected a highly significant inverse relationship was found with a slope of 1.406 pl/min.mV (r=0.503, P<0.001) (Fig. 6).

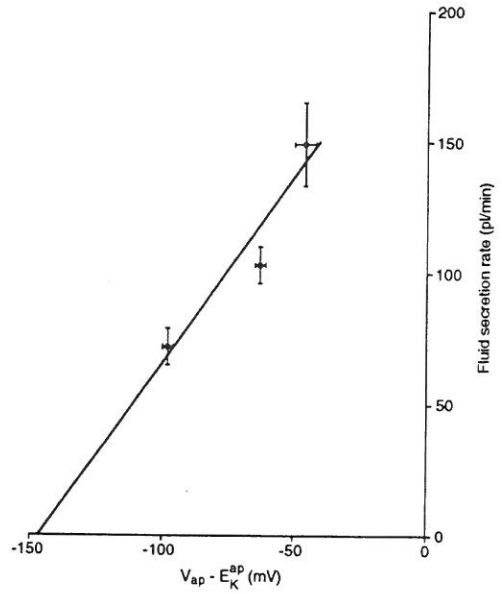

Fig. 6. Fluid secretion rate (original values, n=24 for each K_{bl}, Zhang, unpublished results), plotted against the mean values of V_{ap}-E_K^{ap} (see Table 6) in 57 mM Cl_{bl}. V_{ap} and E_K^{ap} were derived from "single tubule V_{bl} and V_{ap} measurements".

The point of intersection with the abscissa, which gives the value of the electrochemical gradient when transport stops, was -146 mV.

Plotting the relative fluid secretion rate (see above) against the averaged V_{ap}-E_K^{ap} values of the paired experiments (results from Fig. 5) also resulted in a straight line with a similar slope (1.439 pl/min.mV, r=0.498, P<0.001) and point of intersection with the abscissa (-143 mV).

Table 6. "Paired" intracellular and luminal measurements of K^+ concentration and potential differences. Mean value ± SE.

Cl_{bl} (mM)	57			143	
K_{bl} (mM)	4	41	90	5	51
V_{ap} (mV)	- 63 ± 3	- 53 ± 3	- 38 ± 4	- 65 ± 7	- 43 ± 4
K_l (mM)	107 ± 10	115 ± 6	118 ± 6	107 ± 7	134 ± 7
K_c (mM)	28 ± 4	78 ± 2	87 ± 2	71 ± 11	102 ± 6
ratio K_l/K_c	4,41 ± 0,47	1,51 ± 0,08	1,35 ± 0,07	1,63 ± 0,24	1,35 ± 0,08
$Vap-E_K^{ap}$ (mV) [a]	-98 ± 3	-63 ± 2	-46 ± 4	- 76 ± 9	-50 ± 4
n [b]	15	25	9	4	20

a : Apical electrochemical gradient for K^+ with $E_K^{ap} = 58 \log \frac{K_c}{K_l}$, b : n = number of tubules

DISCUSSION

1. The intracellular K^+ concentration in control conditions.

The control solution used in the study of Malpighian tubules of *Formica* is the 51 mM K^+, 57 mM Cl^- Ringer (see Table 1). It mimicks the ionic composition of the haemolymph (Van Kerkhove *et al.*, 1989). The intracellular K^+ concentration measured in tubules incubated in this solution was 82 mM (range: 59 to 128 mM).

This value is an overestimation of the actual free K^+ concentration for two reasons. First of all, it is to be remembered that the term "K^+ concentration" in the present study was defined as the chemical K^+ concentration needed in the 143 mM Cl^- Ringer to obtain the same signal from the ion-sensitive barrel. This solution contains different salts, so the activity coefficient can not easily be calculated. It is estimated to be of the order of 0.7 to 0.8. But measurements in the present study were always referred to the same calibration solution, so they are internally comparable. In order to know the physiologically important free K^+ concentration however, all values should be multiplied by the activity coefficient of the 143 mM Cl^- solution itself.

Secondly an artifact may have been induced by LJP and V_{tip} of the reference barrel (see 'Methods' section). It can be calculated that this would lead to a systematic overestimation of the calculated K_i (see also "Appendix A"). This will be the case in work on other tissues as well (KCl and Na acetate are commonly used filling solutions), so that it makes comparison with the data in the literature possible.

The value for intracellular K^+ in Malpighian tubules of *Formica* falls within the range of intracellular concentrations or activities measured with K^+-sensitive microelectrodes in control conditions in Malpighian tubules of other species and in other invertebrate epithelia. The measurements cover a wide range: from 43 mM in salivary gland of *Planorbis* (Barber, 1987) to 143 mM in Malpighian tubules of *Tenebrio molitor* (O'Donnell and Machin, 1991) (and see Hanrahan and Phillips, 1984; Hanrahan *et al.*, 1986; Wuttke and Berry, 1992; Moffett *et al.*, 1982; Berridge and Schlue, 1978; Palmer and Civan, 1977; Morgan and Mordue, 1983).

Total cellular K^+ concentration was also measured with X-ray microanalysis and gave comparable values in Malpighian tubules (103 mM, *Rhodnius*, Gupta *et al.*, 1976), salivary gland (135 mM, *Calliphora*, Gupta *et al.*, 1978) and midgut cells (130 mM, *Manduca sexta*, Gupta *et al.*, 1985).

2. Does net uptake of K^+ through the basal membrane occur through a conductive pathway ?
Given a basal membrane conductance, mostly due to K^+, of 0.1 S/cm^2 in 51 mM K_{bl} 143 mM Cl_{bl} (Weltens *et al.*, 1992), a basal inwardly directed electrochemical gradient of only 1 mV could account for a K^+-uptake of 1 nmole/s.cm^2. This is sufficiently large to maintain the established fluid secretion rate, measured in the same time period as the ion-sensitive measurements (but not on the same tubules) of 162±12 pl/min or 0.4 nmoles/s.cm^2 K^+ (unpublished results, the secreting part of the tubule was taken to be 1mm long, the luminal diameter 25 µm, K_l 128 mM).
Measurement of such a small gradient is at the limit of the technical possibilities. It is however useful to obtain an idea of the direction of the gradient as this may be of paramount importance in understanding the mechanism of K^+ uptake. If the hypothesis is to be verified (K^+ entry occurs through conductive channels, and needs only a small difference in V_{bl} and E_K^{bl}), the gradients found should be near zero or slightly inwardly directed. If the gradient is indeed too small to be measured accurately, and no decision can be made about its direction (if at all present), other approaches will be necessary. This will be discussed later.
In the literature two methods for the estimation of a K^+ gradient have been used (for references see below): it was calculated as V_{bl}-E_K^{bl} or taken directly from the tracing of the ion-selective barrel on impaling a cell. Intuitively, the latter method seems to provide a direct and straigthforward measure of the electrochemical gradient, and Table 5B shows that in most cases small but inwardly directed gradients were found (except in 4 mM K_{bl} 57 mM Cl_{bl}, where it was not significantly different from zero). A possible source of error however may lie in the slope of the calibration curve. Due to the small tip of the double barrelled electrode the slope of the ion-sensitive barrel (ca 50 mV/decade) deviated from the ideal Nernstian slope (see Tripathi *et al.*, 1985). So when impaling a cell, the recording of the ion-sensitive barrel will be influenced by the difference in slope of the resin and that of the K^+-selective channels in the cell membrane (expected to be Nernstian): if for instance no electrochemical gradient for K^+ was present at all, the ion-selective barrel would "measure" an inwardly directed gradient if its slope was smaller than 58 mV/decade. A systematic error would thus be induced when the electrochemical gradient is estimated directly from the ion-sensitive barrel on impalement. This is indeed what is observed: gradients "read" by the ISE barrel were systematically more inward. These findings confirm the theoretical considerations made by Armstrong and Garcia-Diaz (1980) and have been worked out for the present results in Appendix A.
It can be calculated that the gradient derived from V_{bl} and E_K^{bl} is likely to be closer to the actual gradient (see Appendix A). When these values are considered it is observed that in higher K_{bl} the gradients tend to become zero or inwardly directed (see Fig. 2 and 4). In the low K^+ concentrations (4 and 5 mM) they were outward. As secretion does not completely stop in low K_{bl}, net K^+ uptake does continue and according to the data must then occur against a (small) electrochemical gradient.

In the latter circumstances however, K_C is also low (see Table 3) and interference of the resin with intracellular substances (other than Na^+ for which the resin is not very sensitive, see Fujimoto and Kubota, 1976) may become important. It is known that the Corning 477317 resin is sensitive to choline (selectivity coefficient = 110: Palmer and Civan, 1977) and NH_4^+ (selectivity coefficient = 0.2: Fujimoto and Kubota, 1976). Moreover Thomson *et al.* (1988) found that NH_4^+ could be produced by locust rectum from alanine, an amino acid present in the bathing solutions used in this study. But even if some NH_4^+ was present in the cells, it can probably not explain the entire difference (6 to 7 mM, calculated from the data in table 3) between K_C"sensed" by the ion-selective barrel and the lower K_C calculated from the measured V_{bl}, if V_{bl} were indeed approximately equal to E_K^{bl}. About 30 to 35 mM NH_4^+ would have to be present in the cell. A small quantity of choline (ca 0.1 mM) could account for the discrepancy however. It is not known whether the Malpighian tubule cells do contain quaternary ammonium ions.

A systematic error in the estimate of the electrochemical gradient may be present however, as a different approach points to an inwardly directed K^+ gradient in all conditions tested: when Ba^{2+} was added to the bath solution V_{bl} hyperpolarized in all K_{bl} tested (113, 51 and 5 mM, 143 mM Cl_{bl}, Weltens *et al.*, 1992). In more recent experiments, the hyperpolarization was less pronounced (Leyssens, Dijkstra, unpublished results), but a depolarization was never seen. Unless Ba^{2+} increases the impact of other ionic conductances and/or opens channels for an ion with a highly negative equilibrium potential across the basal membrane, this can only be explained if K^+ movements across this membrane are in the inward direction in all circumstances (see below).

Information about the electrochemical K^+ gradient across the basal membrane of a Malpighian tubule in other species is almost non existent. Only Morgan and Mordue (1983) for *Locusta* (small outward gradient of 3 mV) and O'Donnell and Machin (1991) for the rectal tubule complex in *Tenebrio molitor* (inward in high, outward in low bath K^+) provide some data.

With regard to the interpretation of our results the following interesting observation was made in *Manduca* midgut epithelium : Ba^{2+} decreased the short circuit current and hyperpolarized the basal membrane potential difference in normoxic conditions where the electrochemical driving force for K^+ was found to be inward, whereas, when this force is reversed by exposure of the tissue to hypoxic conditions Ba^{2+} depolarizes the membrane (Chao *et al.*, 1990). And in the short circuited rectum of *Schistocerca* in the presence of 1 mM cAMP, an inward electrochemical gradient for K^+ was present across the basal membrane and Ba^{2+} hyperpolarized the membrane in all cases (Hanrahan *et al.*, 1986). This indicates that the direction of the Ba^{2+} induced potential changes does correlate with the direction of the passive K^+-movements through the membrane.

Also, Chao *et al.* (1990) derived the K^+ gradient directly from the ion-sensitive barrel on impaling cells of the midgut of *Manduca sexta* and found that in normoxic conditions not

only in 32 mM K^+, but also in 11 out of 12 cells in 10 mM K^+, an inwardly directed gradient was detected. This corroborates the prediction (see Appendix A) and our finding (see Table 5) that gradients derived directly from the ion-sensitive barrel (whose slopes mostly deviate to some extent from the ideal Nernstian value) tend to be systematically more inward (see also Greger *et al.*, 1984).

In invertebrate salt secreting epithelia an electrogenic cation pump postulated in the apical membrane may induce a circular current that slightly hyperpolarizes the basal membrane and hence can create an inward driving force for K^+ across this membrane (for theoretical considerations see Leyssens *et al.*, 1992 and Weltens *et al.*, 1992). This seems to be the case, at least in the presence of a relatively high bath K^+ concentration or on stimulation by a hormonal factor (Moffett and Koch, 1988; Berridge and Schlue, 1978; Parker and Civan, 1977; Wuttke and Berry, 1992; Barber, 1987). The increase in the inward gradient in high K_{bl}, accompanied by an increase in basal conductance (Dijkstra, personal communication), is in agreement with the findings for neuronal cells, where the conductance of the inward rectifying K^+ channel was dependent upon V_{bl}-E_K, and the steady state value was approximately equal to the square root of K_{bl} (Hagiwara, 1983). In low bath K^+ concentrations or in certain experimental conditions (e.g. hypoxia), where K^+ secretion is slow but not necessarily zero and where the electrogenic pump also turns at a reduced rate, other mechanisms may be necessary to explain net K^+ uptake by the cells. These may be active: a Na^+/K^+ pump was suggested for salivary gland cells of *Calliphora* (Berridge and Schlue, 1978), *Planorbis* (Barber, 1987), for the midgut of *Glossina* (Peacock, 1982), and for Malpighian tubules of *Locusta* (Anstee *et al.*, 1979; Fogg *et al.*, 1991). However, in Malpighian tubules of *Formica*, 1 mM ouabain in 5 mM K_{bl} 143 mM Cl_{bl} had no effect on secretion (Leyssens, unpublished results) or on V_{bl} (Leyssens *et al.*, 1991b). Or they may be secondarily active: e.g. bumetanide-sensitive cation-anion cotransporters as suggested for Malpighian tubules of *Rhodnius* (O'Donnell and Maddrell, 1984) and *Aedes* (Hegarty *et al.*, 1991). This has to be further investigated for *Formica*.

3. The intracellular K^+ concentration is sensitive to the bath K^+ concentration.

A remarkable feature of K_C in the Malpighian tubule cell of *Formica* is its fast and reversible response to changes in the bath K^+ concentration. The mean value for K_C was 2.5 to 3 times higher when K_{bl} was increased by a factor of about 20 (see Table 3). This phenomenon together with the large variability in K_C (and V_{bl}) is probably not the result of cell damage on impalement with the double barrelled microelectrode. First of all a similar variability and range of values for V_{bl} and comparable transient responses on changing K_{bl} were observed with smaller tipped conventional electrodes. When continuously measured in a constant K_{bl}, V_{bl} recorded with these electrodes remained remarkably stable for as long as 45 minutes after which period the electrode was withdrawn (Van Kerkhove, unpublished observations). Also if an unspecific leak was created, a gradual rundown of K_C (and V_{bl}) would occur. This was not the case. A new steady state was always reached and the concentration changes were

perfectly reversible. In a later section, we will show that this response may play a role in the intrinsic regulation of K^+ secretion by K^+.

Possible basal transport mechanisms, other than a conductive K^+ channel, and that may play a role in determining K_C, are under study (Leyssens, unpublished results).

In several other invertebrate epithelia K_C seems to be regulated to some extent: it changes either as a response to the bath K^+, or when exposed to hormonal factors (e.g. Berridge and Schlue, 1978; Hanrahan and Phillips, 1984). The effects of bath K^+ concentration on K_C observed in other invertebrate epithelial cells may differ from no effect (Nicolson and Isaacson, 1987; Baldrick *et al.*, 1988) to changes differing in the rate and the extent of the responses and also in the K^+ concentrations at which they occur (Berridge and Schlue, 1978; Chao *et al.*, 1990; Wuttke and Berry, 1992; Barber, 1987; O'Donnell and Machin, 1991). It is clear however that when cell K^+ participates in secretion, it changes on application of hormonal factors.

4. The luminal K^+ concentration.

The luminal K^+ increased from 90 to 130 mM depending on the K^+ concentration present in the bath, so K^+ is secreted not only against an electrical but also against a concentration gradient (see Table 4).

In Malpighian tubules, the effect of the bath K^+ (and bath Na^+) on the luminal K^+ concentration in basal conditions varies from species to species and can change on hormonal stimulation (see Berridge, 1968, for *Calliphora*; Maddrell, 1969, for *Rhodnius*; Gee, 1976, for *Glossina*, Nicolson, 1976, for *Pieris*; and Dalton and Windmill, 1980, for *Musca*). As was already inferred from the analysis of the secreted droplets with X-ray analysis (Van Kerkhove *et al.*, 1989), it is clear that in *Formica*, at least in unstimulated conditions, the Malpighian tubules preferably secrete K^+ even in a bath K^+ concentration of only 4 mM (bath Na^+ being 109 mM). It must be kept in mind however that the effect of lowering K_{bl} on K_l may be underestimated in the experimental set-up used in the present study: secretory fluid was not allowed to escape from the lumen. So fluid secreted in a new K_{bl} will mix with the previously secreted fluid. This may obscur larger changes in K_l than actually detected.

A comparison of the values found with the K^+-sensitive electrode can be made with the X-ray microanalysis of the secretory droplets performed previously (Van Kerkhove *et al.*, 1989). The latter method measured the total chemical concentration. The luminal K^+ concentration found was appreciably higher (ca 195 mM) and did not seem to be sensitive to the bath K^+ concentration. A similar discrepancy was observed by Morgan and Mordue (1983) when they measured K^+ concentrations with either an ion-sensitive electrode or by flame photometry of secretion droplets of *Locusta* Malpighian tubules they found 148 mM and 169 mM, respectively. The ratio of the K^+ concentration measured with the ion-sensitive electrode (already referred to the chemical concentration in the 143 mM Cl^- Ringer in the present study) over that determined with X-ray analysis could give some estimate of the relative activity coefficient in the luminal fluid. This would then be rather low compared

to the Ringer solution (0.5 to 0.7). Another explanation may be that some of the secreted K^+ is bound and hence will not be sensed by the electrode: it has been observed that the secretory fluid is often viscous and may contain (charged?) organic substances (unpublished observations). In luminal fluid of 5-hydroxytryptamine stimulated *Rhodnius* Malpighian tubules and in *Calliphora* salivary gland the K^+ concentrations found with both methods were much closer (*Rhodnius*: 90 and 82 mM, respectively, Gupta *et al.*, 1976; *Calliphora*: 127 and 139 mM, respectively, Gupta *et al.* 1978).

5. Electrochemical gradient for K^+ extrusion across the apical membrane. The effect of bath K^+.

From Table 6 it is clear that K^+ extrusion across the apical membrane occurs against an appreciable electrochemical gradient. And as one might expect the K^+ secretion increases in a linear fashion when this gradient decreases (see Fig. 6). This finding is in accordance with the proposed model for K^+ secretion: a lumen to cell directed H^+ concentration gradient, built up by an apical electrogenic H^+ pump, can drive K^+ extrusion to the lumen via a K^+/H^+ antiporter (see Wieczorek *et al.* ,1989; Wieczorek *et al.*, 1991). Weltens *et al.* (1992) provided evidence for the presence of such a pump in the Malpighian tubule of *Formica*. From Fig. 5, it is clear that not only the apical electrical gradient (i.e. V_{ap}) decreases but also the K^+ concentration gradient. The close dependence of K_c on K_{bl} causes a reduction of K_l/K_i as the bath K^+ increases. Consequently an enhanced K^+ transport to the lumen via a K^+/H^+ exchanger may be expected.

Preliminary results obtained from intracellular and luminal H^+-selective measurements in Malpighian tubules of *Formica* suggested that in all bath K^+ concentrations a cell-inward H^+ concentration gradient was present which was large enough to drive K^+ extrusion to the lumen via an electroneutral K^+/H^+ antiporter. The stimulation in fluid secretion on increasing K_{bl} was correlated with an increase in the ratio of H_l/H_c over K_l/K_c (Zhang *et al.*, 1992).

6. Conclusions.

From the data obtained up to now we can conclude that bath K^+ has a double role in the intrinsic regulation of K^+ secretion by K^+. First of all, as was shown in a previous study where only potential differences and resistances were measured, the Nernst potential for K^+ across the basal membrane influences the electrical gradient across the apical membrane (Leyssens *et al.*, 1992): when the basal membrane depolarizes in high K^+, the electrically coupled apical membrane follows and this reduces the electrical barrier to be overcome by the transport mechanisms. Furthermore, in the present study we demonstrate that the bath K^+ also determines the amount of cell K^+ available for apical extrusion and hereby diminishes the concentration step to be overcome for K^+.

Arguments in favour of net entry of K^+ through a conductive channel in the basal membrane are discussed. The electrochemical gradients measured are very small (inward, zero, or even

outward in low K_{bl}). The hyperpolarization of V_{bl} by Ba^{2+}, observed for each K_{bl} studied in 143 mM Cl_{bl}, however seems to favour the idea that passive K^+ movements are practically always in the inward direction.

