CL^--TRANSPORT AND H^+-PUMPING IN THE PRIMARY URINE FORMATION IN THE ANT

Sandra Dijkstra

8 november 1993

Jury members: Prof. Dr. A. de Hemptinne (Rijks Universiteit Gent)
Prof. Dr. R. Greger (Albert Ludwigs Universität Freiburg)
Rector H. Martens (Limburgs Universitair Centrum)
Prof. Dr. P. Steels (Limburgs Universitair Centrum)
Prof. Dr. P.P. van Bogaert (Universitair Centrum Antwerpen)
Prof. Dr. W. Van Driessche (Katholieke Universiteit Leuven)
Prof. Dr. B. Vanheel (Rijks Universiteit Gent)
Prof. Dr. E. Van Kerkhove (Limburgs Universitair Centrum)

Thesis required
to obtain the degree of
Philosophical Doctor
in Biology
from the
Faculty of Sciences
L.U.C. Diepenbeek

Acknowledgement: this research was supported by EC grant SC1-CT90 0480.

Preface

Working with ants for three years has brought some surprising insights. Despite our superior individual intelligence, there is a lot we can learn from ants. For example their total altruism (which, contradictiously is thought to arise from the 'selfish gene'), their ability to work in a group, and some of their conventions: where humans send their able young men to war, the ants send the tough middle-aged females whose role in reproduction is already history. I've spent some pleasant moments leafing through the bible of ant-science ("The ants" by the king in a field where otherwise only queens count: E.Wilson [Hölldobler & Wilson, 1990]).

However, what I enjoyed most in these three years have been my colleagues. Both during and after working hours, most of them no longer mere "colleagues" but friends. The man who shaped my scientific thinking deserves a very special 'thank you', my first advisor Manuel Lohrmann. His patient explaining, his thoroughness, the hours of enlightning discussion, his preference for two components-glue, none will be forgotten. The time invested in me by 'those who are more equal than others': Prof. Greger, Prof. Steels and Prof. Van Kerkhove (Emmy sounds better) is another reason for gratitude. As is the thoroughness with which the rest of the jury has read and commented on my thesis.

Instead of naming explicitly every person I am indebted to for their help and companionableness (family, friends, jury-members, etc.), I dedicate to them the following poem (poor Shakespeare would turn in his grave if he saw how his "Shall I compare thee to a summer's day" has been abused).

Shall I compare thee to a *Manduca*'s midgut?

Shall I compare thee to a *Manduca*'s midgut?
Thou art more plenishful but much thinner.
Rough pH's tester the goblet cells of *Sexta*,
two cell types making it complexer.

Sometimes thou worketh, sometimes thou don't
And oft is thy dancing motion stilled,
But share the workload, that thou won't
Thy single cell type does all that is willed.

Thy workings are as yet perplexing,
Probing them both stimulating and vexing,
But thy secret of secretion shall fade!
Mystery won't brag thou wanderest in his shade,
For as long as man can read and eyes can see
(and money floweth from the EC)
So long work we and we investigate thee.

Sandra.

Table of contents

Objectives

For an effective insect pest-control, fundamental understanding of the physiology of insects is an absolute necessity. In this study we aimed for a better understanding of the Malpighian tubules, the functional equivalent of the kidneys in mammels, of the ant.

The in vitro perfusion technique of tubule segments can be highly informative as many parameters can be measured simultaneously (resistances, voltages) and the composition of both luminal and peritubular fluid is controlled. Therefore a first aim of this study was to establish whether it was at all possible to use this technique on the Malpighian tubules of the ant. If so, then an electrical characterization of the epithelium was our next goal (chapter 3).

In general in Malpighian tubules of insects, high NaCl or KCl gradients are build up (see introduction) and Cl^- is assumed to follow passively along its electrochemical gradient (review: [Phillips, 1981]). If Cl^- follows paracellularly, the shunt must be anion-selective since the tubules exhibit a high lumen positive potential, so as to keep the K^+ and Na^+ in and let the Cl^- through. This hypothesis was tested by biionic replacement experiments (chapter 3). The transcellular Cl^--pathway was further investigated by the measurement of intracellular and intraluminal Cl^--activities (chapter 6). Also specific Cl^--transporter blockers were used to elicit some information about the molecular mechanisms responsible for Cl^--transport (chapters 3 and 6).

The active cation transport in insect epithelia has been postulated to be due to a vacuolar type H^+-ATPase at the luminal membrane in combination with a H^+/K^+-antiporter [Wieczorek et al., 1989]. This hypothesis was further investigated for the Malpighian tubules of *Formica* and a characterization of the apical pump with the aid of specific blockers and pH-sensitivity was made (chapter 4). In an earlier study dinitrophenol (DNP) has been used to learn more about the active step at the luminal membrane [Leyssens et al., in press(b)]. In this study the protonophoric effect of DNP on the Malpighian tubules was investigated (chapter 5).

1 Introduction

"Ants are everywhere, but only occasionally noticed... recent measurements suggest that about one-third of the entire animal biomass of the Amazonian *terra firme* rain forest is composed of ants.." [Hölldobler & Wilson, 1990]. Ants are the most destructive insect pests of Central and South America [Cherrett, 1982]. "Ants alter their physical environment profoundly. They are responsible for a large part of the dispersal of vegetation, they highly enrich deeper laying soil by carrying fresh green into their nest chambers and in most terrestrial habitats they are among the leading predators of other insects and small vertebrates" [Hölldobler & Wilson, 1990]. Ants are thus an insect species well worth researching.

In this electrophysiological study we investigated the mechanism of ion transport in the Malpighian tubules of forest ants (*Formica polyctena* and *F. rufa*).

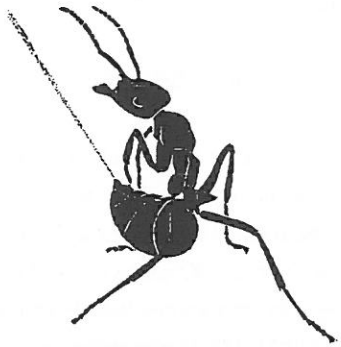
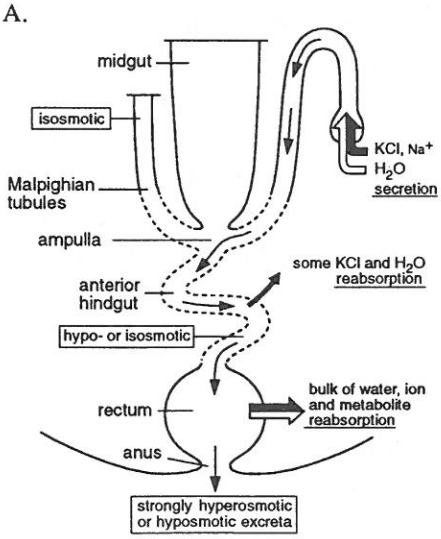


Fig.1.1 Reaction of a forest ant to an oncoming scientist (by Johan Billen)

A.



B.

Fig.1.2 A. Schematic illustration of the Malpighian tubule. Adapted from Phillips (1981). B. Photo of dissected midgut with tubules attached.

The Malpighian tubules of insects are the sites of primary urine formation and haemolymph clearance. They are blind ending tubules of a single cell layer attached to the base of the ampulla. Fig.1.2 schematically illustrates the position and function of Malpighian tubules. In the case of *Formica polyctena* the tubules consist of one cell type only. The cells demonstrate characteristics typical for cells with a transport function: large euchromatic nucleus, several nucleoli, abundant mitochondria, a conspicious brush border and basal infoldings [Garayoa et al., 1992]. Since the environments on the apical (or luminal) and basolateral (or haemolymph) side differ, and since the function of the tubules is to secrete in a specific direction namely into the lumen, the cells are polarized to meet these demands. Besides having different transporters, channels and pumps on the two different membranes (apical and basolateral), electrical properties as conductivities and thus resistances, also differ.

A brief overview of the field will be given here to put this study in perspective.

Since insects have no closed "blood"- or rather haemolymph-circulation, urine formation by pressure-driven filtration, as in mammalian kidneys, is excluded. The primary urine must thus be formed by secretion. In most insects (as in forest ants) secretion is driven by active transport of K^+ since this is the most common ion found in plants, on which the insects feed. In carnivorous insects active transport of Na^+ also plays an important role [Phillips, 1981]. Cl^- is generally assumed to follow passively. The secreted fluid is isotonic with the haemolymph, locally osmotic gradients are thought to be responsible for water transport to the lumen. The numerical details for the Malpighian tubules of ants are shown in fig.1.3. The ion concentrations in the haemolymph exhibit quite large variations (ranges of measurement: K^+ 12-70 mmol/l, Na^+ 105-215 mmol/l, Cl^- 31-43 mmol/l [Van Kerkhove et al., 1989]). In deciding upon a control solution this large tolerance has been taken into account and a Ringer's solution with fairly high K^+- and Cl^--concentrations has been chosen, since this positively influences fluid secretion. The elements generally thought to play a role in the active secretion of KCl (or NaCl, as the case may be) are depicted in fig.1.4 (adapted from [Nicolson, 1993]). It must be noted here that the variety amongst insects is large. Fig.1.4 gives a compilation of different transporters, channels and pumps found in different species. This is not to say all are present in every insect. In fact, while some elements, as for example a basolateral Cl^--conductivity, have been demonstrated in some insects (*Onymacris* [Nicolson & Isaacson, 1987], *Glossina* [Nicolson, 1993]), their absence has been shown in others (*Aedes* [Sawyer & Beyenbach, 1985], *Drosophila* [Wessing et al., 1987]). This variety among species stresses the caution to be taken when extrapolating results from other insects to the species under study.