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Appendix A

If the resin of the K^+-sensitive barrel was perfectly selective for K^+, a calculation can be made of the errors induced in the estimate of K_c and of the transbasal electrochemical gradient for K^+ due to $E_{err}{}^m$ (i.e. ΔV_{tip} and/ or ΔLJP, see Table 1) and to a deviation from the ideal value (i.e. 58 mV/decade at room temperature) of the slope of the ion-sensitive barrel.

Some of these considerations can be found in the paper of Armstrong and Garcia-Diaz (1980) but have been adapted for a better understanding of the present results.

Taking a situation as shown in Fig. 7.

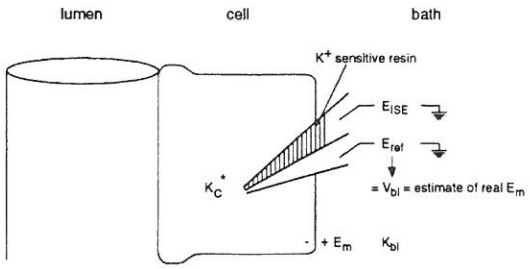

Fig. 7. Impalement of a Malpighian tubule cell from the basal side with a double-barrelled microelectrode. For definition of abbreviations see text.

and defining:

K_c^* : real intracellular K^+ concentration

K_c: value calculated from the measurements (see below)

E_{ISE}^* : signal from the ion-selective barrel expected if S = 58 mV/decade (N.B. this would also be the real electrochemical gradient for K^+)

E_{ISE} : recorded signal from the ion-sensitive barrel

and $E_{ISE} = E_m + S \log K_c^*/K_{bl}$ eq. (1)

[N.B. as the ion-selective barrel doesn't show a tip potential (Leyssens, upublished result) as may be the case for the reference barrel, the ion-selective barrel is expected to sense E_m and not V_{bl}]

V_{bl} : recorded signal from the reference barrel.

K_c and the electrochemical gradient for K^+ were estimated and a comparison with the actual real values was made.

1. Calculation of K_c.

from eq. (1)

$$E_{ISE} - V_{bl} = S \log K_c^*/K_{bl} + (E_m - V_{bl})$$

defining $E_{err}^m = -(E_m - V_{bl})$
i.e. the error in the measurement of the membrane potential difference.

$$K_c^* = K_{bl} \cdot 10^{(E_{ISE} - V_{bl})/S} \cdot 10^{E_{err}^m/S} \qquad \text{eq. (2)}$$

As E_{err}^m is generally expected to be small with respect to V_{bl} and as its exact value is not known for each individual experiment K_c was calculated as

$$K_c = K_{bl} \cdot 10^{(E_{ISE} - V_{bl})/S} \qquad \text{eq. (3)}$$

From eq. (2) and (3) it can be derived that the error induced in K_c by ignoring E_{err}^m (negative, see Table 1) results in an overestimate of K_c (see Table 7).

2. Estimate of the electrochemical gradient for K^+ across the basal membrane.

Estimate from ($V_{bl} - E_K^{bl}$)

with $E_K^{bl} = -58 \log K_c/K_{bl}$
and K_c derived from eq. (3)

$$V_{bl} - E_K^{bl} = (1 - 58/S)\, V_{bl} + 58/S \,.\, E_{ISE} \qquad \text{eq. (4)}$$

The actual gradient is given by:

$$E_m - E_K^{*bl}$$

$$= E_m + 58 \log K_c^*/K_{bl}$$

$$= V_{bl} - E_{err}^m + 58 \log . 10^{(E_{ISE} - V_{bl} + E_{err}^m)/S}$$

$$= (1 - 58/S)(V_{bl} - E_{err}^m) + 58/S\, E_{ISE} \qquad \text{eq. (5)}$$

The difference between eq. (4) and (5)

$$= (1 - 58/S)\ E_{err}^{m}$$

Again with E_{err}^{m} being negative (see Table 1) the error will result in calculated gradient that is a slightly more outward (see Table 7).

Estimate directly from the ion-sensitive barrel.

$E_{ISE} = E_m + S \log K_c^*/K_{bl}$ (= eq. 1)

The actual gradient is given by

$$E_{ISE}^* = E_m + 58 \log K_c^*/K_{bl} \qquad \text{eq. (6)}$$

The difference between eq. (1) and (6)

$$= (S - 58) \log K_c^*/K_{bl}$$

S mostly being smaller than 58 mV/decade the ion-selective barrel will record a gradient, that is more inward than the actual gradient.

An overview of calculated values of errors is given in Table 7 as a function of S, E_{err}^{m} and K_c^*/K_{bl}. Values for these parameters were chosen in a range applicable to our data. It is clear from Table 7 that the electrical gradient derived directly from the ion-selective barrel had almost always a more inward value. The error was not sensitive to E_{err}^{m} (not shown) but increased rapidly with K_c^*/K_{bl} and with a less ideal slope.
The error in $(V_{bl} - E_K^{bl})$ on the other hand was sensitive to E_{err}^{m} and also to S, but to a much smaller extent and for a perfectly selective resin it stayed relatively constant in different K^+ concentrations (not shown). The error was small however even with a

Table 7. Possible errors induced in the estimate of K_c, and of the transbasal electrochemical gradient for K^+. Dependence on S, E_{rr}^m and K_c^*/K_{bl}.

S	E_{err}^m	K_c/K_c^* $10^{-E_{err}^m/S}$	Error in $V_{bl} - E^{bl}_K$ eq (4) - eq (5) $(1 - \frac{58}{S}) E_{err}^m$	Error in E_{ISE} [a] eq (1) - eq (6) $(S - 58) \log \frac{K_c^*}{K_{bl}}$		
mV/decade	-	-	-	$K_c^*/K_{bl} = 1$	$K_c^*/K_{bl} = 2$	$K_c^*/K_{bl} = 10$
58	0	1.0	0	0	0	0
53	0	1.0	0	0	- 1.5 mV	- 5.0 mV
48	0	1.0	0	0	- 3.0 mV	- 10.0 mV
58	- 3 mV	1.13	0	-	-	-
53	- 3 mV	1.14	+ 0.3 mV	-	-	-
48	- 3 mV	1.15	+ 0.6 mV	-	-	-
58	- 6 mV	1.27	0	-	-	-
53	- 6 mV	1.30	+ 0.6 mV	-	-	-
48	- 6 mV	1.33	+ 1.25 mV	-	-	-

S : calibration slope of ion-sensitive barrel (lowest accepted slope was 48 mV/decade, see Methods Section).

K_c^* : real intracellular K^+ concentration.

K_c : calculated intracellular K^+ concentration.

E_{err}^m : $V_{bl} - E_m$ (E_m being the real, V_{bl} the recorded membrane potential difference).

[a] : values chosen for K_c^*/K_{bl} are in the range of the values found experimentally (see Table 3).

high E_{err}^{m} and/or low S, when compared to the deviations in E_{ISE}. It was of the order of magnitude of the experimental error.
The calculated K_c was primarily sensitive to E_{err}^{m} and much less to S. Also as can be derived from the equation K_c/K_c^* stayed constant in different K^+ concentrations (not shown). It should be kept in mind however that the Corning 477317 may be sensitive to other cations, especially when K_c is low, e.g. in 4 mM K_{bl} (see also "Discussion"). Interference with Na^+ should be negligible according to the selectivity coefficient (0.01) found by Fujimoto and Kubota (1976). Work with single barrelled electrodes in our hands (Weltens, unpublished results) allowed us to calculate that for a K_c of about 30 mM and supposing K_c had been replaced by Na_c (ca 70 mM) an overestimate of K_c of about 3 mM is expected.

In conclusion we are confident that the electrochemical gradient calculated from $V_{bl} - E_K^{bl}$ gives relatively reliable values when n = large as in Table 3. K_c however will be slightly overestimated.

PAPER 4

BOTH DINITROPHENOL AND BA^{2+} REDUCE KCL AND FLUID SECRETION IN MALPIGHIAN TUBULES OF *FORMICA* : THE ROLE OF THE APICAL H^+ AND K^+ CONCENTRATION GRADIENT.

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BOTH DINITROPHENOL AND BA^{2+} REDUCE KCL AND FLUID SECRETION IN MALPIGHIAN TUBULES OF *FORMICA* : THE ROLE OF THE APICAL H^+ AND K^+ CONCENTRATION GRADIENT.

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Running title: DNP and Ba^{2+} on Malpighian tubules

ABSTRACT

In the present study, further evidence is presented for the close relationship between fluid secretion and the ratio of the apical H^+ over K^+ concentration gradient.

Two agents with a fast and reversible inhibitory effect on fluid secretion were tested on intracellular and luminal H^+ and K^+ concentrations and on transepithelial and transmembrane potential differences: 6 mM Ba^{2+}, a K^+ channel blocker, and 2.10^{-4} M 2,4-dinitrophenol (DNP), a well-known protonophore and uncoupler of oxidative phosphorylation.

Ba^{2+} hyperpolarized the apical membrane potential difference (V_{ap}, lumen reference) from -59±3 to -90±6mV (n=6) and the basal membrane potential difference (V_{bl}) from -23±3 to -74±7 mV(n=6). At the same time, the cell acidified (H_c increased from 18±4 to 50±21 nM and the lumen alkalinized (H_l decreased from 117±23 to 74±11 nM); H_l/H_c was reduced from 7.7±2.4 to 2.8±0.8 (n=6). On the other hand, K_c dropped from 85±6 mM to 73±6 mM (n=9) and K_l from 143±3 mM to 121±1 mM (n=5); consequently K_l/K_c remained unchanged (i.e. 1.7±0.1). As a result, the H_l/H_c over K_l/K_c ratio decreased from 4.5 to 1.7.

DNP depolarized V_{ap} from -63±7 to -26±3 mV (n=8); V_{bl} slightly depolarized from -21±1 to 20±1 mV. In the presence of 6 mM Ba^{2+}, V_{ap} and the basal membrane

potential difference (V_{bl}) (bath reference) depolarized from -81±5 to 1±2 mV and from -68±6 to 1±1 mV, respectively (n=5) when applying DNP.

Like Ba^{2+}, the addition of 2.10^{-4} M DNP to the control solution caused an acidification of the cytosol (H_c rose from 24±5 to 81±9 nM); H_l was not significantly changed (i.e. 80±12 and 80±9 nM in the absence and presence of DNP, respectively). Consequently, H_l/H_c dropped from 3.0±0.7 to 1.0±0.2 (n=8). K_c diminished from 104±9 to 80±5 mM and K_l from 141±8 to 93±6 mM after the addition of DNP; K_l/K_c was not significantly changed (i.e. 1.4±0.1 and 1.2±0.2 in the absence and presence of DNP, respectively, n=6). The overall result was a reduction of the ratio [H_l/H_c over K_l/K_c] from 2.1 to 0.8. On the other hand, the sensitivity of V_{bl} and V_{ap} to a change in bath K^+ from 5 to 51 mM was virtually unchanged from control in the presence of 2.10^{-4} M DNP: V_{bl} depolarized with 33±1 and 32±1 mV, and V_{ap} with 19±3 and 22±1 mV, in the absence and presence of DNP, respectively. Furthermore, the transient changes of V_{bl} on varying the bath K^+, suggesting a change in K_c, were comparable whether DNP was present or absent.

These findings are consistent with the hypothesis of an apical K^+/H^+ antiporter. Ba^{2+} and DNP reduce the driving force for K^+ secretion to the lumen by slowing down the K^+/H^+ antiporter . Ba^{2+} increases the electrical component of the proton motive force of the electrogenic H^+ pump, thereby decreasing the free energy for building up a proton concentration gradient. DNP inhibits the realization of an apical lumen to cell directed H^+ concentration gradient by a double action mechanism. It depletes the apical H^+ pump from its energy source, and it can dissipate H^+ gradients via its protonophore action at the basal (and apical) cell membrane(s). Further evidence is presented for the electroneutrality of the K^+/H^+ antiporter.

INTRODUCTION

Malpighian tubules of *Formica* secrete KCl-rich primary urine. The fluid secretion rate is strongly dependent upon the basal K^+ concentration. K^+ is always transported against its electrochemical gradient from the hemolymph to the lumen (Van Kerkhove *et al.*, 1989). At the basal side, K^+ entry through highly conductive K^+ channels is suggested (Leyssens *et al.*, 1992a; Leyssens *et al.*, 1993). At the apical side, the K^+ transfer is proposed to be realized via the combination of an electrogenic H^+ pump and a K^+/H^+ antiporter. Weltens *et al.* (1992) provided evidence for the existence of such a H^+ pump in the Malpighian tubules of *Formica*. Furthermore, using double-barrelled H^+-selective electrodes, the existence of a favourable, cell-inwardly directed apical H^+ concentration gradient has been demonstrated by Zhang *et al.* (submitted). Results obtained from K^+-selective measurements suggested that, for each basal K^+ concentration, the transfer of K^+ from the cell to the lumen could be driven by the cell-inward H^+ concentration gradient via a K^+/H^+ antiporter (Leyssens *et al.*, 1993; Zhang

et al., submitted). In the present study, evidence is given for the electroneutrality of the antiporter. This is in contrast to the finding of Wieczorek *et al.* (1991) where an electrogenic K^+/H^+ antiporter, transferring at least 2 H^+ for 1 K^+, was proposed for *Manduca sexta* midgut.

The aim of the present study is to provide further evidence that the proton concentration gradient, built up by the H^+ pump, is closely correlated with K^+ and fluid secretion.

If the pump that develops a proton concentration gradient is electrogenic (see Weltens *et al.* 1992), an increase in electrical potential difference across the apical membrane would hamper the transfer of protons from the cell to the lumen by the pump and hence reduce the cell-inwardly directed H^+ concentration gradient. A hyperpolarization of the apical membrane potential can be realized by adding Ba^{2+} to the bath solution. The primary effect of Ba^{2+} is blockage of the K^+ channels in the basal membrane, slowing down net K^+ uptake by the cell (see Weltens *et al.*, 1992; Leyssens *et al.*, 1993). As a result fluid secretion drops, and at the same time the apical electrical gradient increases (Weltens *et al.*, 1992). In the present study, the effect of Ba^{2+} on the apical H^+ and K^+ concentration gradient in relation to its effect on fluid secretion was studied.

An abolition of the apical H^+ concentration gradient could also be realized by 2,4-dinitrophenol (DNP). Weak-acid uncouplers of oxidative phosphorylation such as DNP can increase the conductance of lipid membranes by several orders of magnitude. In this high conductance state, these membranes appear to be highly selectively permeable to H^+ or OH^- (Bielawski *et al.*, 1966; Finkelstein, 1970). Mc Laughlin and Dilger (1980) proposed a model in which this class of weak acids can transport protons across a membrane by acting as a carrier. By its protonophore action at the inner mitochondrial membrane, DNP will inhibit the ATP synthesis in the inner mitochondrial membrane and deplete the apical H^+ pump from its energy source. Moreover, DNP will also exert its protonophore action at the cell membrane level where it will dissipate H^+ gradients. Since its effects on fluid secretion and on H^+ and K^+ gradients across the apical membrane were very fast and fully reversible (contrary to the V-type ATPase blocker bafilomycin A_1, Weltens *et al.*, 1992), DNP turned out to be an excellent tool in studying H^+ and K^+ gradients.

Part of the results have been published as an abstract (Leyssens *et al.*, 1991; Leyssens *et al.*, 1992b).

MATERIALS AND METHODS

1. Preparation.

Worker ants of the species *Formica polyctena* were collected from natural nests at the periphery of woods and kept in an artificial nest at a constant temperature of 20 °C.
The animals were fed with sugar and water.
After dissection, a single Malpighian tubule (1-2 mm) was transferred to a bathing droplet (50 µl) on the stage of an inverted microscope, continuously perfused at a constant rate of 150 µl/min. The bathing droplet was covered with paraffin oil to avoid evaporation (see Van Kerkhove *et al.*,1989).

2. Experimental set up.

Fluid secretion measurements.

The technique used for measuring secretion rate was described in detail by Van Kerkhove *et al.* (1989). The secreted fluid was collected every 10 min. and blown out of the hydrophobic collection pipette under paraffin oil. By measuring the diameter of the perfectly spherical droplet with a Zeiss monofilar ocular (8x) through two objectives (16x and 40x), the secretion rate could be calculated. In order to test a drug effect, to overcome the variability in the absolute values for secretion rate in different tubules, the absolute value of each secretion period within an individual experiment was expressed as a relative rate, i.e. as a percentage of the value measured during the third collection period in control solution. Furthermore in control conditions, the fluid secretion rate declined with time, and was significantly different from the third collection period after 60 minutes (n=9). To account for this time effect, the ratio of the experimental over the control relative rate for each period in time was calculated. In order to test the statistical significance of a drug effect, the experimental relative rate was compared with the control relative rate during the same time period (unpaired Student's t-test). The drug effect was considered to be fully reversible when no statistically significant difference (unpaired Student's t-test) was found between the relative secretion rate during a wash-out period and the corresponding control time period.

Electrical potential measurements.

The method was described in detail previously (Leyssens *et al.*, 1992a). Measurements were performed with 100 mM KCl filled microelectrodes (resistance about 300 MΩ (Borosilicate filament glass, Hilgenberg, Malsfeld, Germany; O.D. 1.3 mm, I.D. 0.7 mm). The electrode was connected to a high impedance electrometer (Duo 773, WPI) via an Ag/AgCl wire. The reference electrode was a low resistance (1 MΩ) 3 M KCl electrode, connected to the earth via an Ag/AgCl wire. In order to facilitate

impalement, the Malpighian tubule was immobilized in the bathing droplet by two holding pipettes. The secreted fluid remained in the tubule. The measurement was accepted if a sudden negative potential deflection occurred and was stable for at least a few minutes, and if the electrode potential differed maximally 3 mV from the baseline after withdrawal.

The transepithelial potential (V_{te}) was measured by advancing the microelectrode through the cell layer into the lumen of the tubule. All results, obtained with single-barrelled conventional microelectrodes, were obtained from "single tubules V_{bl} and V_{ap} measurements", i.e. intracellular and luminal recordings were made consecutively in the same tubule. The apical potential (V_{ap}) was calculated as the difference between V_{te} and the basal potential (V_{bl}), and was expressed with reference to the lumen [$V_{ap} = -(V_{te}-V_{bl})$].

H- and K^+-selective measurements.

The technique used for the construction of double-barrelled ion-sensitive electrodes was described by Zhang *et al.* (submitted). In summary, the double-barrelled electrodes were drawn from paired filament containing glass capillaries with unequal diameter (OD_1=1.5 mm, ID_1=0.87 mm; OD_2=0.75 mm, ID_2=0.35 mm; Hilgenberg, Malsfeld, Germany) on a horizontal puller with an air jet cooling system (Model-77, Sutter Instrument U.S.A.). A tip diameter of approximately 1 µm was obtained. The inside of the larger barrel was silanized (hexamethyldisilazane; Janssen Chimica), the smaller barrel remained hydrophylic. A short column of the ionselective liquid ion exchanger (H-IIX, proton-cocktail 82500, Fluka or Corning 477317 for the H^+- or K^+-selective electrode, respectively) was introduced into the tip of the larger barrel, i.e. the ion-selective barrel, and the rest of this barrel was backfilled with 1 M KCl. The other barrel, i.e. the reference barrel, was simply backfilled with 1 M KCl or 1 M Na acetate + 10 mM KCl for the H^+- or K^+-selective electrode, respectively. The filled electrodes were bevelled in a rotating polishing alumina solution (No.3 AB Gama Polishing Alumina, Buehler, U.S.A.). After bevelling, the ionsensitive and the reference barrel had tip resistances ranging from 50 to 100 GΩ and from 0.5 to 1 GΩ, respectively.

The experimental set-up for the ionsensitive measurements was described previously by Zhang *et al.* (submitted). The reference and ionselective barrel were connected via Ag/AgCl half-cells to a high impedance electrometer (Duo 773, WPI). Three tracings were recorded on a pen recorder (Sefram or Linseis): the potential of the reference barrel, the potential of the ion-selective barrel and the difference between both, representing the H- or K^+-sensitive signal.

Calibration of the H^+-selective electrode was done in Ringer solutions at pH values of 6.50, 7.25 and 8.00 (accurately titrated with NaOH or KOH). This corresponds to a H^+ concentration of 316, 56 and 10 nM, respectively. Calibration of the recorded K^+-sensitive signal occurred in the normal bathing medium, i.e. a 143 mM Cl^- Ringer

solution, in which the K^+ concentration varied from 5 to 113 mM, using Na^+ as a substitute. The measured intracellular or luminal K^+ signal was always expressed as the chemical K^+ concentration that would be needed in a 143 mM Cl^- Ringer solution to result in the same reading of the K^+-selective barrel. The ion-sensitive electrodes were accepted for use if a stable potential signal was obtained for both the ion-sensitive and the reference barrel, if the K^+ or pH sensitivity of the reference barrel was less than 1 mV, and if the calibration curve could be fitted by a straight line with a slope of at least 48 mV/decade.