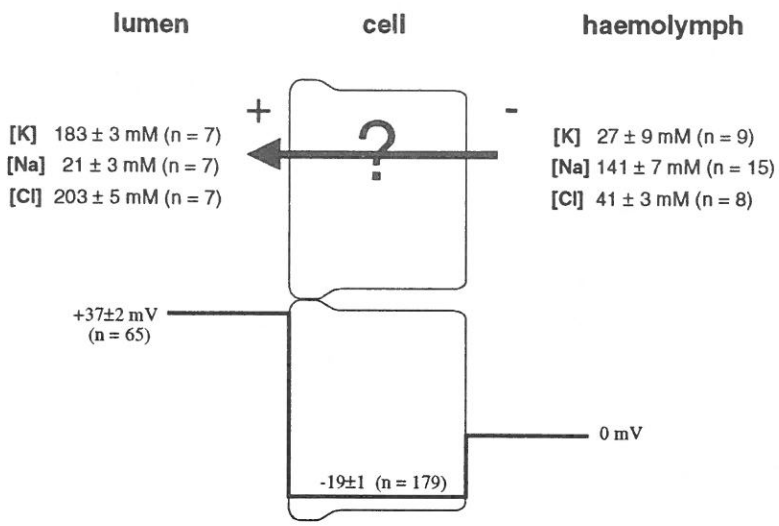


Fig.1.3 Circumstances in which Malpighian tubules secrete. Ion concentrations taken from micro X-ray analysis and flamephotometer data [Van Kerkhove et al., 1989]. Ion concentrations in the lumen determined from the secreted fluid gathered from tubules bathed in control solution (table 2.1, 51 mmol/l K^+, 62 mmol/l Na^+, 57 mmol/l Cl^-). Electrical data from Leyssens et al. [1992]; tubules bathed in the same solution.

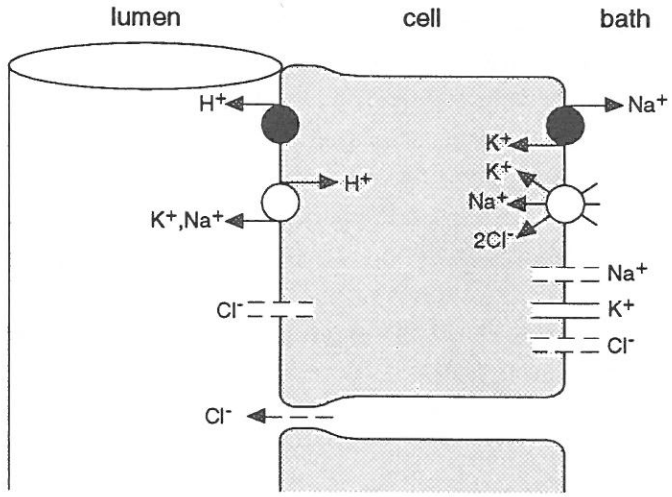


Fig.1.4 General model for K^+-Na^+-Cl^--secretion in Malpighian tubules of insects. Adapted from Nicolson (1993).

Since the electrophysiological evidence in support of these proposed mechanisms has been comprehensively stated in a recent review by Nicolson (1993), we will just discuss the different transport elements in the light of the facts known about ant Malpighian tubules.

An 'alkali metal ion'-pump has been held responsible for the active extrusion of K^+ at the apical membrane [Harvey et al., 1983; Maddrell, 1977]. Intensive research as to the nature of this "common cation pump" has led to the discovery of a vacuolar type H^+-ATPase in the apical membrane of goblet cells of the *Manduca Sexta* midgut, a model for K^+-transporting epithelia [Schweikl et al., 1989; Wieczorek et al., 1989]. In general there were thought to be two types of H^+-ATPases (review: [Al-Awqati, 1986]): 1. the E_1E_2-type or phosphorylated ATPases present in e.g. plant and yeast plasma membranes. The gastric H^+/K^+-ATPase and non-proton pumps such as Na^+/K^+-ATPases are also members of this family. The pumps consist of one large transmembrane protein (±100 kDa) and sometimes another, small subunit; 2. the F_1F_0-type which only catalyzes electronic proton transport. It is present in e.g. mitochondria, chloroplasts and bacteria. The transmembrane portion (F_0) consists of three proteins with stoichiometry a,b,c_{10} and acts as a proton channel. The large catalytic domain (F_1) consists of at least five proteins. In electron microscopic images it protrudes as a sphere on a stalk above the membrane and gives the F_0F_1-pump its "lollipop" appearance. In the second half of the 80's a novel class of H^+-ATPases has been identified: the vacuolar type (review: [Forgac, 1989]). These pumps occur mainly in intracellular organelles such as endosomes, lysosomes, Golgi apparatus, plant vacuoles, etc., but have also been identified in the luminal plasma membrane of kidney cells and now in the plasma membrane of some insect epithelia such as Malpighian tubules, midgut, salivary gland and sensilla. The structure of vacuolar type pumps also exhibits the "ball-and-stalk"-model seen with the F_0F_1-type ATPase, but the two classes differ in their inhibitor specificity. Bafilomycin has so far been the only inhibitor specific for vacuolar type H^+-pumps [Bowman et al., 1988]. Recently the same laboratory published a class of even more effective vacuolar type pump inhibitors: concanamycins [Dröse et al., 1993].

The H^+-pump in Malpighian tubules is parallelled by a H^+/K^+-antiporter thus providing the secondary active K^+-extrusion mechanism. Evidence for the existence of such a pump in the Malpighian tubules of ants is given in chapter 4 and has also been provided by other experimentators in this laboratory [Weltens et al., 1992; Zhang et al., in press]. The existence of the ubiquitous Na^+/K^+-pump of vertebrate epithelia is controversial in the Malpighian tubules of insects. Their presence has been repeatedly suggested to explain the observation that Na^+ stimulates K^+-driven fluid secretion when external K^+-concentrations are low [Phillips, 1981]. However, in the light of the results presented in chapter 6, an increased importance of a basal NaK2Cl-cotransporter under these conditions could also be responsible for this effect. In most insect species no, or only modest, ouabain inhibition of fluid secretion has been observed [review: Anstee & Bowler, 1979]. However, some insects feed on toxic plants containing cardiac glycosides such as ouabain and the possibility of an ouabain insensitive Na^+/K^+-ATPase has been suggested [Phillips, 1981]. In ants its presence cannot be excluded, but the

possible role in fluid secretion appears to be minimal (chapter 4). No effects of ouabain have been observed either on secretion or membrane potentials [Leyssens, 1993].
Possible basal entrance mechanisms for K^+ include K^+-channels, the controversial Na^+/K^+-pump and KCl-cotransporters. A high K^+-conductivity has been established for *Formica polyctena*, the direction of the electrochemical gradient is not always clear since it is small but because the conductivity is so high, a very small inward gradient would suffice for total basal K^+ transport [Leyssens et al., in press (a)]. In chapter 3 this high K^+ conductivity, and the blockage of it by Ba^{2+}, has been used to arrive at estimates of the basolateral and apical membrane and shunt resistances. Pannabecker et al. (1992) have found numerical values for the individual membrane resistances of Malpighian tubules in *Aedes* by the application of 2,4-dinitrophenol (DNP) which uniquely increases the transepithelial resistance (R_{te}). On the basis of some observations it was assumed DNP had completely blocked the transcellular pathway.

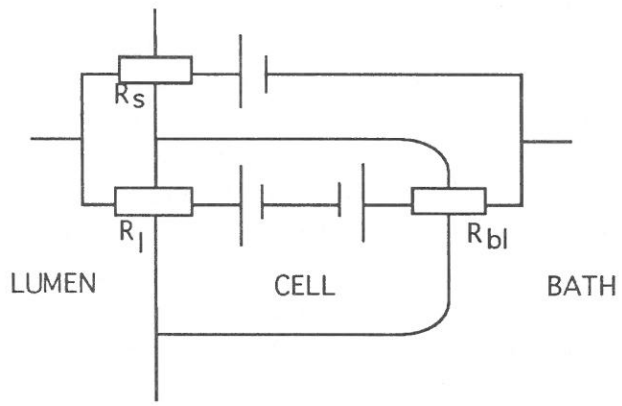


Fig.1.5 The simple electrical model for a polarized epithelium.R_S resistanceof the shunt, R_l resistance of the luminal membrane, R_{bl} resistance of the basolateral membrane.

According to the electrical model for the epithelium (fig.1.5): $1/R_{te} = 1/R_S + 1/R_{cell}$ (where R_S and R_{cell} represent respectively the resistance of the shunt and of the cell), thus if R_{cell} is increased infinitely the measured R_{te} will equal the shunt resistance. With the aid of the R_{te} under control conditions and the voltage divider ratio (VDR, the apical over basolateral membrane resistance), the absolute resistances for the apical and basolateral membrane can then be calculated. This was not possible in the epithelium under study since addition of DNP caused a decrease in R_{te} instead of an increase. This decrease was further investigated in chapter 5.