As was discussed in a previous paper (Leyssens *et al.*, 1993), ion-sensitive measurements are an adequate tool to study changes in intracellular and luminal ion concentrations and to determine electrochemical gradients. Ideally, H^+- and K^+-selective measurements should be performed on the same tubule. However, this was technically not possible. Furthermore, since the calibration curve has a logarithmic scale, the detection of small changes in intracellular and luminal concentrations, especially in the high concentration range is difficult; for instance, when the K^+-sensitive electrode has a slope of 50 mV/decade and the intracellular K^+ is about 120 mM, an increase of the intracellular K^+ with 6 mM represents an increase of the K^+-sensitive signal with only 1 mV. On evaluating the results, one has to take into account that this approaches the limit of detection.

3. Artificial salines and test substances.

A 51 mM K^+ 143 mM Cl^- Ringer solution was taken as control solution. The composition was as follows: 51 mM KCl, 62 mM NaCl, 2 mM $CaCl_2$, 13 mM $MgCl_2$, 12.1 mM Hepes, 2.8 mM alanine, 10.6 mM trehalose, 11.7 mM maltose, 139 mM glucose; pH was adjusted to 7.20 (7.25 for the H^+-selective measurements) with NaOH; the osmolality of the solution was 375 mosm/kg H_2O. Solutions were freshly prepared each week, filtered through 0.22 µm Millipore filters and kept at 2 °C until used.

In one series of experiments, the effect of the bath K^+ concentration (K_{bl}) was tested and 51 mM K^+ was reduced to 5 mM by replacing KCl with NaCl. Substances were tested at the following concentrations: barium ($BaCl_2$; Janssen Chimica): 6 mM; 2,4-dinitrophenol (DNP; UCB): 2.10^{-4} M.

4. Statistics.

Results were presented as mean values±SE (n=number of experiments). The mean values of the H^+ selective measurements were used to calculate the intracellular and luminal pH values. When intracellular and luminal measurements were made consecutively on the same tubules, the ratio of the luminal over intracellular H^+ or K^+ concentration (H_l/H_c or K_l/K_c, respectively) obtained from individual experiments was averaged (see Fig. 3); otherwise (i.e. the K^+-selective experiments in the presence of

Ba^{2+}, see Fig. 2B and Fig. 3A) the ratio of the mean values of the luminal over intracellular (K^+) concentrations, and the corresponding S.E., was calculated. Statistical computations were obtained using Statview II (Abacus Concepts Inc., Berkeley, CA, U.S.A., 1987).

The paired two-tailed Student's t-test was performed, except when indicated differently. A P-value of < 0.05 was accepted as indicating statistical significance.

RESULTS

1. The effect of 6 mM Ba^{2+} on electrical potential differences and ion concentrations.

We studied the effect of Ba^{2+} on the intracellular and luminal H^+ and K^+ concentration (H_c, K_c and H_l, K_l, respectively) and we calculated the H^+ and K^+ gradients. The H^+- and K^+-selective measurements were performed on a different series of tubules. The intracellular and luminal H^+-selective measurements were carried out on the same tubules. In Fig. 1A, an example of the measurement of the

basal membrane potential difference (V_{bl}) and the intracellular pH (pH_c) and of the transepithelial potential difference (V_{te}) and the luminal pH (pH_l) in the absence and presence of 6 mM Ba^{2+} is given. The upper trace shows V_{bl} and V_{te} as measured with the reference barrel.

The lower trace gives the potential difference between the H^+-selective and the reference barrel. These potential differences were converted to H^+ concentrations; the corresponding pH-values are shown.The intracellular pH was alkaline with respect to the bath pH of 7.25.The addition of Ba^{2+} to the bath solution resulted in an acidification of pH_c with 0.05 pH units while V_{bl} strongly hyperpolarized. In the lumen, on adding Ba^{2+}, pH_l became more alkaline while V_{te} depolarized.

After washout, V_{te} returned to a more positive potential difference and pH_l to a less alkaline pH. The effects of Ba^{2+} on V_{bl} and V_{te} are consistent with the results described by Weltens *et al.* (1992). Fig. 1B shows an example of a intracellular and of a luminal K^+-selective measurement.The lower trace gives the potential differences between the K^+-selective and the reference barrel. These potential differences were converted to K_c and K_l values. Due to the logarithmic scale of the calibration curve the determination of the K_c and K_l values present was at the limit of detection. However, small changes upon administration of Ba^{2+} were systematically present. After the addition of Ba^{2+}, V_{bl} hyperpolarized and K_c decreased with 15 mM. Washout resulted in a depolarization of V_{bl} to the control value and an increase of K_i. On repeating the same protocol, an identical potential and K^+ concentration profile was obtained. In the lumen of another tubule, the addition of Ba^{2+} caused a depolarization of V_{te} and on careful examination of the recording a small decrease of K_l with 12 mM corresponding

to 1 mV could be detected. After washout, V_{te} increased and K_l rose with 16 mM. Subsequently, calibration is also shown.

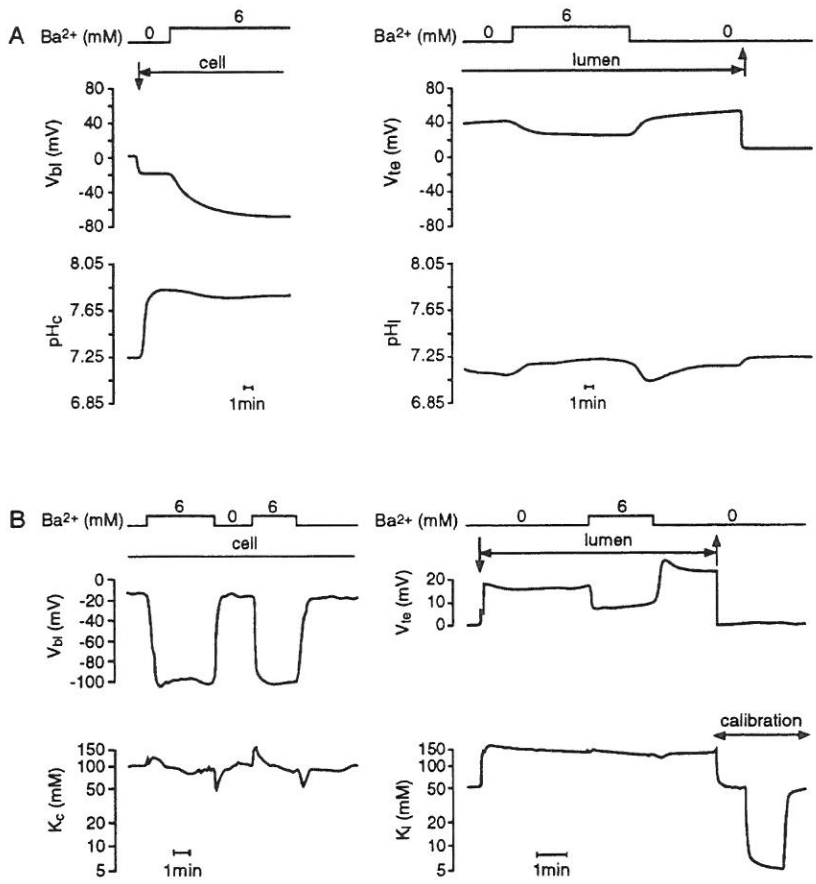

Fig. 1. Effect of the addition of 6 mM Ba^{2+} to the bath on the basal membrane potential difference (V_{bl}), the intracellular pH (pH_c), the transepithelial potential difference (V_{te}) and the luminal pH (pH_l) (A) and on V_{bl}, the intracellular K^+ concentration (K_c), V_{te} and the luminal K^+ concentration (K_l) (B). Bath K^+ concentration was 51 mM. H^+- and K^+-selective measurements were carried out on different tubules; intracellular and luminal measurements were performed on the same tubules for the H^+- but on different tubules for the K^+-selective measurements. The upper trace shows V_{bl} and V_{te} as measured with the reference barrel; the lower trace gives the potential difference between the ion-sensitive and the reference barrel. These potential differences were converted to pH (A) and K^+ (B) concentration values by using a calibration curve. In Fig. 1B (luminal measurement) calibration of the double-barrelled K^+-selective electrode was shown at the end of the experiment.
↓: intracellular or luminal impalement
↑: withdrawal of the electrode.

The results, obtained from the H^+- and K^+-selective measurements are summarized in Fig.2. The results of the H^+-selective measurements are expressed in H^+ concentrations.

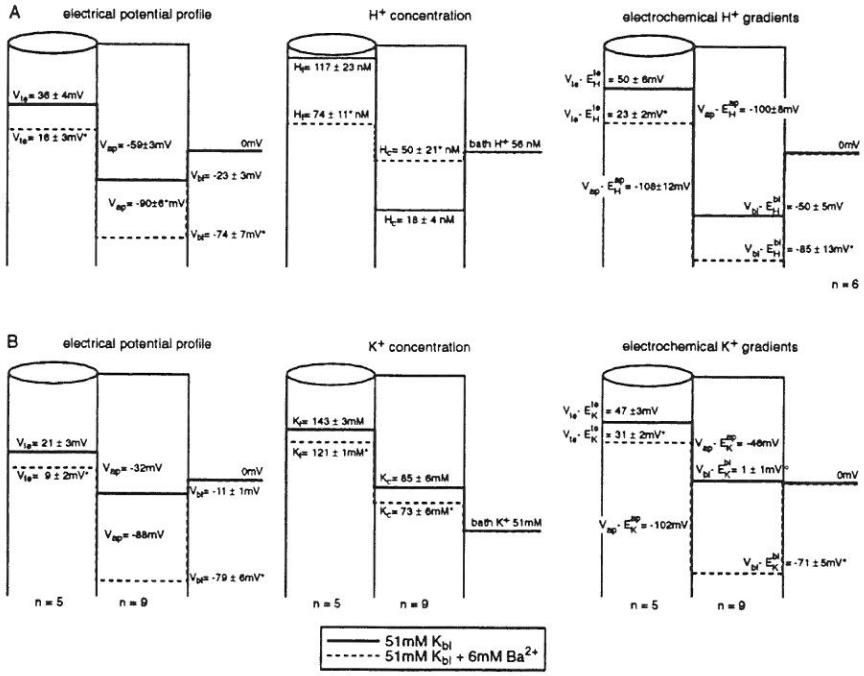

Fig. 2. Effect of the addition of 6 mM Ba^{2+} to the control solution on electrical potentials (V_{te}, V_{bl} and V_{ap}), ion concentrations (H_l and H_c, K_l and K_c), and on the transepithelial (V_{te}-E_H^{te}, V_{te}-E_K^{te}), basal (V_{bl}-E_H^{bl}, V_{bl}-E_K^{bl}) and apical (V_{ap}-E_H^{ap}, V_{ap}-E_K^{ap}) electrochemical $H^+$and K^+ gradients. Summary of intracellular and luminal H^+- (A) and K^+- (B) selective measurements in the absence and presence of 6 mM Ba^{2+}. Mean values ±S.E.. H^+- and K^+-selective measurements were carried out on a different series of tubules; the intracellular and luminal measurements were performed on the same (A) or on different (B) tubules. The apical potential difference was expressed with reference to the lumen [$V_{ap} = -(V_{te}-V_{bl})$] . Intracellular and luminal voltages and ion concentrations were determined after experimental treatment at 3 ± 1 (n = 6) and 3 ± 1 (n = 6) min., and after 3 ± 1 (n = 9) and 3 ± 1 (n = 5) min., for the H^+- (A) and K^+- (B) selective measurements, respectively.
In Fig. 2B, V_{ap} was calculated as the difference between the mean value of V_{te} and V_{bl}.
*: significantly different from control (paired Student's t-test, $P<0.05$)
° **not** significantly different from zero ($P>0.05$).

The addition of 6 mM Ba^{2+} resulted in a strong hyperpolarization of V_{bl}, and in a depolarization of V_{te} (Fig. 2A and B). At the same time, Ba^{2+} caused an increase in H_c and a decrease in H_l (Fig. 2A). This corresponds to an acidification of the cell from pH 7.74 to 7.30 and an alkalinization of the lumen from pH 6.93 to 7.13. Both K_c and K_l decreased after the addition of Ba^{2+} (Fig. 2B). Furthermore, the addition of Ba^{2+}

caused a significant decrease of the transepithelial electrochemical H^+ and K^+ gradient, and a significant increase of the basal cell-inward electrochemical H^+ and K^+ gradient. The apical electrochemical H^+ gradient remained unchanged while the K^+ gradient increased (not statistically tested) (Fig. 2A and B, respectively). All Ba^{2+} effects were fully reversible and statistically significant. All electrochemical H^+ and K^+ gradients (except the basal K^+ gradient in control condition) were significantly different from zero both in the absence and presence of Ba^{2+}.

It is believed that the energy for transfer of K^+ from the cell to the lumen is realized by an electrogenic H^+ pump at the luminal membrane: the pump creates a cell-inward H^+ concentration gradient, driving a K^+/H^+ antiporter (Weltens *et al.*, 1992; Zhang *et al.*, submitted). Therefore, the H^+ and K^+ concentration values were used to calculate the apical H_l/H_c and K_l/K_c ratio, which determines the apical H^+ and K^+ concentration gradient,respectively, in the absence and presence of Ba^{2+} (Fig.3A). In the presence of 6 mM Ba^{2+}, the H_l/H_c ratio dropped significantly; the K_l/K_c ratio remained virtually unchanged. The overall result was that the ratio of H_l/H_c over K_l/K_c was strongly reduced from 4.5 to 1.7.

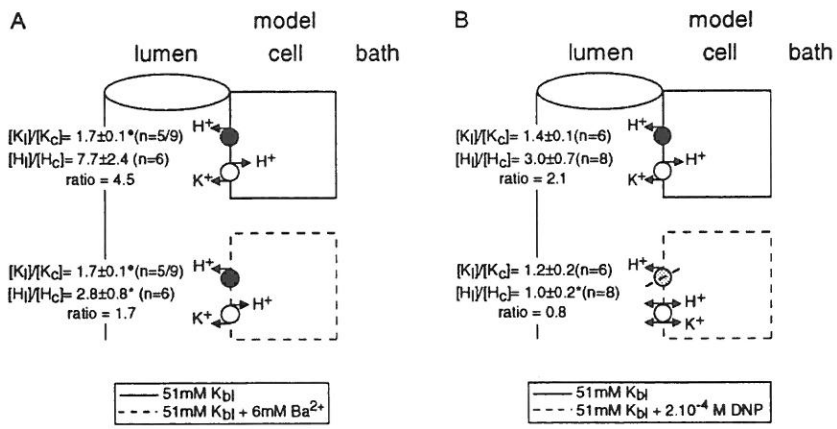

Fig. 3. Model for the apical H^+ and K^+ transport systems in the absence and presence of 6 mM Ba^{2+} (A) or 2.10^{-4} M DNP (B). The apical H^+ and K^+ concentration gradients (H_l/H_c and K_l/K_c, respectively) and their ratio are given. Mean values ±S.E..

*: intracellular and luminal measurements were made on a different series of tubules (see also Fig. 2B), therefore K_l/K_c (and the corresponding S.E.) was calculated as the ratio of the mean value of the luminal over the intracellular K^+ concentration (taken from Fig. 2B)

*: significantly different from control (paired Student's t-test, $P<0.05$).

2. The effect of 2.10^{-4} M DNP on fluid secretion, electrical potential differences and ion concentrations.

The effect of DNP was tested on the fluid secretion rate in the control solution (see Fig. 4). On application of 2.10^{-4} M DNP, fluid secretion rate was severely slowed down and stopped completely within 30 minutes. The effect of DNP was fully reversible and statistically significant.

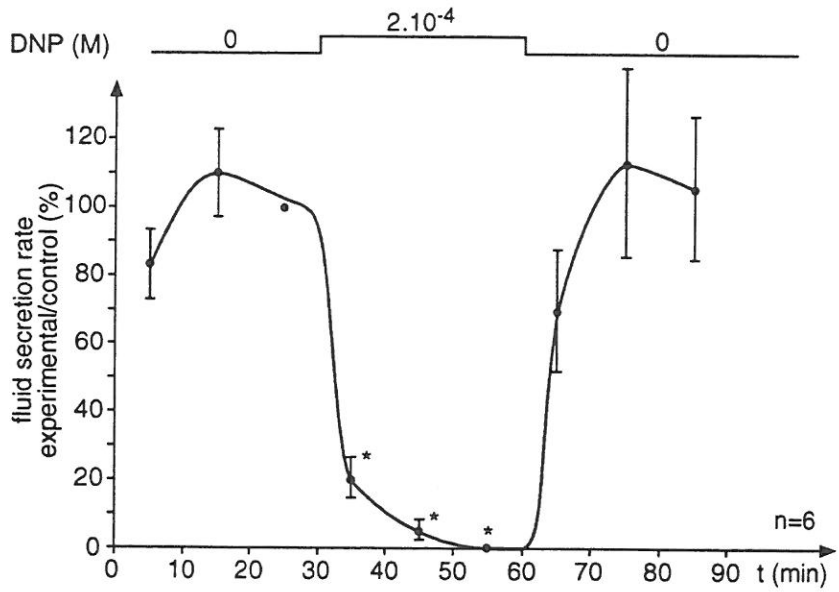

Fig. 4. The effect of 2.10^{-4} M DNP on the fluid secretion rate. To take into account any time effect on fluid secretion, the secretion was expressed as the ratio of the relative rate in the experimental condition over the relative rate in control condition. The relative rate was the absolute rate expressed as a percentage of the absolute rate in the third control period (i.e. the period just before adding a drug was taken to be 100 %). Mean values±S.E.. The absolute value of fluid secretion just before application of DNP was 180 ± 38 pl/min. (n=6).
*: statistically significant difference between the value in the presence of DNP and the corresponding control value (in time) (unpaired Student's t-test, $P<0.05$).

The effects of DNP on H^+ or K^+ concentrations were measured on two different series of tubules. Intracellular and luminal measurements of either H^+ or K^+ concentrations were obtained from the same tubule. Fig. 5A gives an example of an intracellular followed by a luminal recording of potential differences and pH in a spontaneously secreting Malpighian tubule. In this experiment, the effect of DNP was tested on V_{bl} and pH_c, and on V_{te} and pH_l in the same tubule. The addition of 2.10^{-4} M DNP to the bath solution resulted in a very fast acidification with 0.48 pH units. The effect was reversible. In the presence of DNP, the intracellular acidification was accompanied by only a slight depolarization of V_{bl}. On the other hand, after the addition of DNP, V_{te} rapidly dropped with 36 mV. Washout resulted in a fast recovery. In this experiment,

the addition of DNP caused a small reversible luminal alkalinization. However, the effects of DNP on pH_l were not unidirectional for all experiments: in 5 out of 8 experiments, a small acidification occurred.

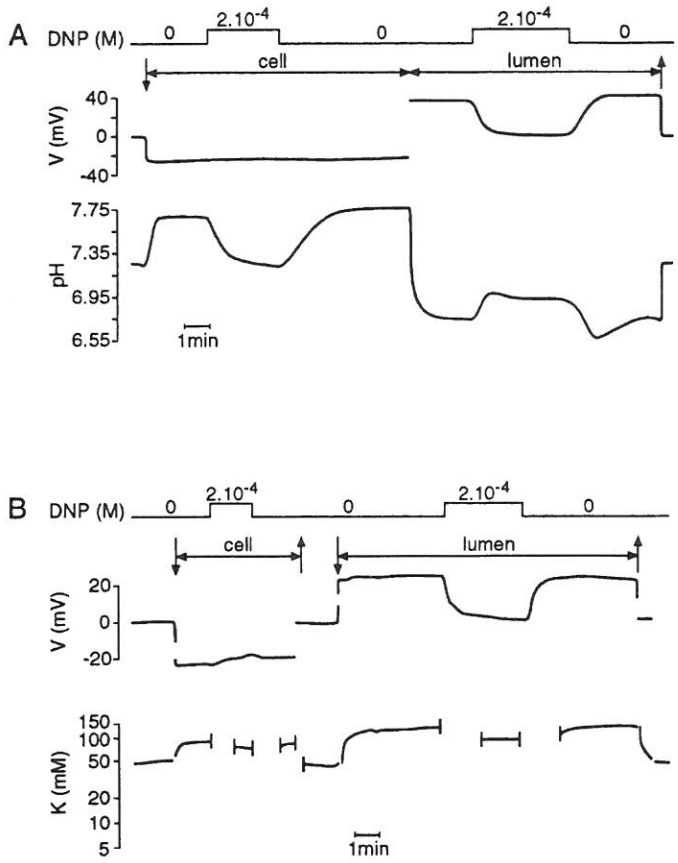

Fig. 5. Effect of the addition of 2.10^{-4} M DNP to the bath solution on the V_{bl}, pH_c, V_{te} and pH_l (A), and on V_{bl}, K_c, V_{te} and K_l (B). H^+- and K^+-selective measurements were performed on different tubules; intracellular and luminal measurements were made consecutively on the same tubule. On changing the bath solution, electrical disturbances were recorded by the K^+-selective (i.e. the most sensitive) barrel and were omitted from the figure for clarity (B).

In Fig. 5B, an example is shown of the effect of DNP on V_{bl} and K_c, followed by the measurement of V_{te} and K_l. The addition of 2.10^{-4} M DNP to the bath solution resulted in a very fast drop of K_c with 24 mM. At the same time, V_{bl} depolarized with 4 mV. After washout, V_{bl} recovered with 2 mV and K_c with 16 mM. In the presence of DNP, K_l decreased with 42 mM while V_{te} dropped with 22 mV. Washout resulted in a fast and complete recovery.

Results are summarized in Fig. 6. Adding DNP to the bath solution significantly increased H_c (Fig. 6A); the cytosol acidified from pH 7.62 to 7.09. The luminal H^+ concentration was not significantly changed; pH_l remained 7.10. The basal potential difference depolarized slightly, but a paired t-test showed that the depolarization was systematic and significant. The significant drop of V_{te} is the result of the strong significant depolarization of V_{ap} after the addition of DNP.

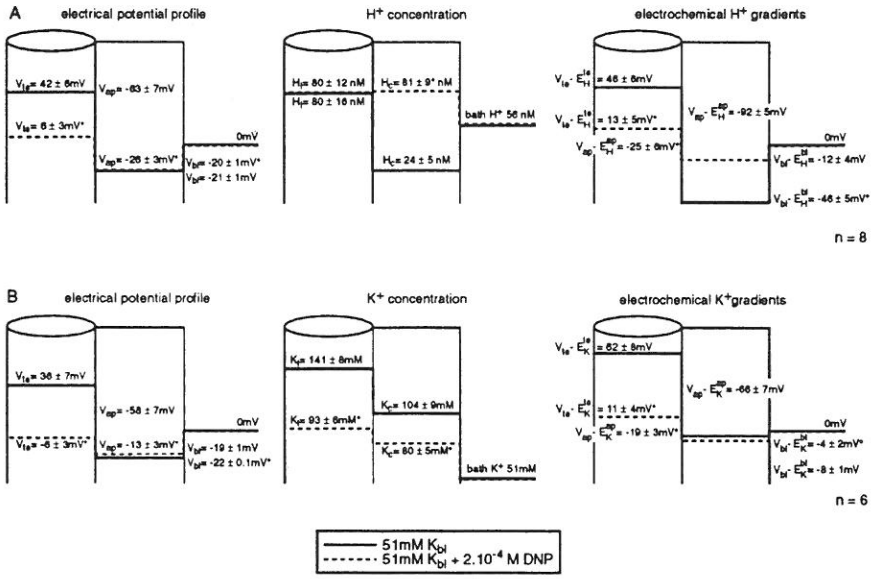

Fig. 6. Effect of 2.10^{-4} M DNP on electrical potentials and ion concentrations, and on electrochemical gradients. H^+-selective (A) and K^+-selective (B) measurements were carried out on a different series of tubules; intracellular and luminal measurements were obtained consecutively from the same tubules. The apical potential difference was expressed with reference to the lumen [$V_{ap} = -(V_{te}-V_{bl})$]. Mean values ±SE. Intracellular and luminal voltages and ion concentrations were determined after experimental treatment at 3 ± 1 (n = 8) and 3 ± 1 (n = 8) min., and after 3 ± 1 (n = 6) and 3 ± 1 (n = 6) min., for the H^+- (A) and K^+- (B) selective measurements, respectively.
*: significantly different from control (paired Student's t-test, $P<0.05$)
° not significantly different from zero ($P>0.05$).

In the batch of tubules where K^+ was measured, the K^+ concentration decreased significantly in the cell as well as in the lumen on applying DNP (Fig. 6B). In this series of tubules, the depolarizing effect of DNP on V_{bl} and V_{ap} was in the same direction as in the previous series; but now, V_{te} became even negative. The effects of DNP were fully reversible.

With the exception of the basal electrochemical K^+ gradient, all K^+ and H^+ gradients were significantly reduced on applying DNP (Fig. 6A and B). Both in the absence and presence of DNP, the mean transepithelial, apical and basal electrochemical H^+ and K^+

gradients (except the basal K^+ gradient in control condition) were significantly different from zero.

The H^+ and K^+ concentration values were used to calculate the apical H^+ and K^+ concentration ratio in the absence and presence of DNP. This is visualized in Fig. 3B. The addition of DNP caused a rise of the intracellular H^+ concentration and abolished the apical cell-inward H^+ concentration gradient. At the same time, the K^+ ratio did not change significantly. The overall result was a drop of the H_l/H_c over K_l/K_c ratio from 2.1 to 0.8.

3. The effect of K_{bl} on electrical potential differences in the presence and absence of 2.10^{-4} M DNP.

In order to investigate the sensitivity of V_{bl}, V_{te} and V_{ap} to a change of K_{bl} both in the absence and presence of DNP, the effect of 2.10^{-4} M DNP was tested with single-barrelled conventional microelectrodes on membrane and transepithelial potentials in the control solution (51 mM K_{bl}) and in a low bath K^+ Ringer solution (5 mM K_{bl}). Intracellular and luminal measurements were performed on each tubule in both 5 and 51 mM K_{bl}. An example of such an experiment is shown in Fig. 7. A decrease in K_{bl} from 51 to 5 mM resulted in a hyperpolarization of V_{bl}. After the addition of DNP, V_{bl}

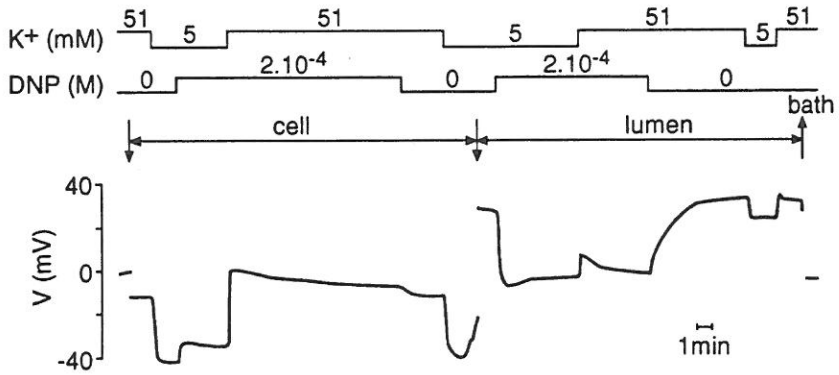

Fig. 7. Effect of a change in K_{bl} (5 and 51 mM K_{bl}) on V_{bl} and V_{te} in the absence and presence of 2.10^{-4} M DNP. Intracellular and luminal measurements were performed on the same tubule.

immediately depolarized with 9 mV. Increasing K_{bl} to 51 mM in the presence of DNP, V_{bl} reached a new steady state at -8 mV after a transient depolarization to +1 mV. Since the basal membrane behaves as a K^+ electrode (Leyssens *et al.*,1992a; Leyssens *et al.*, 1993) this transient change of V_{bl} suggests the persistence of the responsiveness of K_c to a change in K_{bl} in the presence of DNP. In between, DNP was washed out in 51 mM K_{bl}. In 5 mM K_{bl}, the impalement was lost. Subsequently, the lumen of the same tubule was impaled. V_{te} dropped to a negative potential value after the addition of DNP.

In response to an increase of K_{bl} to 51 mM in the presence of DNP, V_{te} first increased to +8 mV. After this transient increase, reflecting a transient change in V_{bl} and consequently an increase in K_c, V_{te} returned to a new steady state value of -1 mV. In the absence of DNP, decreasing K_{bl} to 5 mM resulted in a reversible depolarization of V_{te}. After washing out DNP in 51 mM K_{bl}, V_{te} returned to a positive potential value; decreasing K_{bl} to 5 mM resulted in a reversible depolarization of V_{te}. Results obtained in similar experiments are summarized in Table 1. The effect of K_{bl} on V_{te}, V_{bl} and V_{ap} was significant, both in the absence and presence of DNP. Apparently, the sensitivity of both V_{bl} and V_{ap} to changes in K_{bl}, already described by Leyssens *et al.* (1992a) for control conditions, does not disappear in the presence of DNP.

Table 1. Effect of a change from 5 to 51 mM of the bath K^+ concentration (K_{bl}) on potential differences in the absence and presence of 2.10^{-4} M DNP. Mean ± S.E. (n = 5).

	V_{bl} (mV)		V_{te} (mV)		V_{ap} (mV)	
K_{bl} (mM)	control	2.10^{-4} M DNP	control	2.10^{-4} M DNP	control	2.10^{-4} M DNP
5	- 50 ± 3	- 44 ± 3*	35 ± 5	- 14 ± 4*	- 85 ± 8	- 30 ± 2*
51	-17 ± 2	- 12 ± 2*	49 ± 10	- 4 ± 1*	- 66 ± 10	- 8 ± 1*
Δ (5 - 51)	33 ± 1°	32 ± 1°*	14 ± 4°	10 ± 2°*	19 ± 3°	22 ± 1°

Results were obtained from "paired" cellular - luminal measurements; measurements in 5 and 51 mM K_{bl} were performed on the same tubule.
*: significantly different from control (paired student's t-test, $P < 0.05$)
°: Δ (5 - 51 mM K_{bl}) significantly different from zero ($P < 0.05$)

Comparing the effect of K_{bl} on V_{bl} and V_{te} in the absence and presence of DNP, although significant, the difference was minimal; for V_{ap}, there was no significant difference.

The results in 51 mM K_{bl} confirmed those obtained with the reference barrel of the ion-sensitive electrodes (see 'Results', section 2): DNP caused a small but significant depolarization of V_{bl}, and a more pronounced reduction of V_{te} and V_{ap}. In 5 mM K_{bl}, the addition of DNP also resulted in a strong, significant depolarization of V_{te} and V_{ap}. As was the case in 51 mM K_{bl}, the effect on V_{bl} was smaller but also significant .

4. The effects of 2.10^{-4} M DNP on electrical potential differences in the presence of 6 mM Ba^{2+}.

Ba^{2+} causes a strong hyperpolarization of V_{bl} and V_{ap} (Weltens *et al.*,1992). Weltens *et al.* (1992) concluded that, under Ba^{2+} treatment, electrical events occurring at the apical membrane become more visible, and that the Ba^{2+} induced hyperpolarization of V_{bl} and V_{ap} is due to an active and electrogenic transport mechanism in the apical membrane which is probably a H^+ pump. Therefore, we also tested the effect of 2.10^{-4} M DNP on V_{bl} and V_{te} in the presence of 6 mM Ba^{2+} (experiments with single-barrelled conventional microelectrodes). A typical example of an intracellular and luminal recording (two examples obtained from different tubules) is given in Fig. 8A and B, respectively. In the presence of 6 mM Ba^{2+}, V_{bl} strongly hyperpolarized (Fig. 8A). The addition of DNP caused a very fast drop of V_{bl} to 0 mV.

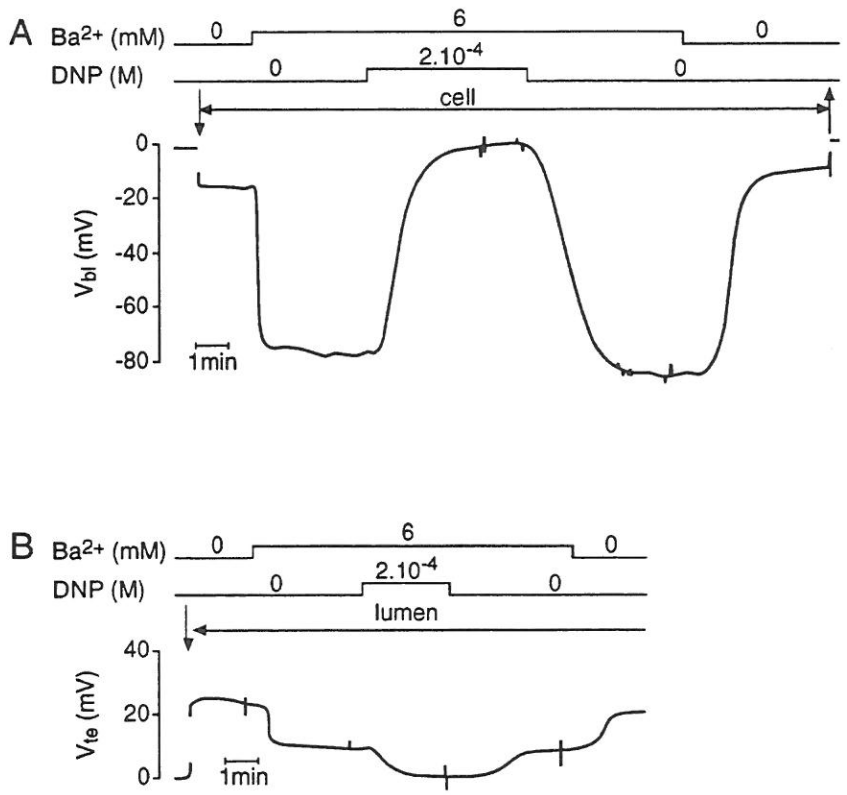

Fig. 8. Effect of the addition of 2.10^{-4} M DNP in the presence of 6 mM Ba^{2+} on V_{bl} (A) and V_{te} (B).

Washout resulted in a fast recovery of V_{bl}. When washing out Ba^{2+}, V_{bl} returned close to its control value. The addition of Ba^{2+} caused a drop of V_{te} to 10 mV (Fig. 8B). After adding DNP, V_{te} became 0 mV. The effect of DNP was reversible.

The results are summarized in Fig. 9. Intracellular and luminal measurements were performed consecutively on the same tubules. Prior to the addition of Ba^{2+}, V_{bl}, V_{te} and V_{ap} were -13±1, +30±4 and -43±4 mV, respectively. Both V_{bl} and V_{ap} depolarized completely to 1 mV, when adding DNP in the presence of Ba^{+}. The effect of DNP in the presence of Ba^{2+} was fully reversible.

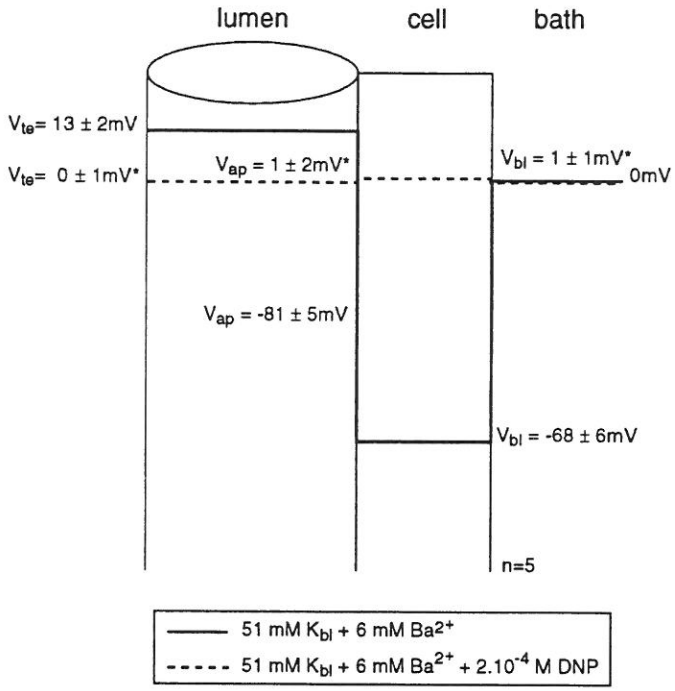

Fig. 9. Effect of the addition of 2.10^{-4} M DNP in the presence of 6 mM Ba^{2+}. Summary of "single tubule V_{bl} and V_{ap} measurements". Mean values ±SE. V_{te} and V_{bl} values were determined at 3 ± 1 and 3 ± 1 min. (n = 5), and at 3 ± 1 and 3 ± 1 min. (n = 5) after Ba^{2+} and Ba^{2+} + DNP treatment, respectively.
*: significantly different from control (paired Student's t-test, $P<0.05$).

DISCUSSION

1. The inwardly directed apical proton concentration gradient can be abolished either by hyperpolarization of the apical membrane or by a protonophore action.

a. Effect of 6 mM Ba^{2+} on H_l/H_c.

Ba^{2+} was found to cause a pronounced hyperpolarization of V_{bl} and V_{ap} in Malpighian tubules of *Formica*, possibly by blocking K^+ uptake through K^+ channels in the basolateral membrane, and subsequently allowing an electrogenic cation pump to build up a higher electrical potential difference across the apical membrane (see Weltens *et al.*, 1992). Ba^{2+} is known to selectively block K^+ channels in a variety of tissues, including insect epithelia (Moffett and Koch, 1985; Hanrahan *et al.*, 1986). The hyperpolarization of V_{ap} should reduce the part of the electromotive force of the hypothetical electrogenic H^+ ATPase that can be used in building up a H^+ concentration gradient. Our results confirm this hypothesis: the increase of V_{ap} in the presence of Ba^{2+} was accompanied by an acidification of the cytosol and an alkalinization of the lumen (Fig. 2A); H_l/H_c dropped from 7.7 to 2.8 (Fig. 3A). The new steady state level of H_l and H_c could be the result of the reduced active transport of protons from the cell to the lumen via the H^+ pump, some backleak of protons via the shunt, and passive movements of H^+ and K^+ via the hypothetical K^+/H^+ exchange system.

b. The effect of 2.10^{-4} M DNP on H_l/H_c.

The fast and pronounced acidification of the cell may be the result of a protonophore effect of DNP across -at least- the basal membrane. To realize rapid crossing of H^+ ions, Mc Laughlin and Dilger (1980) suggested that a dimer ($DNP + DNP^- \leftrightarrow DNP_2^-$) was formed in the mitochondrial membrane. In the same way 2 DNP molecules may enter the cell of a Malpighian tubule and return to the bath as DNP and DNP^-, leaving one H^+ in the cell. That the basal membrane of the Malpighian tubule is still sensitive to K_{bl} as in control conditions implies that $g_K >> g_{DNP2^-}$ and that DNP_2^- would be distributed across the membrane according to V_{bl}, which in this case is close to E_K^{bl}. We expect that the highly lipophilic neutral form of DNP will tend to be evenly distributed across the membrane. If so, it can be calculated that the system attains equilibrium when $K_c/K_{bl} \approx H_c/H_{bl}$. This is in agreement with the results:
K_c/K_{bl} = 80 mM/51 mM and H_c/H_{bl} = 81 mM/56 mM. Due to the intracellular acidification, the gradient across the apical membrane against which the pump is secreting H^+ ions would be lost and as long as ATP is available, the pump would continue transferring H^+ ions at an increased rate rapidly depleting the cell of ATP and eventually stopping the pump. This in turn would result in a depolarization of V_{ap} as was indeed observed. As yet we have no evidence for a protonophore effect across the apical membrane. Dijkstra *et al.* (1993) did not observe an effect of 2.10^{-4} M DNP on the voltage divider ratio, i.e. the ratio of the apical (R_{ap}) over the basal (R_{bl}) membrane

resistance. As the control VDR is very high (ca 40) a protonophore effect -if present in both membranes- is expected to decrease R_{ap} but not R_{bl}, the high g_K masking any new and smaller conductance appearing in the basal membrane.

Similar effects of DNP on V_{te} were reported in other species. Applying DNP at the bath side caused a prompt and reversible decline of V_{te} in non-perfused (Nicolson and Isaacson, 1987) or luminally perfused (Isaacson *et al.*, 1989) Malpighian tubules of the desert beetle *Onymacris plana* (1mM DNP), and in perfused Malpighian tubules of the mosquito *Aedes aegypti* (1 mM DNP, Williams and Beyenbach, 1984; 0.1 mM DNP, Pannabecker *et al.*, 1992). When injected luminally, 1 mM DNP caused a rapid decline of V_{te} in the rectal tubule complex of *Tenebrio molitor L.* This decline was also explained by an inhibitory effect on the apical H^+ pump (O'Donnell and Machin, 1991).