The gradient for Cl^- both over the total epithelium and over the apical membrane generally allows passive entrance of Cl^- into the lumen of the Malpighian tubules of all

species studied sofar. This is confirmed for *Formica* with the experiments with double barrelled Cl^--selective electrodes presented in chapter 6. It is, however, not clear whether the main Cl^- pathway is trans- or paracellular. To address this question the specificity of the shunt for Cl^- was investigated (chapter 3). Concerning the possible transcellular route, the presence of some form of KCl-cotransport in the basal membrane is suggested by the inhibition of fluid secretion by furosemide and/or bumetanide in many species (*Rhodnius, Aedes, Locusta, Glossina, Drosophila*, references in [Nicolson, 1993]), including *Formica polyctena* [Leyssens, 1993]. As is discussed both in chapter 3 and 6 and in the review by Nicolson (1993), the presence of either basal or apical Cl^- channels varies widely among insect Malpighian tubules. To investigate the possible existence of Cl^- channels in the apical membrane of the ant Malpighian tubule, the effect on electrical parameters of the luminal application of Cl^- channel blockers was investigated (chapter 3). If a conductivity was blocked in the apical membrane, one would expect an increase in the VDR and the R_{te}. One might also expect the apical membrane to hyperpolarize since a possible Cl^- conductivity would have a depolarizing effect (intracellular Cl^- is expected to be smaller than luminal Cl^- and indeed found to be so, chapter 6), however the contribution of a Cl^- conductance to the total membrane potential might have been very small in the first place. To investigate the possibility of a basal Cl^- conductance, Cl^- concentrations in the bath were changed and the effect on potentials and intracellular Cl^- registered. Since in general Cl^- channels are often found to be cAMP-sensitive, the effects of cAMP-application on intracellular and luminal Cl^- concentrations and potentials were also investigated (chapter 6). If a Cl^- conductance were enlarged in either of the two membranes, one would expect either the membrane potential or the distribution of the Cl^- ions over the membrane to come closer to Nernst equilibrium. Most likely would of course be a combination of the two.

2 Methodology

2.1 General information

The ants

All experiments were performed on isolated Malpighian tubules of *Formica polyctena* and *rufa* (respectively small and red forest ant; *Hymenoptera, Insecta*). Two ant genera were used because the work was performed in two laboratories and neither genera occurred naturally in both places. These two genera are very closely related, a distinction in their classification has only been made since 1953 [Bernard, 1968]. Their protein maps and chromatogrammes of whole worker ants showed no significant difference [Gößwald, 1989]. The ants were collected from natural nests in the woods and kept at room temperature in artificial nests in the laboratory until use. To imitate natural conditions, humidity was kept high, between 40 and 60 %. In natural surroundings the nests average 1.5 m in height (from which only 0.5 m above ground) and 1.3 m across. The presence of many worker ants in this limited space creates a special micro-climate: the air is relatively warm due to the warmth produced in the course of metabolic and muscle activity and has a high formic acid-content, the CO_2-content is around 40% [Bernard, 1968]. Since these conditions could not be met in laboratory surroundings, the ants from artificial nests were only used for a limited time and replenished regularly. The ants were fed water and sugar *ad libitum.*

Two methods were used to study these tubules: 1. the luminal perfusion technique measuring transepithelial and basolateral potential differences and resistances, and 2. impalement with double barrelled microelectrodes measuring potentials and electrochemical gradients for chloride in non-perfused Malpighian tubules. Furthermore, some additional experiments measuring spontaneous fluid secretion were performed. All experiments were performed at room temperature.

Dissection of the Malpighian tubules

Ants were killed by decapitation, the abdomen opened with fine forceps in a low K^+-Ringer's solution (5K-solution table 2.1). The whole tubules (2-3 mm in length) were removed, the blind end was cut off and the tubule transferred into the perfusion chamber and mounted on the stage of an inverted microscope (Freiburg: Zeiss IM-35, Diepenbeek: Nikon diaphot-TMD) where they were hooked up between a constriction and a collection pipette (constriction diameter 30-40 µm). The tubules were considered acceptable (i) if they writhed spontaneously in a low K^+-concentration due to the contraction of the spiral muscle belt around the tubule, (ii) if an open lumen could be detected when studied under a microscope and (iii) if the tubule cells were optically clear. The dissected tubules under

study had a final length varying between 200 and 2100 µm, an inner diameter of 22±6 µm (n=18) and an outer diameter of 48±5 µm (n=10).
Bath flow rate during experiments was usually such that the bath was exchanged 6 times per minute.

Artificial salines and test substances
The bath was perfused with haemolymph-like solutions (table 2.1). In the luminally perfused experiments 51K57Cl-Ringer's solution was used as control solution, in the experiments measuring Cl^-, 51K143Cl was used as control-solution to minimize interference of other anions with the measurements. In experiments where Cl^- was measured, different Cl^--concentrations were achieved by replacing Cl^- with SO_4^{2-}. Solutions were freshly prepared and kept at 4°C until use. Different NaCl concentrations needed in establishing the specificity of the shunt were achieved by replacing glucose by NaCl and vice versa (table 2.1). A Ba^{2+} containing solution was prepared by adding 6 mmol/l $BaCl_2$ to the control solution. $Ba_3citrate_2$ is water soluble up to a concentration of 32 mmol/l. The Ca^{2+}-activities as measured with a Ca^{2+}-selective electrode, was 3.1±0.2 (n=5) mmol/l in solutions containing 143 mmol/l Cl^- and 0.3±0.1 (n=6) mmol/l in the solutions where Cl^- had been replaced by $citrate^{3-}$. Solutions where Cl^- was replaced by SO_4^{2-} were not measured.

Table 2.1. The composition of the Ringer's solutions.

	control 51K57Cl[1]	5K	high NaCl	low NaCl	control 143 Cl[2]	14 Cl
K^+	51	5	51	51	51	51
Na^+	62	108	127	36	62	62
Cl^-	57	57	122	30	143	14
SO_4^{2-}						64
$Citrate^{3-}$	29	29	29	29		
Ca^{2+}	2	2	2	2	2	2
Mg^{2+}	13	13	13	13	13	13
Alanine	3	3	3	3	3	3
Glucose	139	139	31	183	161	170
Hepes	12	12	12	12	12	12

All concentrations in mmol/l. pH was adjusted to 7.2 by adding NaOH. Osmolality was 350 mosm/l.
[1] Control used in the luminal perfusion experiments. [2] Control used in the experiments with Cl^--selective micro-electrodes.

NPPB, (5-nitro-2-(3-phenylpropylamino)-benzoate), from the laboratory of Prof. Greger (Freiburg, used in a concentration of 10^{-5} mol/l), DIDS (4,4'-diisothiocyano-2,2'-

stilbenedisulfonate, Sigma), bafilomycin A_1 (Prof. Altendorf, Osnabrück) and NEM (N-ethylmaleimide, Sigma, used at 5.10^{-4} mol/l) were presolubilized in DMSO (dimethylsulfoxide, Merck and Sigma), the final concentration of DMSO never exceeding 0.1 %. Omeprazole (used at 10^{-4} mol/l, Hässle, Sweden) was presolubilized in paraffin, the final concentration never exceeding 1 %, S28080 (Schering compound 28080, used at 10^{-4} mol/l, a kind gift from Prof. H. Oberleithner, Würzburg) was kept in a 10^{-2} mol/l stock in 1:1 DMSO/ethanol. DNP (2,4-dinitrophenol, Sigma and UCB), CPT-cAMP (8-(4-chlorophenylthio)-adenosine3'-5'-cyclic monophosphate, Sigma) and vanadate (Na_3VO_4, Janssen Chimica) dissolved readily in the Ringer's solutions after pH readjustment.

The luminal perfusion solution was similar in composition to the bath control solution. However, to diminish growth of bacteria and fungi which clog the perfusion pipette, in one series of experiments (n=7) glucose and alanine were substituted by mannitol (28 mmol/l) and citrate by gluconate (total osmolality was 300±4 mosmol/l). In all other experiments luminal solution osmolality was 350 mosmol/l. Paired tests showed no significant difference between the parameters obtained with the two solutions. Therefore, results with the slightly different luminal control solutions were pooled.

Statistics

Results are presented as mean values ± SEM (n = number of experiments). A paired t-test was used to test for statistical significance of the differences. A P-value of < 0.05 was accepted as indicating statistical significance.