The effect of DNP on the apical H^+ pump activity becomes even more pronounced under Ba^{2+} treatment. Blocking the basolateral K^+ conductance hyperpolarizes V_{bl} and V_{ap}. This large cell negative potential, built up by the electrogenic H^+ pump in the apical membrane (see Weltens *et al.*, 1992), can be abolished by the action of specific V-type ATPase blockers (NEM and bafilomycin A_1) (see Weltens *et al.*, 1992) and as shown also by the action of DNP (see Fig. 9). The fact that V_{bl} and V_{ap} drop to 0 mV in the presence of Ba^{2+} + DNP seems to indicate that electromotive forces other than the basal diffusion potential for K^+ and the apical electrogenic pump are of little importance in determining electrical potential differences across both cell membranes.

Both an inhibition of the apical H^+ pump activity, due to depletion of ATP and a shunting of the active proton transfer, by a protonophore action at the cell membrane, will result in the loss of the actively built up H^+ concentration gradient. Furthermore, if an electroneutral K^+/H^+ antiporter is functional in the apical membrane, changes in intracellular and luminal H^+ and K^+ concentrations occurring upon administation of DNP will be mutually dependent (see also 'Discussion, section 2'): as the apical H^+ concentration gradient is dissipated, the K^+ and H^+ concentration gradient will approach each others value. This is what is observed in the present study: in the presence of DNP H_l/H_c drops from 3.0 to 1.0, and K_l/K_c decreased (but not statistically significantly) from 1.4 to 1.2 (see Fig. 3B).

In conclusion, Ba^{2+} and DNP had opposite effects on the basolateral and apical membrane potential, but both reduced the apical H^+ concentration gradient. Since Ba^{2+} caused a hyperpolarization and DNP a depolarization, and both caused the cell to acidify, pH_c alterations via voltage-dependent changes in basolateral and/or apical H^+ transport are unlikely. These findings are in contrast with the observations in principal cells of frog skin which indicate an important role of a voltage-dependent basal and/or apical H^+ or OH^- conductance pathway in the regulation of pH_c (Lyall *et al.*, 1992).

2. An unfavourable H_l/H_c over K_l/K_c ratio is correlated with a reduction in fluid secretion.

a. Fluid secretion and H_l/H_c over K_l/K_c ratio in the presence of 6 mM Ba^{2+}.

In Malpighian tubules of *Formica*, Ba^{2+} reduced the fluid secretion rate to about 8 % of its control value (Weltens *et al.*, 1992). If one assumes that the apical K^+ extrusion takes place via an electroneutral K^+/H^+ exchange system, the lowered fluid secretion rate should be associated with a reduced H_l/H_c over K_l/K_c ratio. The present data confirmed the hypothesis. The calculated H_l/H_c over K_l/K_c ratio was strongly reduced in the presence of Ba^{2+}; it dropped from 4.5 to 1.7 (see Fig. 3A). Since the secretion was greatly reduced by Ba^{2+}, a ratio closer to 1 would be expected. This discrepancy could be explained by the fact that, due to experimental constraints, H^+- and K^+-selective measurements were performed on a different series of tubules.

The decrease in H_l/H_c over K_l/K_c was primarily due to a drop in H_l/H_c; K_l/K_c remained constant although the absolute values of K_l and K_c significantly decreased in the presence of Ba^{2+}. But they did so to the same extent (see Fig. 2B and 3A). Since we assume that the basolateral K^+channels may be an important pathway for K^+ uptake into the cytoplasm (see Leyssens *et al.*, 1993), it is expected that blocking this pathway will affect the secretion rate and also the intracellular K^+ content. This is in accordance with the reported effects of Ba^{2+} on another K^+ transporting epithelium (i.e. the midgut of *Manduca sexta* , Zeiske *et al.*, 1986; Moffett *et al.*, 1988). The addition of 2 mM Ba^{2+} to tissues bathed in 32 mM K^+ lowered the short-circuit current carried by K^+, and induced both a large basal hyperpolarization and a concomitant reduction of K_c. The luminal K^+ concentration in the Malpighian tubules of *Formica* was also significantly lowered in the presence of Ba^{2+}. This could be explained by the dynamic balance between the residual active K^+ transport into the lumen (which will be dependent on the apical H^+-gradient, see 'Discussion, section 1') and some diffusional backflux via the paracellular pathway.

b. Fluid secretion and H_l/H_c over K_l/K_c ratio in the presence of 2.10^{-4} M DNP.

The abolition of the cell-inward H^+ gradient resulted in an unfavourable H_l/H_c over K_l/K_c ratio (see Fig. 3B): it decreased from 2.1 to 0.8. Consequently, if apical K^+ extrusion occurs via an electroneutral K^+/H^+ antiporter, fluid secretion is expected to stop. This is what was observed (see Fig 4). An inhibitory effect of DNP and other metabolic inhibitors (cyanide, iodoacetate) on fluid secretion has also been described for tubules of *Calliphora* (Berridge, 1966), *Rhodnius* (Maddrell, 1969) and *Musca domestica* (Dalton and Windmill, 1980).

Again, the decrease of H_l/H_c over K_l/K_c was primarily the result of a strong reduction of H_l/H_c. On adding DNP, the K_l/K_c ratio was relatively unchanged: K^+ was reduced but to the same extent in the cell and the lumen. The addition of DNP resulted in an abrupt drop of K_c that was reflected in the small but statistically significant

depolarization of V_{bl} (see Fig. 6B). Until now, the cause for the sudden decrease in K_c remains unknown. An explanation might be the inhibition by DNP of an active K^+ uptake system, for instance a Na^+/K^+ or K^+/H^+ ATPase. The presence of a Na^+/K^+ ATPase was demonstrated biochemically in Malpighian tubules of *Locusta* (Fogg *et al.*, 1991) and functionally in Malpighain tubules of *Locusta* (Anstee *et al.*, 1979; Morgan and Mordue, 1981; Baldrick *et al.*, 1988), *Drosophila* (Wessing *et al.*, 1987; Bertram *et al.*, 1991), *Aedes* (Hegarty *et al.*, 1991) and *Rhodnius* (Madrell and Overton, 1988). On the other hand, unlike in vertebrate renal epithelia (see Okusa *et al.*, 1992; Gifford *et al.*, 1992) no reports have been made upon the presence of an K^+/H^+ ATPase in insect Malpighian tubules. Zhang *et al.* (submitted) however suggested that the extrusion of protons at the basal side via such an active exchange mechanism may be needed to explain the lowering of the proton concentration level in both the lumen and the cell when increasing the bath K^+ concentration in Malpighian tubules of *Formica*.

In the midgut of *Manduca sexta*, the effect of metabolic inhibition was studied via N_2-hypoxia. A decrease in K_c was not observed: hypoxia reduced the transepithelial K^+ transport (I_{sc}) by affecting the apical pump however; V_{bl} depolarized but K_c increased slightly (Moffett and Koch, 1988; Chao *et al.*, 1990). The significant reduction of K_l in Malpighian tubules of *Formica* could be the result of a block of the apical K^+ extrusion via the apical K^+/H^+ antiporter and a backflux via the paracellular pathway, even via the K^+/H^+ antiporter (the H_l/H_c over K_l/K_c ratio became smaller than 1 in the presence of DNP).

On the other hand, the basal membrane properties do not seem to have changed. In the presence of 2.10^{-4} M DNP, R_{bl} is unaffected (Dijkstra *et al.*, 1993). The basal membrane potential is still sensitive to changes in K_{bl} (see Table 1) and V_{bl} is still close to E_K^{bl} (see Fig. 6B). Furthermore, transient changes in V_{bl} on changing K_{bl} still occur in the presence of DNP (see Fig. 7). This suggests that the intracellular K^+ still responds to a change in K_{bl} after adding DNP (see also Leyssens *et al.*, 1992a for control conditions). Therefore, a primary inhibitory effect of DNP on K^+ entry through K^+ channels is unlikely.

In conclusion, although the presence of active basal K^+ uptake systems (i.e. Na^+/K^+ ATPase or K^+/H^+ ATPase) that would also be inhibited by DNP is possible, the complete inhibition of the fluid secretion rate by DNP is probably due primarily to the abolition of the apical H^+ concentration gradient. Also, the intracellular acidification, occurring upon administration of DNP, by itself, may affect the activity of the apical K^+/H^+ antiporter.Brey *et al.* (1980) demonstrated in *Escherichia coli* that the activity of the K^+/H^+ antiporter, reaching a maximum as the pH of the cytosol approaches 8, is greatly reduced at a cytosolic pH of 7.2.

c. Could the apical K^+/H^+ antiporter be electrogenic?

Previous results fitted the hypothesis of an electroneutral exchange system (Zhang *et al.*, submitted; Leyssens *et al.*, 1993), but electrogenicity of the K^+/H^+ antiporter could not be excluded. In the present study, we propose that the K^+/H^+ antiporter in the luminal membrane is electroneutral. Driving forces could be calculated for apical K^+ extrusion via a m H^+/ n K^+ antiporter for a tubule that was secreting normally (control) or not at all (+DNP) (see Fig. 10). Different values for m and n were considered. In control conditions, when tubules are secreting normally, an appropriate driving force must be present to allow apical K^+ extrusion and hence fluid secretion. In the presence of DNP, the calculated driving force should be near zero. As can be seen in Fig. 10, only if the antiporter is electroneutral, a cell-outwardly directed driving force for net K^+ secretion was found in control conditions which tended to zero in the presence of DNP.

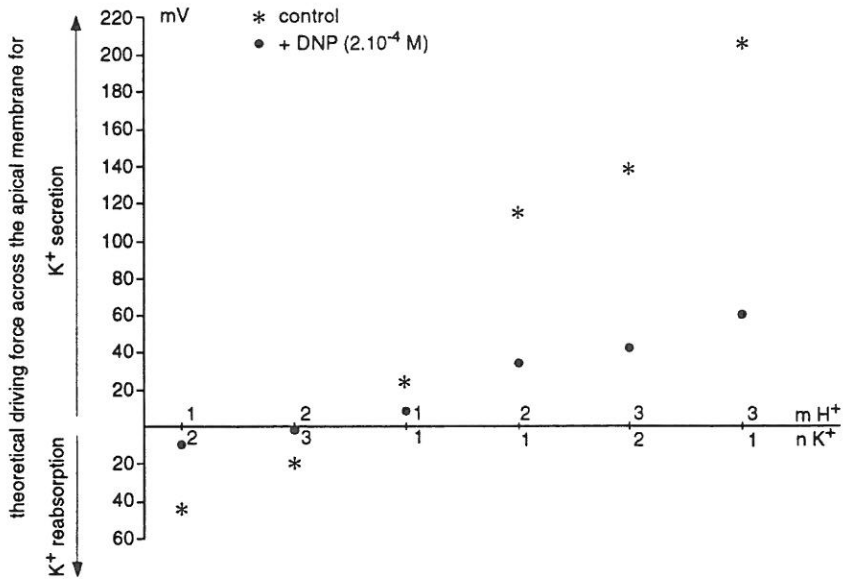

Fig. 10. Calculated driving force for an apical electroneutral or electrogenic mH^+/nK^+ antiporter in the absence and presence of 2.10^{-4} M DNP.

Theoretically (see Aronson, 1981), the free energy change (ΔG) necessary to realize net passive H^+ uptake and K^+ extrusion via a mH^+/nK^+ antiporter is:

$\Delta G = m\,\Delta m_H^{c\text{-}l} - n\,\Delta m_K^{c\text{-}l}$ (with $\Delta m_H^{c\text{-}l}$ and $\Delta m_K^{c\text{-}l}$ the electrochemical gradient across the apical membrane for H^+ and K^+, respectively)

When expressed as a potential difference across the apical membrane:

$\Delta G/F = (m\,V_{ap1} - m\,RT/F\,\ln H_l/H_c) - (n\,V_{ap2} - n\,RT/F\,\ln K_l/K_c)$ (V_{ap} expressed with the lumen as reference)

H_l/H_c and K_l/K_c values were taken from Fig. 3B, V_{ap} values from Fig. 6, V_{ap1} and V_{ap2} were obtained from the H^+- and K^+-selective measurements, respectively);

if $\Delta G/F<0$, K^+ ions are secreted into the lumen in exchange for H^+; if $\Delta G/F>0$, net passive movement of K^+ and H^+ would occur in the opposite direction; if the driving force =0, net apical K^+ or H^+ transport via the antiporter is abolished.

A small driving force for K^+ secretion was still present, but this may be due to the experimental conditions. Since H^+- and K^+-selective measurements were performed on a different series of tubules, the variability in electrophysiological parameters amongst tubules, for instance a different value for V_{ap} for the two series of tubules, will introduce some error in the calculation.

These findings are in contrast with those of Chao *et al.* (1991), presented for *Manduca sexta* midgut: the pH gradient across the goblet cell apical membrane was of the wrong polarity to drive K^+ secretion from cytoplasm to goblet cavity by an electroneutral antiporter. In this K^+ transporting system, an electrogenic antiporter, transferring at least 2 H^+'s for 1 K^+, was proposed by Wieczorek *et al.* (1991).

3. Conclusions.

The realization of a cell-inward H^+ concentration across the apical membrane was inhibited by Ba^{2+} due to the hyperpolarization of V_{ap}, increasing the electrical component of the proton motive force of the electrogenic apical H^+ pump, and by DNP due to depletion of ATP and to a direct protonophore action of DNP at the basal cell membrane. The K^+ concentration gradient remained virtually unchanged. This resulted in an unfavourable H_l/H_c over K_l/K_c ratio which was correlated with a reduced fluid secretion rate. Thus by using Ba^{2+} and DNP, we presented evidence for the role of an apical H^+ pump, creating an apical lumen to cell directed H^+ concentration gradient which is larger than the K^+ concentration gradient present. Furthermore evidence was presented that the K^+/H^+ antiporter using this H^+ gradient to drive K^+ extrusion is electroneutral.

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PAPER 5

MECHANISMS OF K^+ UPTAKE ACROSS THE BASAL MEMBRANE OF MALPIGHIAN TUBULES OF *FORMICA*: THE EFFECT OF EXTERNAL IONS AND INHIBITORS.

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MECHANISMS OF K^+ UPTAKE ACROSS THE BASAL MEMBRANE OF MALPIGHIAN TUBULES OF *FORMICA*: THE EFFECT OF EXTERNAL IONS AND INHIBITORS.

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Key words: Malpighian tubules, K^+ uptake mechanisms, fluid secretion, electrical potential differences, intracellular and luminal K^+ concentrations, ion substitution, dihydro-ouabain, Sch28080, vanadate, bumetanide.

Running title: External ions and inhibitors on Malpighian tubules.

ABSTRACT

In the present study, the existence of active and passive co- and countertransport systems, and their role in K^+ uptake, were investigated in Malpighian tubules of *Formica*. Alterations in the ionic composition of the bathing media were performed, and various well-known pharmacological agents were tested in different bath K^+ concentrations (i.e. 5, 10, 51 and 113 mM) on the fluid secretion rate, transepithelial and transmembranal electrical potential differences, and/or intracellular and luminal K^+ concentrations.

It is shown that in the presence of 6 mM Ba^{2+}, when the K^+ sensitivity of the basal membrane potential (V_{bl}) is lost, indicating a blockage of K^+ channels, the intracellular K^+ concentration (K_c) increases to the same amount and with a similar time course in response to a rise in bath K^+ as in the absence of Ba^{2+}.

The Na^+/K^+ ATPase inhibitor dihydro-ouabain (10^{-3} M) did not affect the fluid secretion rate or the electrical potential differences; nor was an effect of the K^+/H^+ ATPase blocker Sch28080 (10^{-4} M) observed on the fluid secretion. On the other hand, vanadate (10^{-3} M), known as an effective blocker of both active carrier systems, had a strong inhibitory effect on the fluid secretion rate in Na^+-containing but also in Na^+-free conditions. Furthermore, the transepithelial potential difference (V_{te}) depolarized on administration of vanadate; in the presence of Ba^{2+}, the effect was also reflected on V_{bl}; K_c or the luminal K^+ concentration were not affected. Therefore it was concluded that the effects of vanadate must probably be explained by an effect on the apical H^+

pump. On the other hand, in a low K^+, high Na^+ bath solution, the fluid secretion rate was decreased when Na^+ was omitted from the bath solution.
Bumetanide, a blocker of the K^+ (, Na^+) and Cl^- cotransport system, applied at a concentration of 10^{-4} M, did not influence fluid secretion in high K^+, Na^+-free conditions, but significantly diminished the fluid secretion rate in 51 mM K_{bl}. In a low K^+ high Na^+ medium, a concentration of 10^{-5} M was already effective. Substitution of Cl^- by Br^- was used as a criterium to distinguish between a K^+/Cl^- and a $K^+/Na^+/2Cl^-$ cotransporter: on replacing Cl^- by Br^- the fluid secretion was stimulated in 51 mM K_{bl} and inhibited in the low K^+, high Na^+ medium, suggesting the functioning of a K^+/Cl^- and a $Na^+/K^+/2Cl^-$ cotransporter, respectively.
The effect of bumetanide (10^{-4} M) was electrically silent and did not lower K_c. The rise in K_c on increasing K_{bl} was not hampered on adding bumetanide to the bath solution.
A model for transcellular K^+ transport is proposed. The role of a basal active K^+ uptake system, if present, in maintaining the cellular K^+ content and hence the fluid secretion rate is apparently of little importance and limited to a low K^+, high Na^+ medium. Besides the possibility of K^+ influx through K^+ channels, a coupled entry mechanism of K^+ (,Na^+) and Cl^- is proposed. An attempt has been made to distinguish between the relative importance of a K^+/Cl^- and $Na^+/K^+/2Cl^-$ cotransporter. K^+ channels and cotransporters may function at different rates depending on the bath K^+ (and Na^+) concentration and may thereby determine the amount of cellular K^+ available for apical K^+ transport to the lumen. The rise in K_c on increasing the bath K^+ can probably occur both via K^+ channels and via a coupled K^+/Cl^- entry mechanism.

INTRODUCTION

Malpighian tubules of forest ants (*Formica polyctena*) secrete fluid and concentrate K^+ and Cl^- in the lumen. The fluid secretion rate is strongly dependent on the bath K^+ concentration (Van Kerkhove *et al.*, 1989).
Since K^+ is always transported across the epithelium against its electrochemical gradient, K^+ transport has to occur transcellularly. The active step is primarily situated in the apical membrane where the extrusion of K^+ into the lumen is believed to be realized via the combination of an electrogenic H^+ pump and an electroneutral K^+/H^+ antiporter (Weltens *et al.*, 1992; Zhang *et al*; 1992; Dijkstra *et al.*, in press; Leyssens *et al.*, 1993). As an intrinsic regulatory mechanism, the bath K^+ concentration determines the cellular K^+ content available for apical K^+ transport (Leyssens *et al.*, 1992; Leyssens *et al.*, 1993).
Basal K^+ transport systems have to exist to ensure a sufficiently large K^+ uptake to maintain fluid secretion, and to account for the adaptation of the intracellular K^+

concentration to the surrounding bath K^+concentration. K^+ entry may occur passively through K^+ channels. Ion substitution and K^+-selective measurements demonstrated an appreciable K^+ permeability of the basal membrane (Leyssens *et al.*, 1992; Leyssens *et al.*, 1993). The hyperpolarization of V_{bl}, observed when blocking the K^+ channels with Ba^{2+}, in all bath K^+concentrations (Weltens *et al.*, 1992), suggested the existence of an electrochemical K^+ gradient favourable for K^+ uptake. On the other hand, the calculated electrochemical gradient for K^+ across the basal membrane was very small and less inward as the bath K^+ concentration decreased (Leyssens *et al.*, 1993). Furthermore, in the presence of Ba^{2+}, when the basal electrical resistance was largely increased and the K^+ sensitivity of the basal membrane potential was lost, indicating a blocked state of the basal K^+ channels, the fluid secretion rate was reduced but not completely abolished (Weltens *et al.*, 1992). These findings suggested that, as in other K^+ transporting Malpighian tubules (for a review see Nicolson, 1993), alternative pathways for K^+ uptake may be present in the basal membrane, and that the relative rate of these hypothetical K^+ transport systems may vary in different transport rate conditions.

The present study focuses on these alternative routes for K^+ transport across the basal membrane in different bath K^+ concentrations.

First of all, the existence and functioning of an active K^+ uptake system is investigated. To detect any Na^+/K^+ ATPase activity, Na^+ substitution experiments were performed, and the effect of ouabain on fluid secretion rate and electrical potential differences was studied. Zhang *et al.* (submitted) suggested that the extrusion of protons at the basal side via a K^+/H^+ ATPase might be necessary in some conditions in Malpighian tubules of *Formica*. Therefore the specific inhibitor Sch28080 was tested on fluid secretion. Vanadate, a possible blocker of both active systems, was tested on fluid secretion, electrical potential differences and intracellular and luminal K^+ concentrations. It tended to have an effect on the apical H^+ pump however.