2.2 Luminal perfusion technique

Experimental set-up

The in vitro perfusion of isolated tubule segments as first described by Burg and coworkers [Burg, 1972] has been extensively modified by Greger and Hampel (1981). The set-up is illustrated schematically in fig.2.1. In these experiments the tubules were hooked between a collection and a constriction pipette. The constriction of the pipette had a diameter of about 30 µm widening again to around 60 µm at the tip. This 'funnel' was kept as short as possible (usually around 50 µm). The collection pipette was slightly wider: around 50 µm and judiciously fire polished but with no constriction so as not to impede perfusion. Both the collection and the perfusion set-up were mounted on Leitz manipulators in Freiburg and on electromotor driven SMI Luigs & Neumann manipulators in Diepenbeek. Within the constriction pipette a double barrelled perfusion pipette was centered. This was made of theta shaped borosilicate glass with a 1.2 mm outer diameter and 0.1 mm wall thickness (Hilgenberg, Malsfeld, Germany). It was either

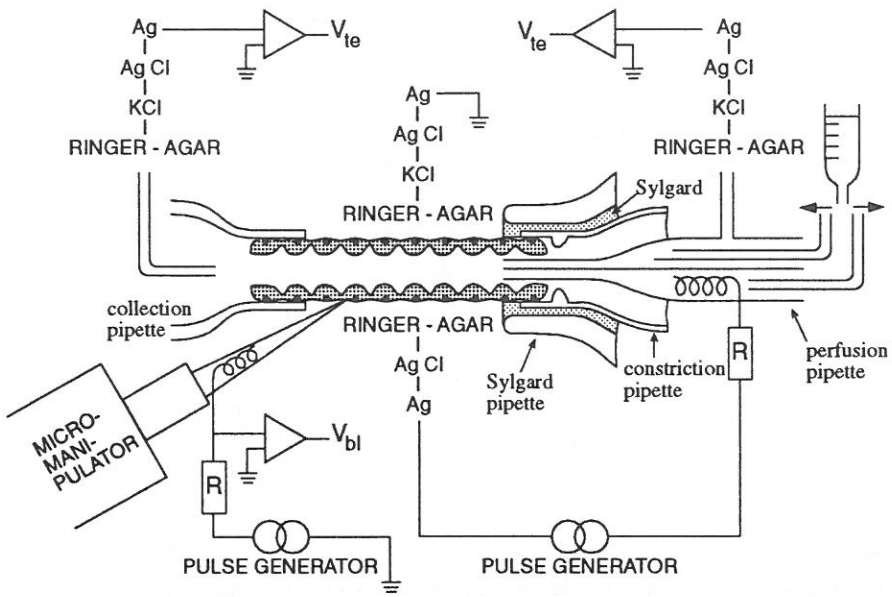


Fig.2.1 Schematic illustration of the experimental set-up for the luminal perfusion, resistance and potential measurements of isolated tubules.

manually made on a home made puller (Freiburg) or pulled on an automatic two-steps puller (Zeitz, DMZ Universal puller, Diepenbeek). Manually, the glass was carefully drawn over a length of around 3000 µm to a diameter near 15 µm. Then a length of 1500-2000 µm was drawn with a constant 15 µm diameter. Because of this long 15 µm diameter-part, the tip of the perfusion pipette could be broken off a few times after the pipette had already been built into the set-up to combat clogging of the tip. In one series of experiments (chapter 3) the perfusion pipette tip was slightly fire polished to lessen damage to the luminal side of the cells. Consequently, the perfusion pipette had to be taken out of the system every time the tip was clogged and subsequently broken. The pipettes that had been drawn on the two-steps puller ended in a sharp tip which was polished off with the aid of a rotating fine-grain wetstone until the tip diameter was also around 15 µm.

The holder by which the perfusion pipette is centered and held, is fastened onto another holder which could be moved over the supporting rails by an electric motor (Luigs & Neumann). It allowed careful insertion of the perfusion pipette into the lumen. A relatively large Sylgard pipette (final diameter 200-500 µm) coated with hardened Sylgard (No.184) and filled with Sylgard oil (attached to a different holder that could be moved separately by the same motor system) was moved over the tubule for electrical insulation. One of the barrels of the perfusion pipette contained a fluid-exchange pipette (see fig.2.2). This fluid-exchange pipette was significantly easier (and thus cheaper) to produce than the

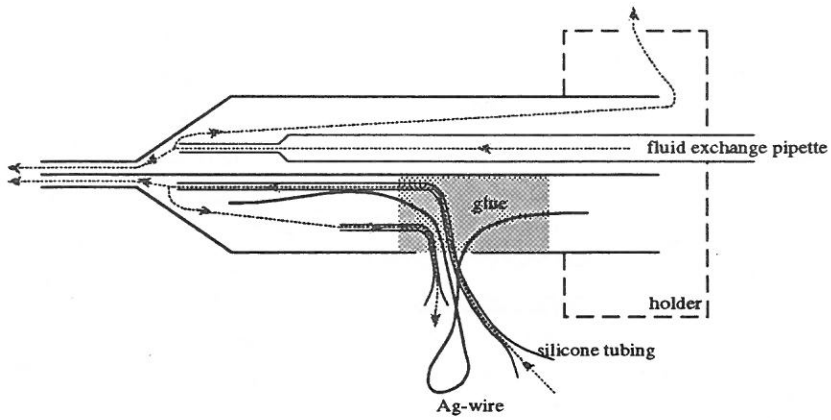


Fig.2.2 Schematic illustration of the perfusion pipette and the possible pathways for flow of the perfusion solutions.

perfusion pipette, and it could be built into and out of the pipette system keeping the rest of the system intact, as opposed to the perfusion pipette. Thus the tip of the fluid-exchange pipette was brought down to 50-80 µm so larger debris in the perfusion fluid would get caught here rather than in the perfusion pipette. Bringing the diameter down further would hamper an efficient exchange of the fluid reservoir in the perfusion pipette during a luminal fluid exchange. This thinner part of the fluid-exchange pipette was prolonged for roughly 400 µm so the tip could be brought as close to the tip of the perfusion pipette as possible. The perfusion pipette-barrel containing the fluid-exchange pipette was connected over a 5% agar bridge made from control Ringer's solution, to a 1 mol/l KCl solution in which the chlorated end of an Ag-wire was inserted, that was connected to an electrometer (Keithley model 602). The other pole of the electrometer was grounded. Since the bath-solution was grounded (over a 5% agar bridge from control Ringer's solution and a chlorated Ag-wire in a 1 mol/l KCl-solution) the transepithelial potential difference (V_{te}) could thus be measured. A similar construction at the collection side principally allowed a double check of the measured V_{te}. However, due to difficulties at the collection end in obtaining adequate electrical insulation, a systematically underestimated V_{te} was registered there. Therefore V_{te}'s presented here are those measured at the perfusion side only. The other barrel of the perfusion pipette contained an Ag-wire connected to a constant current pulse stimulator (Diepenbeek: Grass Instruments, Quincy, MA, USA, Freiburg: home made). This pulse stimulator was connected to the bath over a separate 5% control Ringer's solution agar bridge, so as to keep the circuit off ground and thus no corrections for a current pulse induced voltage deflection across the (low, smaller than 1 MΩ) resistance of the agar bridge connecting the bath to earth were necessary. Herewith 1 second current pulses were injected into the lumen every 10 seconds which resulted in a deflection of the measured V_{te}'s (ΔV_{te}). From the height of

these deflections, the specific transepithelial resistance, R_{te} ($\Omega.cm^2$) and the length constant λ (μm) were calculated by cable analysis (see derivation of cable equations). The current used was limited to 20 - 50 nA because it was noticed that V_{te} decreased extensively when higher currents were injected. The equivalent short circuit current, I_{sc}, was calculated as V_{te}/R_{te}. This I_{sc} is thus not a measured current when V_{te} is clamped to zero. I_{sc} was assumed to be proportional to the rate of net salt secretion in symmetrically perfused tubules. Since the isotonic fluid secretion in Malpighian tubules is thought to occur by locally osmotic gradients created by primarily KCl-secretion [Phillips, 1981], the fluid secretion is in turn assumed proportional to net salt secretion. This hypothesis seems to be confirmed by the results: addition of Ba^{2+} (chapter 3), NEM and vanadate (chapter 4) all diminish I_{sc} significantly, DNP even decreases I_{sc} to 0 (chapter 5). The same substances have been shown to significantly decrease spontaneous fluid secretion [Weltens et al., 1992; Leyssens et al., 1993] and even completely abolish it in the case of DNP [Leyssens et al., in press(b)]. A direct, quantitative comparison of these results is impossible because of the large intrinsic differences between the two experimental set-ups. E.g. the time resolution in the case of the perfusion technique is in the order of seconds whereas with the spontaneous secretion experiments samples are taken only every 10 minutes. Additionally, the sample of the first 10 minutes-period during the application of the drug is distorted by the fluid still present in the lumen from the control period.

In the luminal perfusion set-up single cells were impaled from the basolateral (bath) side with glass micro-electrodes pulled from filament capillaries (1.5 mm OD, 1.0 mm ID, Hilgenberg) and backfilled with 1 mol/l KCl-solution, mounted on a manipulator (Freiburg: Leitz; Diepenbeek: Narishige). The potential measured represents the basolateral potential difference, V_{bl}. The apical or luminal potential difference V_l, was calculated as $V_{te} - V_{bl}$. The electrode resistances varied between 100 and 300 MΩ. The voltage divider ratios, VDR's, were calculated from the deflections of V_{bl} upon injection of current into the lumen (see theoretical considerations). The individual experiments lasted until V_{te}, which fell with time and after several luminal fluid exchanges, dropped below the limit set at 20 mV. The total experimental period varied among tubules and lasted up to 1.5 hours.

Generally, criteria usually used to establish whether the lumen of a tubule actually is perfused entail: 1. a noticable opening of the lumen once luminal perfusion pressure is applied; 2. closure of the lumen when the pressure is removed and low suction is applied at the collection end; 3. movement of small debris in the lumen. However, experience showed that these optical criteria for perfusion of the lumen could be deceiving in the case of Malpighian tubules and a functional test had to be performed in every experiment. For this purpose the luminal pH was lowered during a short period (±2 min) to values between 4.4 and 4.7. This should result in a significant and reversible decrease of the V_{te}

(chapter 4, i.e. [Dijkstra et al., in press]). If this was not the case, the tubule was discarded.