Secondly, a reduction of the basal Cl^- concentration, replaced by $citrate^{3-}$ or SO_4^{2-}, had an inhibitory effect on the fluid secretion rate (Van Kerkhove *et al.*, 1989). Failing to observe a relative Cl^- conductance in the basal membrane (Leyssens *et al.*, 1992), we have examined this epithelium for evidence of electroneutral coupled transport of K^+, Na^+ and Cl^-, i.e. via a K^+/Cl^- or $K^+/Na^+/2Cl^-$ cotransport system. In order to identify and distinguish these hypothetical systems in Malpighian tubules of *Formica* in different bath K^+ concentrations, different approaches have been made: the sensitivity of the fluid secretion rate to bumetanide, its Na^+ dependence, and the potency of Br^- ions to inhibit or stimulate fluid secretion. The effects of bumetanide have been further investigated on electrical potential differences and on intracellular K^+ concentrations.

It is shown that the rise in intracellular K^+ concentration on increasing the bath K^+ still occurs on blocking the basal K^+ channels. The existence of a basal coupled entry mechanism for Cl^- and K^+ is clearly demonstrated and its contribution to K^+ uptake in

different transport rate conditions is discussed. At the same time, the role of Na^+ in transcellular ion transport is addressed. The presence of a primary active K^+ uptake system is critically evaluated. Based on these and previous findings, a transport model for transepithelial ion transport is proposed.

METHODS

1. Preparation.

Worker ants of the species *Formica polyctena* were collected from natural nests at the periphery of woods and kept in an artificial nest at a constant temperature of 20 °C. The animals were fed with sugar and water.

After dissection, a single Malpighian tubule (1-2 mm) was transferred on the stage of an inverted microscope to a bathing droplet (50 µM). The bathing droplet was covered with paraffin oil to avoid evaporation (see Van Kerkhove *et al.*, 1989).

2. Experimental set-up.

Fluid secretion experiments.

The technique used for measuring secretion rate was described in detail by Van Kerkhove *et al.* (1989). The secreted fluid was collected every 10 min. by means of a collection pipette with a hydrophobic tip, and blown out under paraffin oil. By measuring the diameter of a perfectly spherical droplet with a Zeiss monofilar ocular (8x) through two objectives (16x and 40x), the secretion rate could be calculated.

The effect of external ions and inhibitors on fluid secretion rate was tested in 143 mM Cl^- Ringer solutions, containing varying K^+ concentrations (see "Artificial salines" and "Results"). A fluid secretion experiment consisted of a control, test and wash-out period. The bathing fluid could be renewed using a perfusion and suction pipette. When a change in bathing solution was needed, the new solution was added via the perfusion pipette at a rate of 150 µl/min during a 3 min. period. Perfusion was then stopped until the next change in bath solution. As described previously (Weltens *et al.*, 1992), in each individual experiment secretion rate was expressed as a percentage of the rate during the last control period before changing the experimental solution. Furthermore in control experiments (not shown), i.e. experiments where fluid secretion was followed in the control solution over the entire duration of the experiment, on average the fluid secretion rate declined with time. For all control solutions tested (bath K^+= 5, 10, 51, 113 mM), the difference with the reference period (i.e. the same period in time as in the experimental series) became statistically significant at the level of 5 %, not after a period of 60 minutes, i.e. the minimal duration of the test and wash-out period of a secretion experiment. Therefore the mean values of a series of experiments were expressed as relative rates of the experimental series over a control series.

In order to test the statistical significance of a drug effect, the experimental relative rate was compared with the control relative rate at the same moment in time (unpaired Student's t-test). The drug effect was considered to be fully reversible when no statistically significant difference (unpaired Student's t-test) was found between the relative secretion rate during a wash-out period and the corresponding control time period. Although we realize that this method was not ideal (the change in fluid secretion with time in the absence of a test solution had to be evaluated on a different set of tubules), it seemed the most suitable way to distinguish drug effects from time effects. Corrected results, taking into account the spontaneous decline of the fluid secretion rate, are shown in the figures and tables (see "Results").

Electrical potential difference measurements.

The method was described in detail previously (Leyssens *et al.*, 1992). Measurements were performed with microelectrodes filled with 0.1 M KCl (resistance about 300 MΩ) (Borosilicate filament glass, Hilgenberg, Malsfeld, Germany; O.D. 1.3 mm, I.D. 0.7 mm). It was demonstrated that these electrodes showed little change in tip and liquid junction potential when exposed to changing K^+ and Cl^- concentrations as may occur when impaling a cell (Leyssens *et al.*, 1993). The electrode was connected to a high impedance electrometer (Duo 773, WPI) via an Ag/AgCl wire. The reference electrode was a low resistance (1 MΩ) 3 M KCl electrode, connected to the earth via an Ag/AgCl wire. In order to facilitate impalements, the Malpighian tubule was immobilized in the bathing droplet by two holding pipettes. The secreted fluid remained in the tubule. The measurement of the basal membrane potential difference (V_{bl}) was accepted if a sudden negative potential deflection occurred and was stable at least a few minutes, and if the electrode potential differed maximally 3 mV from the baseline after withdrawal. The transepithelial potential difference (V_{te}) was measured by advancing the microelectrode through the cell layer into the lumen of the tubule; V_{bl} and V_{te} were expressed with reference to the bath side. The apical potential difference (V_{ap}) was calculated as the difference between the transepithelial and transbasal potential difference and expressed with reference to the lumen ($V_{ap} = -(V_{te}-V_{bl})$).

The bathing droplet was continuously perfused at a constant rate of 150 µl/min.

K^+-selective measurements.

The technique used for the construction of double-barrelled ion-sensitive electrodes was described previously (see Leyssens *et al.*, 1993). In summary, double-barrelled electrodes were drawn from paired filament containing glass capillaries with unequal diameter (OD_1=1.5 mm, ID_1=0.87 mm; OD_2=0.75 mm, ID_2=0.35 mm; Hilgenberg, Malsfeld, Germany) on a horizontal puller with an air jet cooling system (Model-77, Sutter Instrument U.S.A.). A tip diameter of approximately 1 µm was obtained. The

inside of the larger barrel was silanized (hexamethyldisilazane; Janssen Chimica), the smaller barrel remained hydrophylic. A short column of the ion-selective liquid K^+ exchanger (Corning 477317) was introduced into the tip of the larger barrel, i.e. the ion-selective barrel, and the rest of this barrel was backfilled with 1 M KCl. The other barrel, i.e. the reference barrel, was simply backfilled with 1 M Na acetate + 10 mM KCl.

The filled electrodes were beveled in a rotating polishing alumina solution (No. 3 AB Gama Polishing Alumina, Buehler, U.S.A.). After beveling, the ion-sensitive and the reference barrel had tip resistances ranging from 50 to 100 GΩ and from 0.5 to 1 GΩ, respectively.

As described previously (Leyssens *et al.*, 1993), the reference and ionselective barrel were connected via Ag/AgCl half-cells to a high impedance electrometer (Duo 773, WPI). Three tracings were recorded on a pen recorder (Sefram or Linseis): the potential of the reference barrel, the potential of the ion-selective barrel and the difference between both, representing the K^+-sensitive signal.

The K^+ -sensitive signal was calibrated in the 143 mM Cl^- Ringer solution, in which the K^+ concentration was varied from 5 to 113 mM, using Na^+ as a substitute. The measured intracellular (K_C) or luminal (K_L) K^+ "signal" was always expressed as the chemical K^+ concentration that would be needed in a 143 mM Cl^- Ringer solution to result in the same reading of the K^+-selective barrel. The ion-sensitive electrodes were accepted for use if a stable potential signal was obtained for both the ion-sensitive and the reference barrel, if the K^+ sensitivity of the reference barrel was less than 1 mV, and if the calibration curve could be fitted by a straight line with a slope of at least 48 mV/decade. As was discussed in a previous paper (Leyssens *et al.*, 1993), ion-sensitive measurements are an adequate tool to study changes in intracellular and luminal ion concentrations. However, since the calibration curve has a logarithmic scale, in the high concentration range (>100 mM) small changes in cellular and luminal concentrations (i.e. less than 10 mM), correspond to minor changes in the K^+-sensitive signal (i.e. 1-2 mV). On evaluating the results, one has to take into account that this approaches the limit of detection.

Except if the effect of a certain test solution on the fluid secretion rate or on electrical potentials or concentrations was not clearly reversible, the value of a specific electrophysiological parameter, registered before and after ion substitution or drug application, was averaged and taken as control value. In this way, possible time dependent changes in potentials or concentrations were minimized.

3. Artificial salines.

The different control solutions containing varying K^+ and Na^+ concentrations (see "Results") were obtained by mixing a K^+-containing, Na^+-free and a Na^+-containing, K^+-free standard Ringer solution (composition: 2 mM $CaCl_2$, 13 mM $MgCl_2$,

12.1 mM Hepes, 2.8 mM alanine, 10.6 mM trehalose, 11.7 mM maltose, 139 mM glucose and 113 mM KCl or NaCl, respectively; pH was adjusted to 7.20 with KOH or NaOH, respectively; osmolality of the solution was 375 mosm/kg H_2O).

Solutions were freshly prepared each week, filtered through 0.45 µm Millipore filters and kept at 2 °C until used.

In some experiments, the following alterations in ion composition were performed: Na^+ was completely replaced by N-methyl-D-glucamine (NMDG, Sigma), Cl^- by Br^-; 6 mM $BaCl_2$ was added to the bath solution.

The following pharmacological substances were tested: dihydro-ouabain (Sigma), Na-orthovanadate (Janssen Chimica), bumetanide (Leo Pharmaceutical Products), Schering 28080 (Schering-Plough; solvent 0.05 % methanol 0.05 % dimethylsulfoxide without any effect on fluid secretion, unpublished results).

4. Statistics.

Results were presented as mean values ± S.E.. Statistical computations were obtained using Statview II (Abacus Concepts Inc., Berkeley, CA, U.S.A., 1987). The fluid secretion measurements and electrophysiological measurements were evaluated using the unpaired and paired two-tailed Student's t-test, respectively, except when indicated differently. A P-value of <0.05 was accepted as indicating statistical significance.

RESULTS

1. Effects of Ba^{2+}.

K^+-selective measurements.

In the presence of Ba^{2+}, the K^+ sensitivity of V_{bl} is lost and V_{bl} and V_{ap} hyperpolarize. It was concluded by Weltens *et al.* (1992) that Ba^{2+} is an effective blocker of the K^+ channels in the basal membrane of the Malpighian tubule cells of *Formica*, possibly reducing an inward current. As a consequence and due to the activity of the electrogenic apical H^+ pump both apical and basal membrane hyperpolarize. In the present study, we compared the effects of a change in the bath K^+ concentration (K_{bl}) on K_c in the absence and presence of 6 mM Ba^{2+}. In Fig. 1, an example is shown. In the absence of Ba^{2+}, an increase in bath K^+ concentration (K_{bl}) from 5 to 113 mM resulted in a prompt depolarization of V_{bl} and a concomitant rise in K_c (see also Leyssens *et al.*, 1993). When K_{bl} was decreased to 51 mM K_{bl}, V_{bl} hyperpolarized and K_c remained at the same value. On returning to 5 mM, V_{bl} and K_c changed and approached a slightly higher K_c and more negative V_{bl} value than at the start of the experiment. In the presence of 6 mM Ba^{2+}, the response of V_{bl} to an increase in K_{bl} was greatly reduced: V_{bl} depolarized with only 7 mV for an increase in K_{bl} from 5 to 113 mM. On the other hand, the rise in K_c on increasing K_{bl} was comparable to the response of K_c to a change in K_{bl} in the absence of Ba^{2+}.

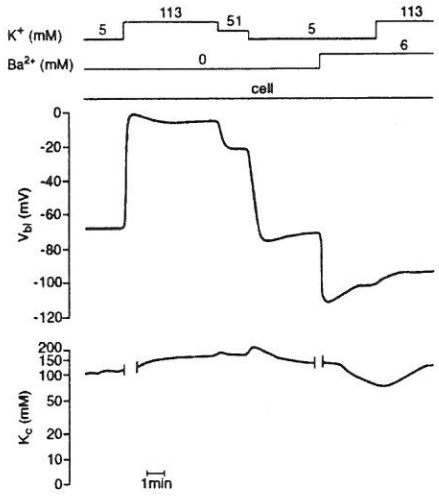

Fig.1. Effect of a change in bath K^+ concentration (5 and 113 mM K_{bl}) on the basal membrane potential (V_{bl}) and the intracellular K^+ concentration (K_c) in the absence and presence of 6 mM Ba^{2+}. In the absence of Ba^{2+}, V_{bl} and K_c were also measured in 51 mM K_{bl}. The upper trace shows V_{bl} as measured with the reference barrel of the K^+-selective electrode; the lower trace gives the potential difference between the ion-sensitive and the reference barrel. These potential differences were converted to K^+ concentrations by using a calibration curve. On changing the bath solution, electrical disturbances were recorded by the K^+-selective (i.e. the most sensitive) barrel and were omitted from the figure for clarity.

In this experiment, the addition of 6 mM Ba^{2+} by itself caused a considerable decrease of K_c both in 5 and 113 mM K_{bl}. This type of behaviour was observed in 2 out of the 4 experiments. On average, both in 5 and 113 mM, K_c was not altered significantly on adding Ba^{2+} to the bath solution (see Table 1). Furthermore, the summarized results demonstrate that, although the K^+ sensitivity of V_{bl} was lost under Ba^{2+} treatment, suggesting a blockage of basal K^+ channels, the rise in K_c on increasing K_{bl} was not affected.

Table 1. : Effect of a change in bath K^+ (K_{bl}) on the basal membrane potential (V_{bl}) and the intracellular K^+ concentration (K_c) in the presence and absence of 6 mM Ba^{2+}.

	5 mM K_{bl}		113 mM K_{bl}		Δ 5 - 113 mM K_{bl}		n
	V_{bl} (mV)	K_c (mM)	V_{bl} (mV)	K_c (mM)	V_{bl} (mV)	K_c (mM)	
control	- 64 ± 2	67 ± 7	- 3 ± 1*	105 ± 7 *	+ 61 ± 5	38 ± 10	4
+ 6 mM Ba^{2+}	- 92 ± 4°	71 ± 11	- 95 ± 6°	114 ± 12*	- 3 ± 1° #	43 ± 11	4

The effect of a change in K_{bl} was tested on the same tubules in the absence and presence of Ba^{2+}.
*: 113 mM K_{bl} significantly different from 5 mM K_{bl}.
°: significantly different from control (paired Student's t-test, $P < 0.05$)
#: not significantly different from zero (Student's t-test, $P > 0.05$)

2. Effects of substitution of Na^+ by $NMDG^+$.

Fluid secretion measurements.

In order to reveal the existence of any Na^+-dependent transport system, the effect of omission of Na^+ from the bath solution was tested using $NMDG^+$ as a substitute (see "Methods, section 3"). In 51 mM K_{bl}, a significant effect was absent

(Fig. 2A, Table 2A). In a low K^+ solution (10 mM K_{bl}), the substitution of Na^+ by $NMDG^+$ caused a small but reversible inhibition of the fluid secretion (Fig. 2B, Table 2A). However, the difference between the last test period and the corresponding time period in the control series was only statistically significant at the level of 10%, probably due to the large variability present in both the experimental and the control series. On the other hand, in the experimental series a statistically significant (paired Student's t-test) increase of the fluid secretion rate was found between the last test period and the first wash-out period (from 68±8 % to 102±16 %, respectively, values not corrected for time effect), suggesting a fast recovery on readmitting Na^+.

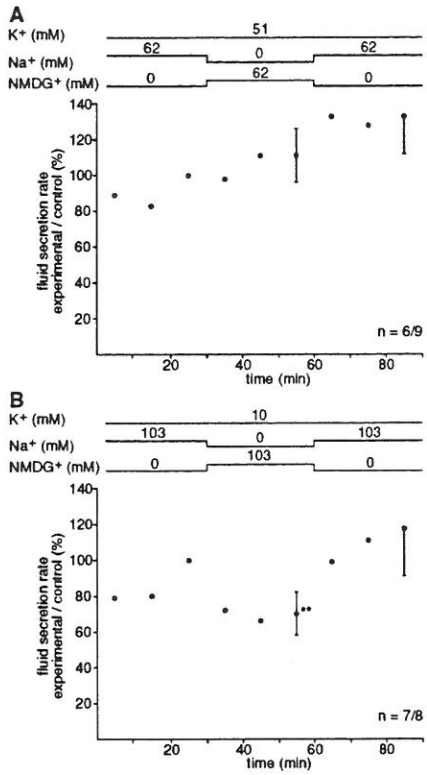

Fig. 2. Effect of the substitution of Na^+ by $NMDG^+$ on the fluid secretion rate in 51 (A) and 10 (B) mM K_{bl}. To take into account any time effect on fluid secretion, the secretion was expressed as the ratio of the relative rate in the experimental condition over the relative rate in control condition. The relative rate was the absolute rate expressed as a percentage of the absolute rate of the third 10 min. control period (i.e. the period just before the change to the test solution). Mean values ± S.E..
**: statistically significant difference (paired Student's t-test, $P<0.05$) between the last test period and the first wash-out period of the experimental series (from 68±8 % to 102±16 %, respectively, values not corrected for time effect).

3. Effects of substitution of Cl^- by Br^-.

Fluid secretion experiments.

According to Ellory and Hall (1988), in human red cells, $Na^+/K^+/2Cl^-$ and K^+/Cl^- cotransport systems display a different anion dependence: a substitution of Cl^- by Br^- would inhibit a $Na^+/K^+/2Cl^-$ and stimulate a K^+/Cl^- cotransport system. Although this criterium is not specific and its applicability on insect epithelia is not known, it could support the idea of separate systems functioning in certain conditions.

Table 2A : Effect of ion substitution on the fluid secretion rate.

ion substitution	bath K^+concentration (mM) #	n	fluid secretion rate experimental/control (%) during last test period
Na^+ by $NMDG^+$	10	6/9	70 ± 12 (a)
	51	7/8	111 ± 15
Cl^- by Br^-	10	6/8	71 ± 14*
	51	8/8	198 ± 57*

Table 2B : Effect of various drugs on the fluid secretion rate.

drug	conc. (M)	bath K^+ concentration (mM)	n	fluid secretion rate experimental/control (%) during last test period
Dihydro-ouabain	10^{-3}	5	8/8	74 ± 24
Sch 280 80	10^{-4}	10	6/9	130 ± 15
		51	8/8	129 ± 18
Vanadate	10^{-4}	51	8/9	108 ± 18
	10^{-3}	51	6/9	8 ± 3*
		113	6/7	30 ± 12*
Bumetanide	10^{-5}	5	8/8	37 ± 11*
		51	8/9	111 ± 17
	10^{-4}	5	8/8	22 ± 8*•
		51	7/9	46 ± 8*
		113	8/7	97 ± 13
	10^{-3}	51	6/9	7 ± 3*•

n : number of tubules experimental/control

: the control bath Na^+ concentration was 0, 2, 103 and 108 mM when the bath K^+ concentration was 113, 51, 10 and 5 mM, respectively; the control bath Cl^- concentration was always 143 mM.

* : statistically significant difference between the experimental relative rate and the control relative rate during the same time period (unpaired student's t-test, $P<0.05$)

• : statistically significant difference (unpaired student's t-test) between the relative secretion rate of the experimental and the control series during a wash-out period; this means that the effect [of ion substitution (A)/drug application (B)] was not completely reversible.

(a) : in the experimental series (not corrected for time effect) the difference between the last test (i.e. 68 ± 8 %) and the first wash-out (i.e. 102 ± 16 %) period was statistically different (paired Student's t-test, $P < 0.05$)

When Cl^- was omitted from the bath solution and replaced by Br^-, fluid secretion rate was significantly stimulated in 51 mM K_{bl} but inhibited in 10 mM K_{bl} (see Fig. 3A and B, respectively , Table 2A).

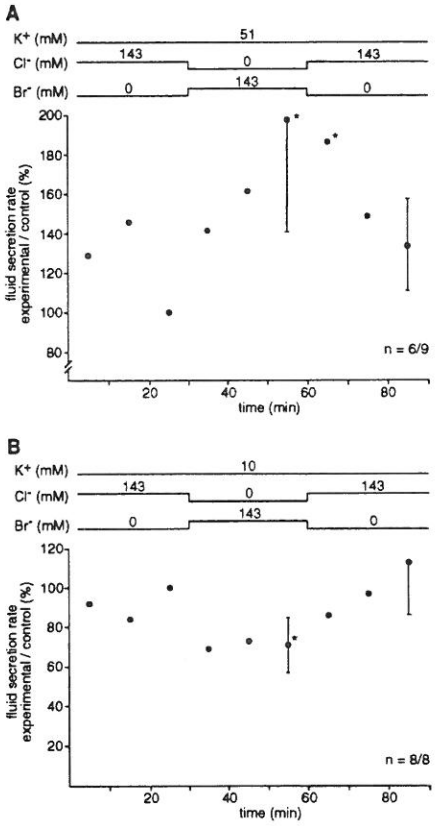

Fig. 3. Effect of the substitution of Cl^- by Br^- on the fluid sectretion rate in 51 (A) and 10 (B) mM K_{bl}. Mean values ± S.E..
*: statistically significant difference between the experimental relative fluid secretion rate and the rate in the control series at the same point in time (unpaired Student's t-test, $P<0.05$).

4. Effects of dihydro-ouabain.

Fluid secretion experiments.

Since K^+ and ouabain, a well-known specific Na^+/K^+ ATPase blocker, are competitive for the same binding site, a high K_{bl} will decrease the ouabain effect (see Baker and Willis, 1970). Therefore the effect of dihydro-ouabain was tested in a low K_{bl} (5 mM). However, no significant effect of 10^{-3} M dihydro-ouabain was observed during the 30 min. application of the drug (Table 2B). However, in the control solution (5 mM K_{bl}), fluid secretion rate was very low before the application of DHO

(i.e. 76±19 pl/min, n=8) and the time course highly variable. Consequently, it was difficult to distinguish a genuine drug effect from time-dependent changes.

Electrical potential difference measurements.

A lowering of K_c and hence a depolarization of V_{bl} may be expected on blocking an active basal K^+ uptake mechanism. Therefore the effect of dihydro-ouabain was tested on V_{bl} (Fig. 4A). However, in 5 mM K_{bl}, ouabain treatment (10^{-3} M) for at least

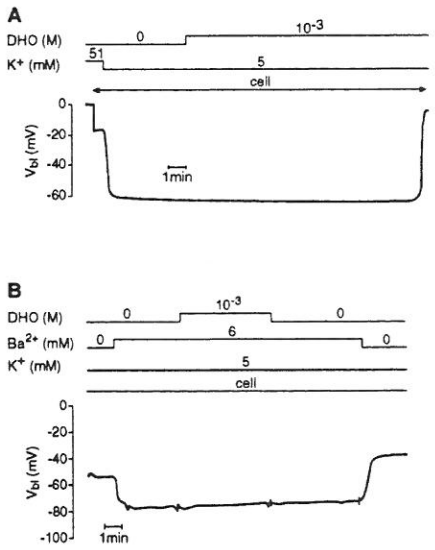

Fig. 4. Effect of 10^{-3} M dihydro-ouabain in the absence (A) and presence (B) of 6 mM Ba^{2+} (5mM K_{bl}) on V_{bl}.

10 minutes did not have a depolarizing effect on V_{bl}. Since a small electrogenic activity might be masked by the high K^+ permeability of the basal membrane, potential measurements were also performed in the presence of 6 mM Ba^{2+} (for theoretical considerations see also Weltens *et al.*, 1992). In Fig. 4B, such an experiment is visualized. The addition of 6 mM Ba^{2+} hyperpolarized V_{bl}. In the presence of Ba^{2+}, following exposure to 10^{-3} M dihydro-ouabain, V_{bl} slightly decreased; after wash-out, V_{bl} further declined with 1 mV, suggesting a small time-dependent decay of V_{bl}. After wash-out of Ba^{2+}, V_{bl} depolarized to approximately the initial value. The potential measurements are summarized on Table 3A. Neither in the absence nor presence of 6 mM Ba^{2+} significant effects of 10^{-3} M dihydro-ouabain on V_{bl} were detected.

Table 3. : Effect of various drugs on electrophysiological parameters.

3A. Intracellular and luminal electrical potential measurements

drug	conc. (M)	bath solution	control			+ drug		
			V_{bl} (mV)	V_{te} (mV)	V_{ap} (mV)	V_{bl} (mV)	V_{te} (mV)	V_{ap} (mV)
Dihydro-ouabain	10^{-3}	5 mM K_{bl}	- 61 ± 1 (8)	-	-	- 60 ± 1 (8)	-	-
		5 mM K_{bl} + 6 mM Ba^{2+}	- 93 ± 4 (8)	-	-	- 90 ± 4 (8)	-	-
Vanadate	10^{-4}	51 mM K_{bl}	-	25 ± 4 (5)	-	-	22 ± 2 (5)	-
	10^{-3}	5 mM K_{bl}	- 61 ± 2 (4)	14 ± 3 (7)	- 75	- 62 ± 3 (4)	8 ± 1* (7)	- 70
		51 mM K_{bl}	- 16 ± 1 (8)	23 ± 2 (9)	- 39	- 17 ± 2 (8)	12 ± 1* (9)	-29
		51 mM K_{bl} + 6 mM Ba^{2+}	- 54 ± 4 (10)	-	-	- 30 ± 7 (10) *	-	-
Bumetanide	10^{-4}	5 mM K_{bl}	-	29 ± 6 (4)	-	-	27 ± 5 (4)	-
		51 mM K_{bl}	-	35 ± 3 (11)	-	-	33 ± 3 (11)	-

3B. : Intracellular and luminal K^+-selective measurements.

drug	conc. (M)	bath solution	control				+ drug			
			cell		lumen		cell		lumen	
			V_{bl} (mV)	K_c (mM)	V_{te} (mV)	K_l (mM)	V_{bl} (mV)	K_c (mM)	V_{te} (mV)	K_l (mM)
Vanadate	10^{-3}	51 mM K_{bl}	- 21 ± 2 (4)	119 ± 13 (4)	22 ± 3 (7)	113 ± 23 (7)	- 21 ± 1 (4)	120 ± 12 (4)	12 ± 1 (7)*	120 ± 16 (7)
Bumetanide °	10^{-4}	5 mM K_{bl}	- 52 ± 3 (5)	52 ± 5 (5)	-	-	- 55 ± 5 (5)	59 ± 7 (5)	-	-
		51 mM K_{bl}	- 11 ± 1 (5)	88 ± 6 (5)	-	-	- 12 ± 2 (5)	89 ± 4 (5)	-	-

* : significant drug effect (paired Student's t- test, $P < 0.05$)
(n) : number of tubules
° : the effect of bumetanide was tested on the same tubules in 5 and 51 mM K_{bl}

5. Effects of Sch28080.

Fluid secretion experiments.

Sch28080 is a specific inhibitor of the K^+/H^+ ATPase; the degree of inhibition of the enzyme is dependent upon the bath K^+ concentration; lower concentrations increase the potency of Sch28080 (see Scott *et al.*, 1987). At a concentration of 10^{-4} M, the activity of the Na^+/K^+ ATPase or the V-type H^+ ATPase (see Froissart *et al.*, 1992) is not affected. Neither in a high (51 mM) nor in a low (10 mM) K_{bl}, there was a significant effect of Sch28080 (10^{-4}M) on fluid secretion rate within 30 min. (Table 2B).

6. Effects of vanadate.

Fluid secretion experiments.

In 51 mM K_{bl}, at a concentration of 10^{-4} M, vanadate, known as an inhibitor of E_1E_2 (P-type) ATPases (i.e. the Na^+/K^+ and K^+/H^+ ATPase) (see Nechay *et al.*, 1986), did not affect the fluid secretion rate; but at a concentration of 10^{-3} M, the fluid secretion was almost completely abolished within 40 min. (Fig. 5A, Table 2B). In a high K^+, Na^+-free solution, vanadate (10^{-3} M) caused a comparable inhibitory effect on fluid secretion (Fig. 5B, Table 2B). There was no statistically significant difference between the inhibitory effect in 51 and 113 mM K_{bl}. In both cases, the vanadate effect was reversible.

Electrical potential difference measurements.

The effect of vanadate was tested on cellular and luminal potentials in a different series of tubules (see Table 3A). In 5 and 51 mM K_{bl}, at a concentration of 10^{-3} M, vanadate did not affect V_{bl} but significantly depolarized V_{te}; within 5 minutes, a steady state was reached. A 10 times lower concentration had no significant effect on V_{te}. The depolarizing effect of 10^{-3} M vanadate on V_{te} suggested the inhibition of an electrogenic transport system (for theoretical considerations, see also Weltens *et al.*, 1992). Therefore, it was of interest to examine the effects of vanadate on the Ba^{2+}-induced hyperpolarization of V_{bl}. A typical example is shown in Fig. 5C. In contrast to the absence of any effect on V_{bl} in the absence of Ba^{2+}, vanadate now caused a depolarization of V_{bl} within a time course of 5 minutes. The reduction of the Ba^{2+}-induced hyperpolarization was statistically significant (see Table 3A). The effects of vanadate on electrical potentials were poorly reversible within the experimental time course (i.e. approximately 15 min. washout).

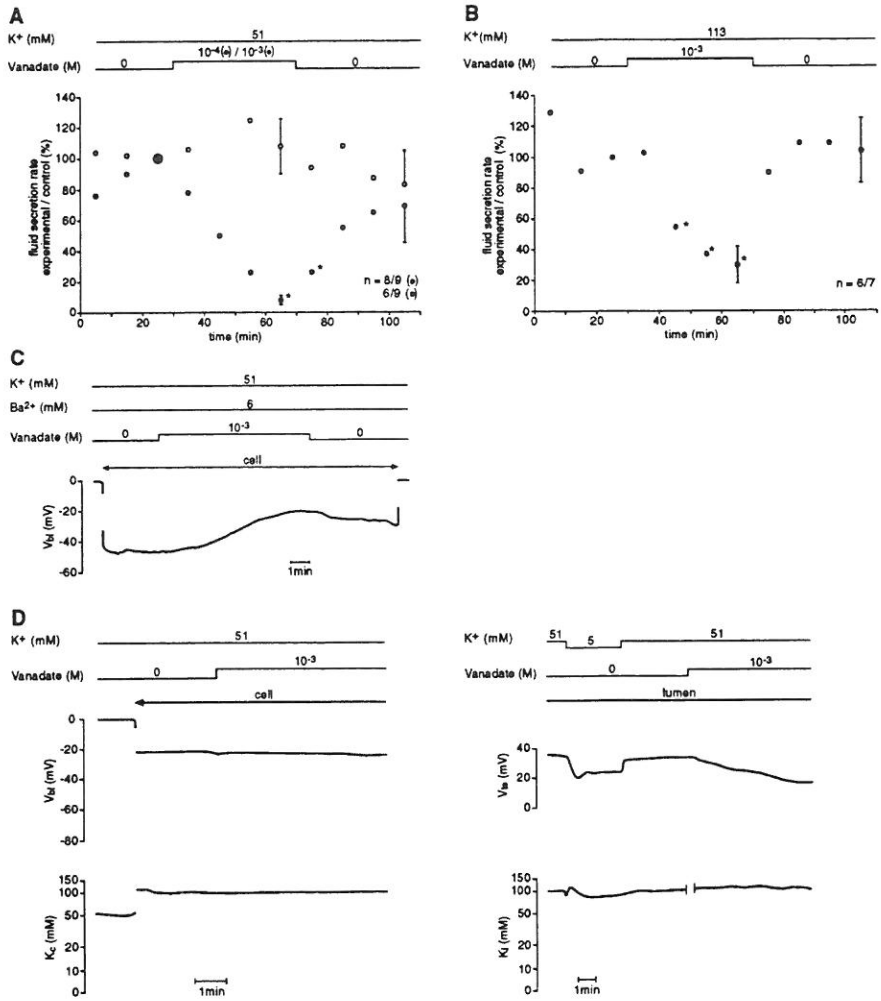

Fig. 5. Effect of vanadate on the fluid secretion rate in 51 mM K_{bl} (at a concentration of 10^{-4} and 10^{-3} M, A) and 113 mM K_{bl} (Na^+-free) (at a concentration of 10^{-3} M, B) mM K_{bl}. Mean values ± S.E..

*: statistically significant difference between the experimental relative fluid secretion rate and the rate in the control series at the same moment in time (unpaired Student's t-test, $P<0.05$).

There was no statistically significant difference between the inhibitory effect of vanadate in 51 and 113 mM K_{bl} (unpaired Student's t-test, $P>0.05$). Effect of 10^{-3} M vanadate on V_{bl} in the presence of 6 mM Ba^{2+} (51 mM K_{bl}) (C). Effect of 10^{-3} M vanadate on V_{bl} and K_c, and on V_{te} and the luminal K^+ concentration (K_l) in 51 mM K_{bl} (D). Intracellular and luminal K^+-selective measurements were performed on different tubules.

K^+-selective measurements.

Since 10^{-3} M vanadate did not affect V_{bl} in the absence of Ba^{2+} within a time course of 5 minutes, a drastic effect on K_c (or K_l) was not expected. This was verified in 51 mM K_{bl}. In Fig. 5D, an example of a cellular and luminal measurement is given. The addition of 10^{-3} M vanadate did not introduce significant changes in V_{bl} or K_c. In the lumen of another tubule, first of all, the reversible response of V_{te} and K_l to a decrease in K_{bl} in control conditions was confirmed. Upon administation of 10^{-3} M vanadate, V_{te} began to decrease within the first minute; after 6 minutes V_{te} was diminished with 18 mV. Within the same time course, K_l was not affected. The experimental data were summarized in Table 3B. There was no significant effect on K_c and K_l.

7. <u>Effects of bumetanide.</u>

Fluid secretion experiments.

The loop diuretic bumetanide inhibits K^+/Cl^- and $Na^+/K^+/2Cl^-$ cotransport systems (see Ellory and Hall, 1988). A concentration of 10^{-5} M is usually sufficient for maximal inhibition of the $Na^+/K^+/2Cl^-$ cotransporter, much higher concentrations are generally needed to affect the K^+/Cl^- cotransporter (see Ellory and Hall, 1988; Palfrey and O'Donnell, 1992). Bumetanide was tested in 5, 51 and 113 mM K_{bl}. In 51 mM K_{bl}, 10^{-4} M bumetanide significantly diminished fluid secretion rate; the application of 10^{-3} M almost completely abolished it (Fig. 6A, Table 2B). Only at a concentration of 10^{-4} M, the drug effect was reversible. In the lower K_{bl} (5 mM), bumetanide reversibly inhibited fluid secretion when applied at a concentration of 10^{-5} M. The inhibitory effect of 10^{-4} M bumetanide was more pronounced and the fluid secretion did not recover within the 30 minutes wash-out period (Fig. 6B, Table 2B). In the high K^+, Na^+-free solution on the other hand, bumetanide (10^{-4} M) had no significant effect (Fig. 6C, Table 2B).

Electrical potential measurements.

Since bumetanide (10^{-4} M) diminished the fluid secretion rate in 51 and 5 mM K_{bl}, electrophysiological effects were tested in these bath solutions. As is shown in Fig. 6D, in 51 mM K_{bl} V_{te} remained constant after the addition of 10^{-4} M bumetanide, in 5 mM K_{bl} V_{te} was 4 mV higher compared to control. On average (see Table 3A), neither in 5 nor 51 mM K_{bl}, a significant effect on V_{te} could be detected. In 2 out of the 11 experiments performed in 51 mM K_{bl}, the drug was applied during more than 20 minutes without observing any clear change in potential difference. Possibly, small drug-induced increases in V_{te} were obscured by a (small) time-dependent decrease in V_{te}, observed in control experiments (unpublished results).

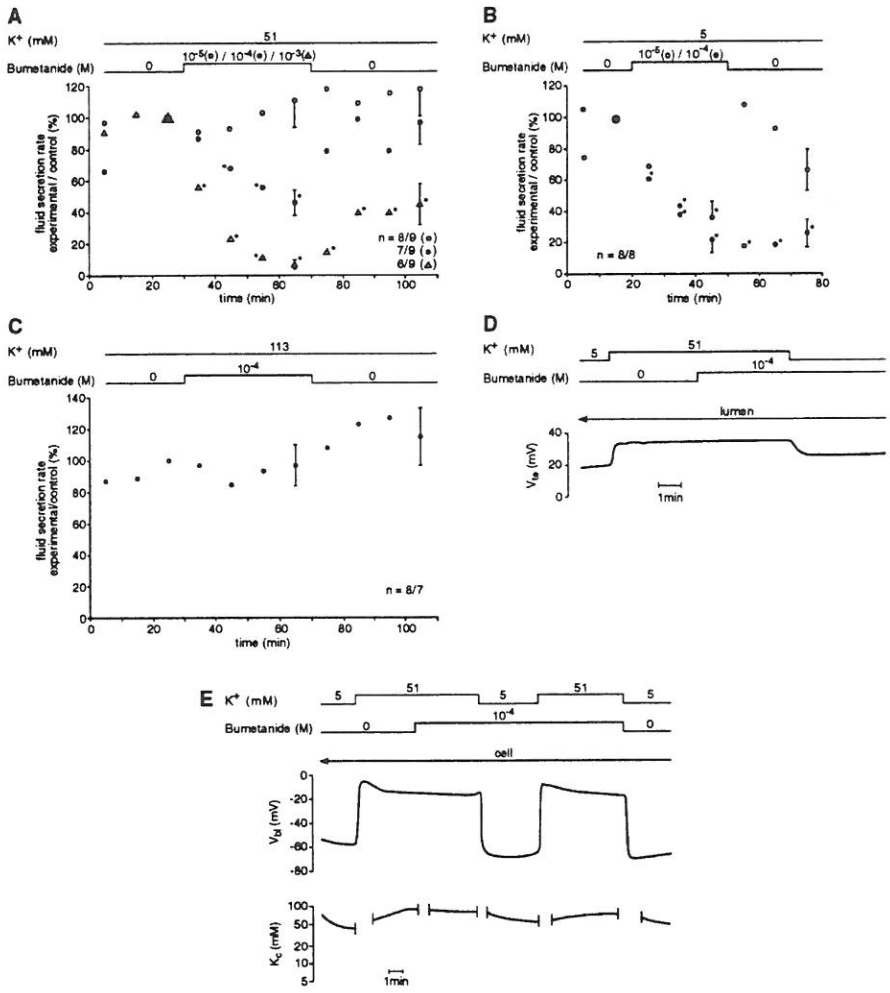

Fig. 6. Effect of bumetanide on the fluid secretion rate in 51 mM K_{bl} (at a concentration of 10^{-5}, 10^{-4} and 10^{-3} M, A), 5 mM K_{bl} (at a concentration of 10^{-5} and 10^{-4} M, B) and 113 mM K_{bl} (Na^+-free) (at a concentration of 10^{-4} M, C). Mean values ± S.E..
*: statistically significant difference between the experimental relative fluid secretion rate and the rate in the control series at the same point in time (unpaired Student's t-test, $P<0.05$).
Effect of 10^{-4} M bumetanide on V_{te} (D), and on V_{bl} and K_c (E) in 51 and 5 mM K_{bl}.

K^+-selective measurements.

The effect of 10^{-4} M bumetanide was tested on V_{bl} and K_c in 5 and 51 mM K_{bl} on the same tubule. Fig. 6E shows an experiment. In control conditions, an increase in K_{bl} from 5 to 51 mM caused a depolarization of V_{bl} and an increase in K_c. On introducing the bumetanide containing bath solution in 51 mM K_{bl}, V_{bl} increased slightly, K_c remained virtually unaffected. When K_{bl} was lowered to 5 mM in the presence of the drug, V_{bl} hyperpolarized and K_c decreased. On returning to 51 mM K_{bl} in the presence of bumetanide, V_{bl} depolarized again while K_c approached its previous value in 51 mM K_{bl}. Thus bumetanide did not inhibit the rise of K_c on increasing K_{bl}. Wash-out of bumetanide in 5 mM K_{bl} did not change the V_{bl} and K_c values registered in the presence of bumetanide. The small (irreversible) changes in V_{bl} and K_c occurring in this experiment during administration of bumetanide, were not systematically observed in other experiments. On average, there was no statistically significant effect of 10^{-4} M bumetanide on V_{bl} or K_c (see Table 3B). The lack of an effect on V_{bl} was confirmed in 8 experiments performed in 41 mM K_{bl} 57 mM Cl_{bl} (Cl^- substituted by $citrate^-$): even after 20 minutes application of 10^{-3} M bumetanide, a concentration which effectively inhibited fluid secretion (Verhulst *et al.*, 1988), V_{bl} did not change significantly (Weltens, personal communication).

DISCUSSION

1. Basal K^+ channels are not the only K^+ entry mechanism.

In the present study, we showed that in the presence of 6 mM Ba^{2+} in response to a rise in bath K^+, K_c increased to the same extent and with a similar time course as in the absence of Ba^{2+} (Fig. 1 and Table 1). This and previous findings (see "Introduction" for references) strongly argue for the role of other K^+ uptake systems besides entry through conductive channels in maintaining fluid secretion and in adapting the cytosolic K^+ concentration to the surrounding K^+ concentration.