The rate of luminal perfusion was 10-20 nl/min, i.e. a factor 100 times higher than spontaneous secretion [Van Kerkhove et al., 1989], thus preventing changes in luminal concentrations of the major ions K^+ and Cl^-. Luminal solutions were exchanged by flushing the barrel of the perfusion pipette in use at that moment with the outlet open, thus exchanging the reservoir in the pipette whilst putting as little extra pressure as possible on the tubule (see fig.2.2). To this end a fluid exchange pipette was inserted in one of the barrels of the perfusion pipette. In the other barrel two pieces of silicone tubing pulled over a bunsen-burner were inserted through a hole in the side of the perfusion pipette together with the Ag-wire needed to inject the current. The hole was sealed with glue (Freiburg: UV-hardening dentist glue; Diepenbeek: 10 sec-glue) after insertion of the tubes. To fully exchange the fluid in the tip of the perfusion pipette and so much of the fluid reservoir as influences the fluid perfused into the lumen, about one ml of fluid was needed when exchanging on the fluid exchange pipette-side, and 0.2-0.4 ml on the silicone tubing side (depending on the resistances of the manually drawn tubes in that perfusion pipette). The same barrel was always used within one experiment to prevent artefacts induced by different luminal pressures, since the barrels are differently traversable.

Derivation of cable equations

The general derivation and the specific description of method 1 (see below) is largely based on the work of Sackin (1978).

Definitions (see also fig.2.3):

$R_{te} = 2\pi r \cdot r_m$ [$\Omega.cm^2$] eq.(1)

R_{te} = specific epithelial resistance per area [$\Omega.cm^2$]

r_m = specific resistance of the epithelium per tubule length [$\Omega.cm$]

L = length of the tubule [cm]

r = tubule radius [cm]

R_{core} = core resistance per tubule length [Ω/cm]

$$R_{core} = \frac{r_{sol}}{\pi r^2} \qquad \text{eq.(2)}$$

r_{sol} = volume resistivity of luminal solution [$\Omega.cm$]

i_m = transverse membrane current [A/cm]

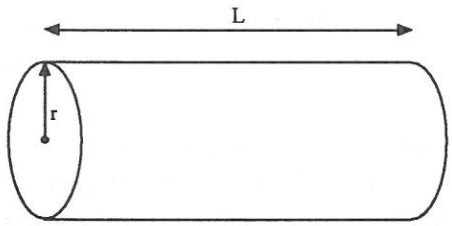
i_{core} = longitudinal current along the axis of the tubule [A]

c_m = membrane capacitance [F/cm]

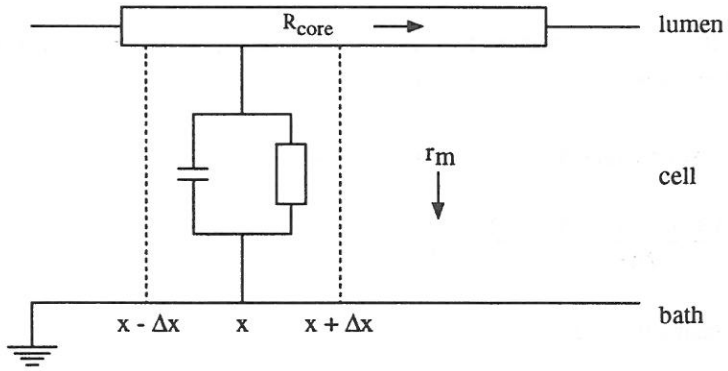
$R_{input} = \Delta V_{te}^0 / I_0$ [Ω]

R_{input} = total resistance of the system.

A model



B resistances



C currents

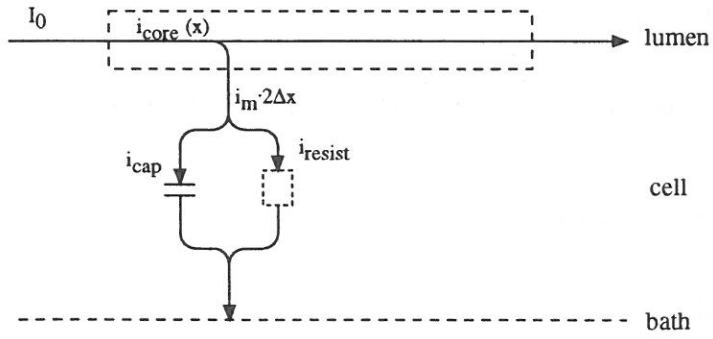


Fig.2.3 Model used in deriving the cable equations, adapted from [Sackin, 1978].

$\Delta V_{te}(x)$ = voltage deflection measured at point x from the tip of the perfusion pipette, upon current injection

ΔV_{te}^{0} = voltage deflection measured at the perfusion side upon current injection.

ΔV_{te}^{L} = voltage deflection measured at the collection end upon current injection.

Kirchoff's first or "loop"-law: the algebraic sum of the electromotive forces in any loop equals the algebraic sum of the I*R products in the same loop, i.e. $\Sigma V = \Sigma I \cdot R$. Applying this to the part of the tubule between the points x and (x+Δx) (see fig.2.3):

$\Delta V_{te}(x) - \Delta V_{te}(x+\Delta x) = i_{core}(x+\Delta x) \cdot R_{core} \cdot \Delta x$

taking the limit as $\Delta x \rightarrow 0$:

$$\frac{\delta(\Delta V_{te}(x))}{\delta x} = -i_{core} \cdot R_{core} \qquad \text{eq.(3)}$$

Kirchoff's second or "branch"-law dictates: the algebraic sum of the currents towards any branch point is zero, i.e. $\Sigma I = 0$. Thus

$i_{core}(x-\Delta x) = i_{core}(x+\Delta x) + i_m \cdot 2\Delta x \quad <=>$

$$\frac{i_{core}(x+\Delta x) - i_{core}(x-\Delta x)}{2\Delta x} = -i_m$$

Again taking the limit as $\Delta x \rightarrow 0$

$\frac{\delta i_{core}}{\delta x} = -i_m$ combining this with eq.(3):

$$-i_m = -\frac{1}{R_{core}} \cdot \frac{\delta^2(\Delta V_{te}(x))}{\delta^2 x} \qquad \text{eq.(4)}$$

The current flow across the membrane can be divided into a capacitative and a resistive current:

$i_m \cdot 2\Delta x = i_{cap} + i_{resist}$

where $i_{cap} = c_m \cdot 2\Delta x \frac{\delta(\Delta V_{te}(x))}{\delta t}$ and $i_{resist} = \frac{\Delta V_{te}(x)}{r_m} \cdot 2\Delta x$

therefore $i_m = c_m \cdot \frac{\delta(\Delta V_{te}(x))}{\delta t} + \frac{\Delta V_{te}(x)}{r_m}$

This combined with eq.(4) yields the basic 1-dimensional cable equation:

$$\frac{\delta^2(\Delta V_{te}(x))}{\delta^2 x} - \frac{R_{core}}{r_m} \Delta V_{te}(x) = c_m R_{core} \frac{\delta(\Delta V_{te}(x))}{\delta t}$$

Since we evaluated the experimental data gathered once a steady state was reached: $\frac{\delta(\Delta V_{te}(x))}{\delta t} = 0$ and the equation simplifies to $\frac{\delta^2(\Delta V_{te}(x))}{\delta^2 x} - \frac{R_{core}}{r_m}\Delta V_{te}(x) = 0$

Defining the length constant λ as $\sqrt{r_m / R_{core}}$ we can write

$$\frac{\delta^2(\Delta V_{te}(x))}{\delta^2 x} - \frac{1}{\lambda^2}\Delta V_{te}(x) = 0 \qquad \text{eq.(5)}$$

The most general solution to the integration of this equation is:

$\Delta V_{te}(x) = Ae^{x/\lambda} + Be^{-x/\lambda}$ where A and B are constants. eq.(6)

We can define the system further with the aid of the boundary conditions. There are four ways of calculating R_{te}, depending on the boundary conditions observed. Two were used in this study.

Method 1 (yields R_{te1}) where the current induced deflections at the collection side (ΔV_{te}^{L}) are used to calculate λ_1 (i.e. λ calculated according to method 1). The optically measured radius of the tubule is then redundant. And method 2 (R_{te2}) where the cable is assumed to be of infinite length and λ_2 (i.e. λ calculated according to method 2) is calculated using the optically measured radius (r) rather than the deflections measured at the collection end.

Method 1. Assuming the Malpighian tubule is well insulated at the side of the perfusion pipette (x=0) and thus no voltage "leaks" away, leads to

$\Delta V_{te}(x) = A + B$ eq.(7)

Assuming insulation at the collection end is also optimal, and thus no axial current can leave the tubule, we write: $i_{core}(x=L) = 0$

Recalling equation 3:

$$i_{core} = \frac{-1}{R_{core}} \cdot \frac{\delta(\Delta V_{te}(x))}{\delta t} = \frac{-1}{R_{core}}\left\{\frac{A}{\lambda}e^{x/\lambda} - \frac{B}{\lambda}e^{-x/\lambda}\right\} \qquad \text{eq.(8)}$$

and substituting $i_{core}(x=L) = 0$ we find:

$$0 = \frac{-1}{R_{core}}\left\{\frac{A}{\lambda}e^{L/\lambda} - \frac{B}{\lambda}e^{-L/\lambda}\right\}$$

therefore $\frac{A}{\lambda}e^{L/\lambda} = \frac{B}{\lambda}e^{-L/\lambda}$ eq.(9)

Combining equations 7 and 9 we can calculate the constants A and B as

$$A = \frac{\Delta V_{te}^0}{1+e^{2L/\lambda}} \quad ; \quad B = \frac{\Delta V_{te}^0 \cdot e^{2L/\lambda}}{1+e^{2L/\lambda}}$$

Substituting these expressions back into equation 6 and multiplying top and bottom by $e^{-L/\lambda}$ we find:

$$\Delta V_{te}(x) = \frac{\Delta V_{te}^0}{e^{L/\lambda}+e^{-L/\lambda}}\left\{e^{(x-L)/\lambda}+e^{-(x-L)/\lambda}\right\} \qquad \text{eq.(10)}$$

Knowing $\cosh a = \frac{e^a + e^{-a}}{2}$ equation 10 can be rewritten as

$$\Delta V_{te}(x) = \Delta V_{te}^0 \cdot \frac{\cosh\frac{x-L}{\lambda}}{\cosh L/\lambda} \qquad \text{eq.(11)}$$

Applying equation 11 to the position x=L:

$$\Delta V_{te}^L = \Delta V_{te}^0 \cdot \frac{\cosh 0}{\cosh L/\lambda} = \frac{\Delta V_{te}^0}{\cosh L/\lambda} \quad \text{from which we derive}$$

$$\frac{L}{\lambda} = \cosh^{-1}\frac{\Delta V_{te}^0}{\Delta V_{te}^L} \quad <=> \quad \lambda = \lambda_1 = L \cdot \cosh\frac{\Delta V_{te}^0}{\Delta V_{te}^L} \qquad \text{eq.(12)}$$

Another consequence of optimal insulation at the perfusion end is that all of the injected current, I_0, enters the tubule at x=0: $I_0 = i_{core}(x=0)$. Substituting this in equation 3 we write $I_0 = -\frac{1}{R_{core}} \cdot \left(\frac{\delta(\Delta V_{te}(x))}{\delta t}\right)_{x=0}$

Since V_{te} has been defined as a function of x in equation 10, we can write:

$$I_0 = -\frac{1}{\lambda \cdot R_{core}}\Delta V_{te}^0 \cdot \frac{\sinh\frac{0-L}{\lambda}}{\cosh L/\lambda} = +\frac{\Delta V_{te}^0}{\lambda \cdot R_{core}} \cdot \tanh L/\lambda \qquad \text{or}$$

$$R_{core} = \frac{\Delta V_{te}^0}{\lambda \cdot I_0} \cdot \tanh L/\lambda = \frac{R_{input}}{\lambda} \cdot \tanh L/\lambda \qquad \text{eq.(13)}$$

Combining the definition of R_{te} (eq.1) and the definition of λ, we can write

$R_{te} = 2\pi r R_{core}\lambda^2$ eq.(14)

Substituting equation 13 into 14:

$$R_{te} = 2\pi r \lambda R_{input} \tanh L/\lambda \qquad \text{eq.(15)}$$

Referring back to the definition of R_{core} (equation 2) we can substitute this into equation 14:

$$R_{te} = 2\pi r \frac{r_{sol}}{\pi r^2} \cdot \lambda^2 = \frac{2 r_{sol} \lambda^2}{r} \qquad \text{eq.(16)}$$

or $$r = \frac{2 r_{sol} \lambda^2}{R_{te}}$$

Substituting this equation in equation 13 we have eliminated the radius from the necessary input to calculate R_{te}:

$$R_{te} = \frac{4\pi r_{sol} \lambda^3 R_{input}}{R_{te}} \cdot \tanh L/\lambda \quad \Leftrightarrow$$

$$R_{te} = 2\sqrt{\pi r_{sol} \lambda^3 R_{input} \cdot \tanh L/\lambda} \quad \text{with} \quad \lambda = \lambda_1 = L \cdot \cosh \frac{\Delta V_{te}^0}{\Delta V_{te}^L} \quad \text{as in eq.(12)}$$

$$R_{te} = 2\sqrt{\pi r_{sol} L^3 \cdot \cosh^3 \frac{\Delta V_{te}^0}{\Delta V_{te}^L} \cdot R_{input} \cdot \tanh\left(\cosh^{-1} \frac{\Delta V_{te}^0}{\Delta V_{te}^L}\right)} = R_{te1} \qquad \text{eq.(17)}$$

Method 2. If the cable is infinitely long: $\tanh L/\lambda = 1$. For practical purposes this only has to be a few times the length constant (λ) (if $L = 2\lambda$ the error is 3.6%, if $L = 3\lambda$ the error is 0.5%). Applying the border conditions for this case we can derive the second way of calculating λ. If the cable is infinitely long, no current will leave at the collection side: $\lim_{x\to\infty} i_{core}(x) = 0$. Applying this to equation 8:

$$\lim_{x\to\infty}\left[-\frac{1}{R_{core}}\left\{\frac{A}{\lambda} e^{x/\lambda} - \frac{B}{\lambda} e^{-x/\lambda}\right\}\right] = 0$$

since $\lim_{x\to\infty} e^{-x} = 0$ this equation has two solutions: A=0 or $\lambda = \infty$. Clearly the last solution has no physiological relevance thus A=0, hence

$$i_{core} = \frac{B}{R_{core} \cdot \lambda} e^{-x/\lambda} \qquad \text{eq.(18)}$$

The second border condition again assumes perfect insulation at the perfusion side, thus $i_{core}(x=0) = I_0$. Substituting this in equation 18 we find a value for the constant B:

$$I_0 = \frac{B}{R_{core} \cdot \lambda} \quad \Leftrightarrow \quad B = R_{core} \lambda I_0$$

So in this case V_{te} as a function of x (eq.6) is $\Delta V_{te}(x) = R_{core} \lambda I_0 \cdot e^{-x/\lambda}$

At the tip of the perfusion pipette $\Delta V_{te}(x=0) = \Delta V_{te}^0$ thus (with the aid of the definition of R_{core} in eq.2)

$$\Delta V_{te}^0 = R_{core}\lambda I_0 \quad \Leftrightarrow \quad \lambda = \frac{\Delta V_{te}^0}{R_{core} \cdot I_0} = \frac{R_{input}}{R_{core}} = \frac{R_{input}\pi r^2}{r_{sol}} = \lambda_2 \qquad \text{eq.(19)}$$

Combining this with eq.(16) where the definitions of R_{te} and R_{core} were rewritten, we can eliminate λ and find the definition for R_{te2}:

$$\lambda^2 = \frac{r \cdot R_{te}}{2r_{sol}} = \frac{R_{input}^2 \pi^2 r^4}{r_{sol}^2} \quad \Leftrightarrow \quad R_{te} = \frac{2R_{input}^2 \pi^2 r^3}{r_{sol}} = R_{te2} \qquad \text{eq.(20)}$$

Note: in the derivation of method 1, perfect insulation at both ends of the tubule was assumed. As mentioned on page 14, this was not achieved at the collection end. Increasing tubule length decreases the amount of current that can leak out at the collection end and thus decreases the error caused by imperfect insulation at the collection side. Results presented in chapter 3 demonstrate that indeed R_{te} calculated according to method 1, is dependent on the length of the tubule under study. However, in the derivation of method 2, infinite tubule length is assumed and the insulation at the collection end is unimportant since no current is assumed to arrive there. Again the reasoning can be applied that increasing tubule length decreases the amount of current that can leak out at the collection end and thus decreases the error caused by the fact that the tubule is not infinitely long. In this case however, results presented in chapter 3 demonstrate that R_{te} calculated according to method 2, is independent of the length of the tubule under study. Apparently in practice, the infinite-length assumption is not so important compared to the relatively large standard deviations on the measurements.

Theoretical considerations

The voltage divider ratio (VDR) was calculated according to the optically measured distance of impalement from the perfusion pipette (x) from which the current induced deflections of V_{te} at distance x were derived according to $\Delta V_{te}(x) = \Delta V_{te}^0 \cdot e^{-x/\lambda}$ and the current pulse induced deflections of V_{bl} (ΔV_{bl}) according to $VDR = (\Delta V_{te}(x) - \Delta V_{bl}) / \Delta V_{bl}$. The high VDR found under control conditions (chapter 3 i.e. [Dijkstra et al., 1994]) implies that the major part of the cellular resistance resides in the luminal membrane. The major paracellular resistance is also assumed to be on the luminal side, i.e. at the tight junction, as is generally assumed for most epithelia and has been shown to be so in the case of the proximal tubules of *Ambystoma tigrinum* and *Necturus maculosus* [Maunsbach & Christensen, 1992]. Thus the use of the luminal

radius as "the" cable radius is justified. In situations with strongly elevated basolateral resistance (as is the case in the presence of basolateral Ba^{2+}) the weighted average of the two cellular resistances will spatially be somewhere between the two membranes. However, since the calculated R_{te} is so sensitive to the radius (to the third power, see equation 20 above) a relatively small increase in the value of the optical radius would already result in a marked increase in R_{te}. Therefore it is safer to persist in using the luminal radius and be aware that this will lead to an underestimation of R_{te} in situations where R_{bl} is increased relative to R_l.