2. Basal primary active K^+ transporting ATPases

A Na^+/K^+ ATPase does occur in insect Malpighian tubules, but only in a few species does its activity affect fluid secretion in any significant way (for a review see Nicolson, in press).

If a Na^+/K^+ ATPase was important for K^+ uptake, omission of Na^+ from the bath solution would result in an intracellular Na^+ depletion and hence in an inhibition of the Na^+/K^+ ATPase. However, the highest secretion rates were found in high K^+ (113 mM), Na^+-free conditions. Buffering the solution with NaOH instead of KOH (addition of approximately 3 mM) had no further stimulatory effect on fluid secretion (see Van Kerkhove *et al.*, 1989). The omission of Na^+ did not affect fluid secretion in

51 mM K_{bl} (Fig. 2A, Table 2A). The latter findings do not favour a significant role of any Na^+-dependent K^+ uptake mechanism in fluid secretion of Malpighian tubules of *Formica*, at least in the presence of relatively high K^+ concentrations.

If a Na^+/K^+ ATPase is present, its action is obscured during fast fluid secretion and is most likely to be seen only in low secreting conditions (see also Maddrell and O'Donnell, 1992; Nicolson, in press). Indeed, in Malpighian tubules of *Formica*, Na^+ omission from the bath solution seemed to decrease the fluid secretion rate in 10 mM K_{bl} (see Fig 2B, Table 2A). However, in 5 mM K_{bl} a significant effect of the specific Na^+/K^+ ATPase inhibitor ouabain on fluid secretion rate could not be distinguished from time-dependent changes (Table 2B). Furthermore, an effect of ouabain on V_{bl} was absent (Fig. 4A, Table 3A). Since a decrease in K_c would have been seen as a depolarization of V_{bl} (see also Leyssens *et al.*, submitted) an essential role for the Na^+/K^+ pump in maintaining the cellular K^+ level, at least in the short run, is unlikely. Neither could ouabain unmask any electrogenicity of a Na^+/K^+ ATPase in the presence of Ba^{2+} (Fig. 4B, Table 3A; for theoretical considerations, see Weltens *et al.*, 1992). Thus although insensitivity of Na^+/K^+ ATPase to ouabain in *Formica* tubules, or even excretion and sequestration of ouabain (for a review see Vaughan and Jungreis, 1977; Anstee and Bowler, 1979) can not be excluded, our results suggest that, even in a low K^+ medium, an active Na^+/K^+ exchange system is of little consequence for transepithelial K^+ transport in Malpighian tubules of *Formica*. The small Na^+ dependence of the fluid secretion rate in low K_{bl}, can probably be explained by the presence of another Na^+-dependent transport system, i.e. a $Na^+/K^+/2Cl^-$ cotransport system (see below).

In other insects, the results vary. The presence of a Na^+/K^+ ATPase was demonstrated biochemically in Malpighian tubules of *Locusta* (Fogg *et al.*, 1991) and functionally in Malpighian tubules of *Locusta* (Anstee *et al.*, 1979; Morgan and Mordue, 1981; Baldrick *et al.*, 1988), *Drosophila* (Wessing *et al.*, 1987; Bertram *et al.*, 1991), *Aedes* (Hegarty *et al.*, 1991) and *Rhodnius* (Maddrell and Overton, 1988). In other species, evidence for the presence of a Na^+/K^+ ATPase was absent: ouabain did not affect the fluid secretion rate in Malpighian tubules of *Glossina* (Gee, 1976); no effect of Na^+ substitution or ouabain application on electrical parameters could be observed in Malpighian tubules of *Onymacris* (Isaacson *et al.*, 1989).

Another candidate for active K^+ uptake could be a K^+/H^+ ATPase. Until now, unlike in vertebrate renal epithelia (see Okusa *et al.*, 1992; Gifford *et al.*, 1992) no reports have been made upon the presence of such an ATPase in insect Malpighian tubules. In Malpighian tubules of *Formica*, the fluid secretion rate was not significantly changed after the addition of the specific inhibitor Sch28080 in either 51 or 10 mM K_{bl} (Table 3B). Zhang *et al.* (submitted) however suggested that the extrusion of protons at the basal side via such an active exchange mechanism may be needed to explain the

lowering of the proton concentration level in both the lumen and the cell when increasing the bath K^+ concentration in Malpighian tubules of *Formica*. In view of the latter finding, the presence of a (basal) K^+/H^+ ATPase can not be excluded entirely, but its relevance for maintaining secretory activity seems to be minimal.

Since vanadate interacts with both Na^+/K^+ and K^+/H^+ ATPases, no significant effect on fluid secretion was expected on adding vanadate to the bath solution. Surprisingly, when applied at a concentration of 10^{-3} M the fluid secretion rate was strongly inhibited by vanadate, the inhibitory effect becoming statistically significant after 20 minutes (Fig. 5A). Both in 5 and 51 mM K_{bl}, V_{te} significantly depolarized to a new steady state level within 5 min. after the addition of vanadate; V_{bl}, K_c and K_l remained unaffected (Fig. 5D and Table 3A and B).

Thus, in contrast to Malpighian tubules of *Locusta*, where both vanadate and ouabain caused a slow depolarization of V_{bl} and V_{ap} (Baldrick *et al.*, 1988), there was a large discrepancy between the vanadate and the oubain or Sch28080 effects in Malpighian tubules of *Formica*. Furthermore, since the inhibitory effect of vanadate on fluid secretion rate in Na^+-containing (i.e. 51 mM K_{bl}) and Na^+-free (i.e. 113 mM K_{bl}) conditions was comparable (Fig. 5A and B, Table 2B), its strong inhibitory effect was probably not due to an interaction with a hypothetical Na^+/K^+ ATPase. Since a major role for a hypothetical K^+/H^+ ATPase in fluid secretion is apparently unlikely (see above), we had to consider other possible sites of action. A complex indirect effect of the molecule was suggested for Malpighian tubules of *Drosophila* (Bertram *et al.*, 1991). A multitude of stimulatory and inhibitory cellular action mechanisms of vanadate have been reviewed by Chasteen (1983) and Nechay *et al.* (1986). In our hands, the effects of vanadate on fluid secretion rate and electrophysiological parameters were comparable to, and followed a similar time course as the effects of the V-type ATPase inhibitors bafilomycin A_1 and NEM reported by Weltens *et al.* (1992) for the same preparation. In the latter study, the reduction of the Ba^{2+}-induced hyperpolarization was attributed to an electrogenic current generated by the apical H^+ ATPase and blockable by bafilomycin A_1 and NEM. Unlike ouabain (in 5 mM K_{bl}), vanadate (10^{-3} M in 51 mM K_{bl}) did significantly reduce the Ba^{2+}-induced hyperpolarization of V_{bl} (Fig. 5C, Table 3A).

Reports on a direct effect of vanadate on the apical H^+ pump are controversial. According to Forgac (1989) vacuolar type H^+ ATPases are insensitive up to at least 10^{-3} M. On the other hand, Chatterjee *et al.* (1992) revealed the presence of a novel type of V-type H^+ ATPase in osteoclast plasma membrane vesicles with a unique pharmacology and specific isoforms of two subunits in the catalytic portion of the enzyme; the H^+ ATPase activity was completely inhibited by 10^{-3} M vanadate. Vanadate (10^{-4} M) affected to a small but significant extent (±20 % inhibition) the

cation stimulated ATPase activity in purified goblet cell apical membranes of *Manduca sexta* (Wieczorek *et al.*, 1986), later identified as a V-type H^+ ATPase (Wieczorek *et al.*, 1989).

Summarizing, no conclusive evidence was found for the existence of basal active K^+ uptake mechanisms in exchange for either Na^+ or H^+ ions. Their role in maintaining an asymmetric ionic distribution across the basal membrane and a certain rate of fluid secretion is apparently minimal. The strong inhibitory effect of vanadate on fluid secretion can probably be explained by a direct effect on the apical H^+ pump.

3. Coupled entry mechanisms for K^+, Cl^- (and Na^+) in the basal membrane.

The existence of coupled entry mechanisms for K^+, Na^+ and Cl^- has been investigated in Malpighian tubules of other insects, but only in a low bath K^+ concentration (for review see Nicolson, in press). In Malpighian tubules of *Formica* different approaches (see "Introduction" and "Results") were used to find evidence for an electroneutral coupled transport of K^+ in different bath K^+ concentrations.

In high K^+ (113 mM) Na^+-free conditions, K^+ entry via a $Na^+/K^+/2Cl^-$ cotransporter can be excluded. K^+ uptake via a K^+/Cl^+ cotransporter is not very likely either, as bumetanide had no effect at a concentration as high as 10^{-4} M (Fig. 6C, Table 2B).

In 51 mM K_{bl}, the fluid secretion rate was not affected by omitting Na^+ from the bath solution (Fig. 2A, Table 2A). Although bumetanide binding to a $Na^+/K^+/2Cl^-$ cotransporter should be favoured in the 51 mM K^+ 62 mM Na^+ bath solution (see Haas and Forbush III, 1986), no change in fluid secretion rate was observed after the addition of 10^{-5} M bumetanide (Fig. 6A, Table 2B). These findings do not favour K^+ entry via a $Na^+/K^+/Cl^-$ cotransporter. On the other hand, the significant reduction of the fluid secretion rate after the addition of 10^{-4} M bumetanide suggests that a large portion of the basal K^+ uptake may occur via a K^+/Cl^- cotransporter (Fig 6A, Table 2B). Since at this concentration no effect of bumetanide was observed in 113 mM K_{bl}, aspecific effects are unlikely. The observation that Br^- has a stimulatory effect on fluid secretion in 51 mM K_{bl} corroborates the hypothesis of a K^+/Cl^- cotransport system (Fig. 3A, Table 2A; see Ellory and Hall, 1988). At a concentration of 10^{-3} M, the inhibitory effect of bumetanide was much more pronounced. However, at this high concentration, interaction with other Cl^--dependent transport mechanisms can not be excluded; in whole cell recordings of the rat lacrimal gland, it was shown that 4.10^{-4} M bumetanide inhibited Cl^- channels for instance (Evans *et al.*, 1986).

In a low K^+, high Na^+ medium (i.e. 10 or 5 mM K_{bl}) on the other hand, both Na^+ omission and Cl^- substitution by Br^-, caused a reversible reduction of the secretion rate (Fig. 2B and 3B, respectively, Table 2A), and bumetanide clearly inhibited fluid secretion at a concentration of 10^{-5} M (see Fig. 6B, Table 2B). These findings argue for the presence of a $Na^+/K^+/2Cl^-$ cotransporter. Evidence for a $Na^+/K^+/2Cl^-$

cotransporter (Maddrell, 1969; O'Donnell and Maddrell, 1984; Hegarty *et al.*, 1991) and/or Cl^+-dependent K^+ uptake (Wessing *et al.*, 1987; Baldrick *et al.*, 1988) is present for Malpighian tubules of other species as well.

That bumetanide (and/or another loop diuretic furosemide for other species) affects electroneutral transport mechanisms is confirmed by the lack of a significant effect on electrical potential differences (Table 3 A and B) (cfr. V_{bl} in *Locusta*, Baldrick *et al.*, 1988; V_{te} in *Onymacris*, Isaacson and Nicolson, 1989; V_{ap} in *Aedes*, Hegarty *et al.*, 1991). Long term application however (several minutes) might change the intracellular K^+, Cl^- and/or Na^+ concentrations and hence V_{bl} (cfr. *Rhodnius*, O'Donnell and Maddrell, 1984; *Aedes*, Hegarty *et al.*, 1991). In Malpighian tubules of *Formica*, K_c didn't change much, as V_{bl} didn't alter.

An important observation was that bumetanide did not hamper the response of K_c to a change in K_{bl} from 5 to 51 mM K_{bl} (Fig. 6E, Table 3B). This means that other pathways, probably K^+ channels, can ensure basal K^+ uptake and adapt the cellular K^+ content to the surrounding K^+ concentration. This finding indirectly points to the existence of an inwardly directed basal electrochemical K^+ gradient in the latter conditions.

To summarize, in a high K^+ (113 mM) Na^+-free condition, a coupled entry mechanism of K^+ and Cl^- seems to be of little importance. In 51 mM K_{bl}, a substantial portion of K^+ entry can occur via a K^+/Cl^- cotransporter. In low K_{bl}, a $Na^+/K^+/2Cl^-$ cotransporter seems to become important.

4. Transport model for transcellular K^+ transport in Malpighian tubules of Formica.

Several transport mechanisms hypothesized by Leyssens *et al.* (1992, Fig. 10) have now been investigated. Previous papers showed that at the apical side, K^+ extrusion into the lumen is realized via the combination of an electrogenic H^+ pump and an electroneutral K^+/H^+ antiporter (see Weltens *et al.*, 1992; Dijkstra *et al.*, in press; Zhang *et al.*, 1992; Leyssens *et al.*, submitted). The present study shows that the pathways for basal K^+ entry seem to be conductive and secundary active and that their relative importance varies depending on the bath K^+ (and Na^+) concentration. Primary active K^+ uptake systems, i.e. a Na^+/K^+ or K^+/H^+ ATPase, if present, do not seem to contribute significantly to transepithelial transport or to the maintenance of intracellular K^+ (and Na^+) levels. Fig. 7 shows the proposed transport model for Malpighian tubules of *Formica*.

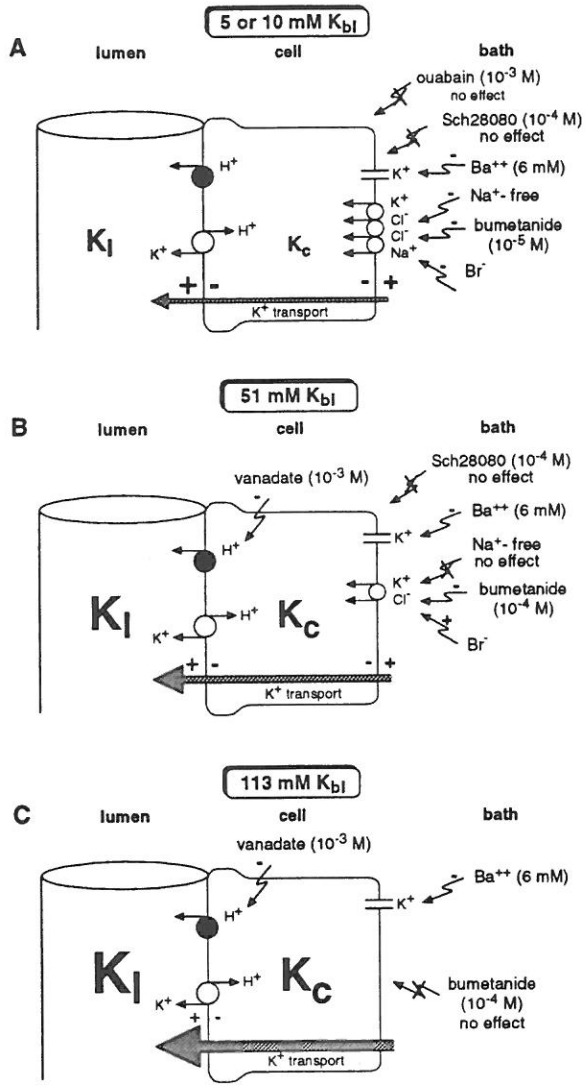

Fig. 7. Model for transcellular K^+ transport in 5 or 10 (A), 51 (Fig. B) and 113 (C) mM K_{bl}, summarizing the results in the present study. It was shown previously that

1) K^+ transport increases (larger arrows) in a higher K_{bl} (Van Kerkhove *et al.*, 1989).
2) that the apical and basal membrane potential differences decrease (smaller + and - signs in the figure) when K_{bl} increases (Leyssens *et al.*, 1992).
3) K_l and K_c increase (indicated by larger symbols in the figure) when K_{bl} increases (Leyssens *et al.*, submitted).
4) a H^+ pump is present in the apical membrane in parallel with a K^+/H^+ antiporter (Weltens *et al.*, 1992; Zhang *et al.*, submitted; Leyssens *et al.*, submitted)

In high K^+ Na^+-free conditions, a coupled entry of K^+ and Cl^- seems to be of little importance. K^+ uptake probably occurs via basal K^+ channels, driven by a favourable electrochemical gradient (Fig. 7A) (see also Leyssens *et al.*, submitted). In more physiological conditions, i.e. 51 mM K_{bl}, when the basal electrochemical K^+ gradient seems to be less inward (see Leyssens *et al.*, submitted), K^+ entry via channels may become less important and a K^+/Cl^- cotransport system probably accounts for a large portion of K^+ uptake (Fig. 7B). In 5 or 10 mM K_{bl} (and high Na_{bl}) little K^+ is available for transepithelial transport; the basal electrochemical K^+ gradient is even smaller compared to 51 mM K_{bl} and possibly outward (see Leyssens *et al.*, submitted). The fluid secretion rate is low but still present. In this condition, a $Na^+/K^+/2Cl^-$ cotransporter may take over a large part of the basal K^+ uptake (Fig. 7C). Furthermore, the different basal transport systems for K^+ entry may function at different rates depending on the basal K^+ (and Na^+) concentration and may thereby determine the amount of K^+ available to transport across the apical membrane via the K^+/H^+ antiporter. The rise of the cellular K^+ in response to an increased basal K^+ concentration can occur both via K^+ channels or via a coupled K^+/Cl^- entry mechanism.

5. Questions remaining to be answered.

In the low K^+ high Na^+ solution, the intracellular K^+ content is seriously decreased (see Leyssens *et al.*, submitted). Consequently, intracellular K^+ has to be replaced by another cation, most likely Na^+. A Na^+ conductance could not be detected (see Leyssens *et al.*, 1992) but the uptake of Na^+ may occur via a $Na^+/K^+/2Cl^-$ cotransporter. The luminal K^+ concentration also decreased on lowering K_{bl} but not to the same extent as K_c (see also Leyssens *et al.*, submitted). This implies that K^+ is still the major cation secreted. Consequently, although the apical K^+/H^+ antiporter may have some affinity for Na^+ ions when intracellular Na^+ levels rise, secretion of K^+ is still predominant. This means that net basal K^+ uptake has to exceed net Na^+ uptake. Additional uptake via a K^+/Cl^- cotransporter seems unlikely: it would need a very low intracellular Cl^- concentration. Preliminary results show that this is not the case (Dijkstra, personal communication). As no evidence was found for a Na^+/K^+ ATPase we have no indication up to now on how intracellular Na^+ is regulated in our preparation. Theoretically, in view of the high intracellular pH found in Malpighian tubules of *Formica* (Zhang *et al.*, 1991), a Na^+/H^+ antiporter in the basal membrane, working in reverse mode and unexpectedly functioning at an alkaline pH, may extrude Na^+ in exchange for H^+.

Until now, the transepithelial Cl^- pathway still awaits further investigation. In high K_{bl} (113 mM), the electroneutral K^+/Cl^- cotransporter didn't seem to be functional and no relative Cl^- conductance was found. This seems to suggest that Cl^- transport is primarily paracellular. The lack of a relative Cl^- conductance in the basal membrane

(Leyssens *et al.*, 1992) points to a paracellular pathway for Cl^- down an electrical gradient in 113 mM K_{bl}. In 51 and 5 mM K_{bl}, a K^+/Cl^- or a $Na^+/K^+/2Cl^-$ cotransporter seems to be functional and at least part of the Cl^- transport may occur transcellularly, hence Cl^- channels are postulated to ensure Cl^- exit across the apical membrane. In a few experiments in 5 mM K_{bl}, a small hyperpolarization of V_{ap} was observed when applying bumetanide (Fig. 6D) (cfr. *Rhodnius*, O'Donnell and Maddrell, 1984; *Locusta*, Baldrick *et al.*, 1988). If a Cl^- conductance was present in the apical membrane, this might be explained by a drop in intracellular Cl^- concentration due to inhibition of basal Cl^- influx. The existence of a transcellular Cl^- pathway was also suggested by a study on symmetrically perfused *Formica* tubules (Dijkstra *et al.*, 1993). Cl^- channels have been detected by a patch clamp study on the apical membrane of *Aedes* tubules (Wright and Beyenbach, 1987). Apical Cl^- channels (as in rectal gland; Greger *et al.*, 1989) and/or bumetanide-sensitive basal cotransporters (as in *Aedes* tubules, Hegarty *et al.*, 1991) could be a site of regulation by hormones and second messengers to accelerate fluid secretion rate even in lower bath K^+ conditions.
Cl^--selective measurements are planned in order to estimate the intracellular and luminal Cl^- concentration in different bath K^+ conditions and to see whether the calculated gradients for Cl^- and K^+ are consistent with the transport mechanisms for K^+ and Cl^- proposed in the model.

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