With the results published so far on this epithelium [Leyssens et al., 1992] the two methods used in this study to calculate R_{te}, could not be brought to agreement. One of the aims of the present study was to optimize the technique until agreement between the two methods was found. A series of 23 experiments was performed where extra care was taken to obtain electrical insulation of the collection side (chapter 3). The constriction and collection pipettes were electrically isolated by means of hardened Sylgard (of variable hardness since 'hardness' is not easily assessed during local heat application and the Sylgard will slowly harden further in the course of days), a Sylgard pipette was moved over both the ends of the tubule. The shape of the collection pipette was adjusted to a longer, tighter fitting model and constriction of the tip due to fire-polishing was reduced to a minimum. It was adapted to the size of the tubule under study. The tip of the perfusion pipette was fire-polished to minimize damage to the inside of the tubule. In this way the two methods of calculating R_{te} could be brought to agreement, i.e. in this well isolated series there was no statistically significant difference between R_{te1} and R_{te2}.

However, all these measures taken to optimize insulation, were very laborious and markedly reduced the chance of successful luminal perfusion. Therefore the resistances obtained from tubules without these extra insulation measures were compared to those obtained from the extra insulated series (results chapter 3). It was found that the R_{te}-values calculated according to method 2, R_{te2}, did not significantly differ between these two groups. Thus R_{te2}-values were taken in the rest of the study. The first series also led us to use $R_{te2} > 100\ \Omega.cm^2$ as an additional selection criterium for acceptable tubules.

2.3 Double barrelled Cl^--selective micro-electrodes

The electrodes were pulled from paired filament containing glass capillaries (borosilicate) with unequal diameter (OD 1.5 and 0.75 mm, ID 0.87 and 0.35 mm respectively, Hilgenberg, Malsfeld, Germany) on a horizontal puller (DMZ-Universal puller, Zeitz Instrumente, Augsburg, Germany). The inside of the larger barrel was made hydrophobic by silanization (hexamethyldisilazane; Janssen Chimica), 10 min in the presence of vapour inside the barrel at 160 °C followed by an hour baking at 160 °C without silazane vapour present. The electrodes were kept in an oven at 100 °C until filling. After filling the electrodes were used within 24 hours. The technique used in this study for the filling of double barrelled ion-sensitive electrodes was described by Weltens & Pirotte (1987). The hydrophobic tip was filled with a few hundred µl of anion selective liquid ion exchanger (Chloride Ionophore I-Cocktail A, Fluka No 24902) thus the final column length of the liquid ion exchanger was usually between 200 and 800 µm. This was done by inserting a very fine, manually pulled filling-capillary by means of a home made microforge inside the electrode as close as possible to the tip. The rest of this barrel was backfilled with 1 mol/l KCl with a different filling capillary. The small air-bubble often remaining between the liquid ion exchanger and the 1 mol/l KCl was removed by applying local heat. The smaller, reference barrel was filled with 1 mol/l KCl. Leakage of this high concentration of Cl^- into the cell appeared to be small since after depletion of Cl^- in the bath, intracellular Cl^- concentrations of 3±2 mmol/l (n=3) were measured. To obtain higher sensitivity and lower tip resistances the electrodes were bevelled in a rotating polishing alumina solution (No.3 AB Gama Polishing Alumina, Buehler, U.S.A.). The electrodes used had tip resistances ranging from $8\text{-}34*10^{10}$ Ω for the ion-sensitive barrel and from $7\text{-}900*10^{7}$ Ω for the reference barrel. The barrels were connected over Ag/AgCl half-cells to an electrometer (Duo 773, WPI , input resistances 10^{15} and 10^{11} Ω for the ion-sensitive and reference barrel signal, respectively). The potential of the reference barrel (V_{te} or V_{bl}), the potential of the Cl^--sensitive barrel (the electrochemical gradient for Cl^-) and the difference between both representing the Cl^--activity were recorded on a Linseis pen recorder (model LS4).
The properties of the electrodes are summarized in table 2.2.

Table 2.2. Properties of the double barrelled Cl^--selective electrodes

slope (mV/decade)	48.8 ± 0.6 (n=33)
resistance Cl^--selective barrel (Ω)	$1.9 \pm 0.1 * 10^{11}$ (n=19)
resistance reference barrel (Ω)	$9.1 \pm 4.6 * 10^{8}$ (n=20)
selectivity for Cl^- over SO_4^{2-} (*)	0.003

(*) from [Kondo et al., 1989]

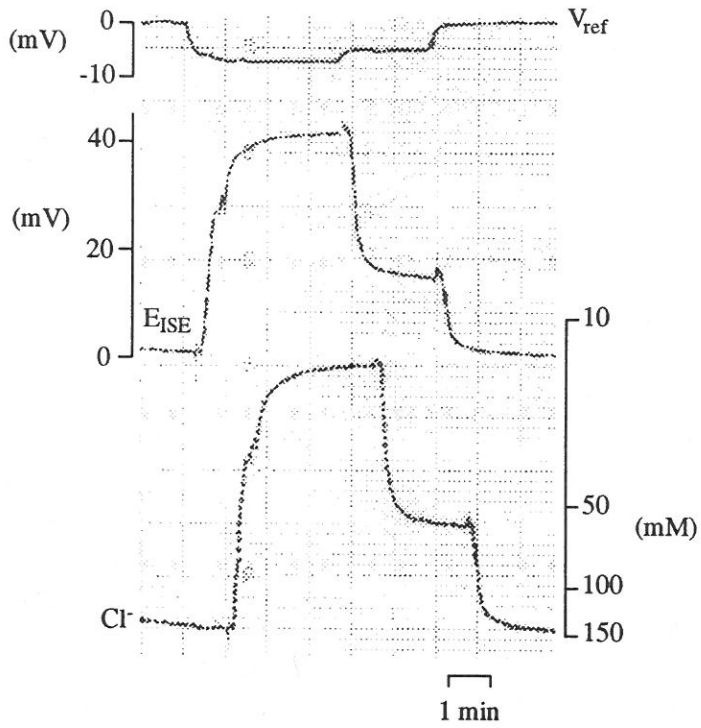


Fig.2.4 Example of a calibration. Solution first changed from 143 mmol/l Cl^- to 14 mmol/l Cl^-, then to 57 mmol/l Cl^- and back to 143 mmol/l Cl^-. Due to the characteristics of the penrecorder, the lines lag half a minute behind one another. V_{ref} is the potential registered by the reference barrel, E_{ISE} is the potential registered by the ion-selective barrel. "Cl^-" is the difference between the two and is expressed as a concentration.

Calibration of the electrode was done in Ringer's solutions containing 143, 57 and 14 mmol/l Cl^-. It was assumed that the activity coefficients are the same in cellular fluids, luminal fluids and the calibration solutions. The Cl^--activity dependent signal was always expressed as the chemical concentration that would be needed in a 51 mmol/l K^+ Ringer's solution to result in the same reading of the Cl^--selective barrel. An example of such a calibration is given in fig.2.4. Cl^- was replaced by SO_4^{2-} in the lower Cl^--concentration solutions. The electrodes were accepted for use when a stable potential signal was obtained for both the reference and ion-sensitive barrel, when the Cl^--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 42 mV/decade. The average slope of the electrodes used in this study was 48.8±0.6 mV/decade (n=33, table 2.2). Since this slope is not so high in comparison with the electrodes made by Kondo et al. [1989] who reported an average slope of 52.4 mV when introducing this ligand, considerable work

was done to improve the electrodes (see below). The resistances of the Cl^--selective barrel were in the same order of magnitude as those made by Kondo et al. ($6.7\pm0.6*10^{11}\Omega$ (n=11), as opposed to $1.9\pm0.1*10^{11}\Omega$ (n=19) in this study) but the resistances of the reference barrels of the electrodes used in this study were higher (Kondo et al.: $1.8\pm0.2*10^8\ \Omega$ (n=7), this study: $9.1\pm4.6*10^8\ \Omega$ (n=20)). N.B. the resistances of the separate barrels were not always measured again after beveling, since it took several minutes for the signal to stabilize again after a current pulse had been injected. Thus the number of measured resistances does not equal the number of measured slopes of the electrodes used.

Measures tried to improve double barrelled electrodes

Work with double barrelled ion-selective electrodes appears to be subject to a multitude of objective and less objective rules of thumb. Most investigators have personal rules concerning the fabrication of electrodes ranging from the pre-washing of the glass to the filling procedure, to storage protocol, to silanization methods. Many papers concerning aspects of the fabrication have been published in the course of years, as has a comprehensive manual reviewing the different types of electrodes and fabrication methods [Thomas, 1978]. A more recent overview of the advantages, disadvantages and applications of the method is given in [Alvarez-Leefmans et al., 1990]. The factors one can change in the fabrication procedure are limited:

1. shape of the tip of the electrode
2. glass (type, washing)
3. ligand
4. silanizing agent
5. silanization- and baking times
6. silanization method
7. polishing and tip-breakage
8. responsible scientist

Except for point 8, these different factors have been adjusted as described below, in our efforts to improve the electrodes. Since there is quite a variability in electrodes even when using the same procedure, a few electrodes per procedure had to be tested.

ad.1. The shape of the tip is probably the most important aspect as, amongst others, it influences whether microelectrodes can penetrate the cell membrane at all, whether the membrane will seal around the electrode preventing leaky impalements, whether the resistance and capacitance of the tip is limited enough to allow relevant physiological measurements, whether the electrolyte filling solution of the reference barrel will leak out, affecting the intracellular environment and whether the silanizing agent used can easily penetrate to the extreme tip or will even 'flow over' in the reference barrel tip. Unfortunately the shape of the tip is also the most volatile determinant. To achieve non-

leaky impalements the tips are in general accepted to be less than 1 µm. This is already on the border of visibility with a normal light microscope, thus tips cannot be properly assessed by eye and functional experiments have to take place. This takes time. Then considering the amount of parameters one can change to influence the pulling of an electrode (13 on the 2-steps horizontal puller used in this study) and the fact that the effects of these parameters on the tip is interdependent on the value of the others, one can imagine that, although a lot of empirical work has been done, not all possibilities have been systematically investigated.

ad.2. Besides the glass capillaries with unequal diameter (OD 1.5 and 0.75 mm, ID 0.87 and 0.35 mm respectively, borosilicate glass, Hilgenberg, Malsfeld, Germany) from which finally all experiments stem, three other (borosilicate) glass types have been tested: theta glass with a thick walled division (WPI No TST150 Kwik-Fil glass capillaries), double barrelled with equal diameter (O.D. 1.5 mm, WPI No 2B150F-6) and single-barrelled filament glass (O.D. 1.3 mm, I.D. 0.7 mm, Hilgenberg)
Washing the glass beforehand seemed to make no difference in our hands. Besides unwashed glass we used glass which had been boiled in water for an hour and glass which had been soaked in 1 mol/l HCl and then boiled for an hour in water. Both then dried in an oven at 100 ^{o}C overnight.

ad.3. Both the Corning 477913-ligand and two batches of Chloride Ionophore I-Cocktail A, Fluka, have been used. All experiments presented were achieved with the Fluka-ligand. The 1 mol/l KCl filling solution for the electrodes has been buffered with 10 mmol/l Hepes and adjusted to a pH of 7.2 since the ligand has been shown to be less reliable at pH>7.6, pH<6.2 was not tested [Kondo et al., 1989].

ad.4. Two different silanizing agents have been tried: 1,1,1,3,3,3-hexa-methyldisilazane (Janssen Chimica) and N,N-dimethyl-trimethylsilylamin (TMSDMA, Fluka). The last agent never brought forth an acceptable electrode in our hands.

ad.5. The times for applying silanization vapour have been varied: 30 and 50 seconds, 2, 3, 5, 5.5, 6, 7, 10, and 15 minutes. The duration of the baking period seemed to be of less influence but has also been varied: 0.5, 0.75, 1, 2, 4 hours and overnight.

ad.6. The method (started out with and) finally opted for, to silanize just one of the two barrels was as follows: before pulling, the first 2 cm of the smaller, future reference barrel was pinched off. The micro-electrodes were pulled and put upright in a special holder (fig.2.5) so that the larger, longer barrel was in contact with the space under the holder and the smaller barrel only with the space above the holder.The holder could now be screwed tight making the special rubber fitting around the larger barrel airtight to prevent gas from under the holder leaking through. A cap was screwed on top of the holder sealing the reference barrel from the surroundings, creating a 'top-chamber'.

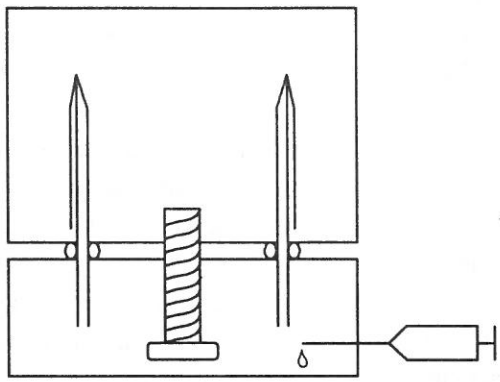


Fig.2.5 Schematic illustration of the holder used to silanize the electrodes.

The pot which could be screwed to the bottom, was climatized in the oven at the baking temperature. The holder was screwed on, 0.1 ml of silanizing agent injected into the thus created 'bottom chamber' and the total put in the oven for the time assigned to apply the vapour. The tip of the larger barrel was assumed to be so small in relation to the total space in the upper chamber, that the vapour possibly entering through the electrode and then silanizing the reference barrel, was assumed to be negligible. After this time period the bottom was screwed off and the holder placed in the oven again to bake. Modifications made to this method were: 1. applying N_2-pressure to the top chamber during vapour application and baking (3 and 4 bar) and applying vacuum (12 and 25 cm Hg); 2. changing the humidity in and around the electrodes before and during silanization (drying overnight in an oven at 100 ^{o}C, acclimatizing at 30-40, 50 and 70 % air-humidity, acclimatizing the larger barrel at 70 % humidity whilst the smaller barrel was sealed from the surroundings after pre-drying); 3. filling the reference barrel with water before the silanization and baking procedure to prevent oversilanization (the watervapour trying to get out should prevent the silane vapour from getting in).

The method of applying the silanization agent was also varied. 1. A drop of silane was injected into the larger barrel (both before and after pulling of the electrode resulted in the same liquid in the tip) and the electrode baked with the tip down. 2. The reference barrel was filled with acetone and the future ion-selective barrel with an acetone-silane mixture (0.2 and 1%, both silanizing agents tried), the electrode baked with the tip down. 3. Dipping the tip of single-barrelled electrodes into the same mixtures before baking. 4. Blowing air through the future ion-selective barrel which had been led over a handwarmed flask containing TMSDMA (boiling point 25 ^{o}C) whilst applying local heat to the tip of the microelectrode.

Other methods tried to seal the reference barrel during the silanization procedure included 1. sealing by melting just the reference barrel, and cutting the end off again after

silanization; 2. melting the end of the theta glass and making a hole in the side of the future ion-selective barrel to let the silanizing agent in; 3. melting a drop of glass as a stop in the back of the reference barrel.

ad.7. As described earlier, the electrodes nearly always needed polishing by bevelling in a rotating alumina solution to obtain an acceptable slope/decade. Tip breakage by stroking over either aluminium foil or very fine grained paper was tried both before the silanization procedure and after filling of the electrode. Neither seemed to be as effective as bevelling in the alumina solution.

2.4 Fluid secretion measurements

To measure spontaneous fluid secretion a modification of Ramsay's method [Ramsay, 1953] was used as described by Van Kerkhove et al. (1989). This was necessary because the Malpighian tubules of the ant are smaller than the tubules for which the technique was originally described. On a plastic petri-dish a small square of glass was stuck with vacuumgrease. On this glass a small bathing droplet (~50-100 µl) was placed which was covered by paraffin oil. The small piece of (hydrophilic) glass assured the droplet stayed in place and did not wander through the oil. The dissected tubule, cut as close to the midgut as possible, was transferred to the droplet. Near the cut end the tubule was sucked into a constriction pipette, thus the end was folded double and sucked strongly against the constriction so no luminal fluid was allowed to leave the tubule at this end. This resulted in a swelling of the lumen of the tubule. This end of the tubule was pulled into the paraffin oil over a distance of 0.3-0.4 mm. A hole was made with a broken glasstip in the

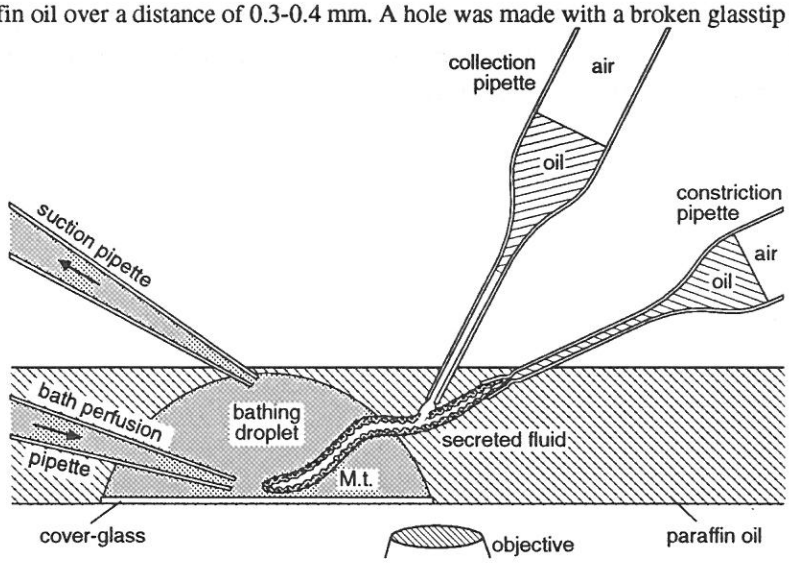


Fig.2.6 The secretion set-up. See text for explanation

short length now exposed to the oil and a droplet appeared, forced out by the secretion pressure (fig.2.6). The droplet was collected with a glass collection pipette made hydrophobic by silanization. This first droplet was discarded. Then droplets were collected every ten minutes and blown out under oil. The fluid stayed attached to the pipette tip and formed a perfect spherical droplet. The diameter of this droplet was measured with an eyepiece and from this the volume and thus the secretion rate, could be calculated. Since secretion rates in control conditions show large variabilities, each tubule was used as its own control. In general, the protocol consisted of 3 collection periods in control solution followed by 3 experimental periods and again 3 periods of control solution to assess reversibility of the tested compound. The secretion rate was expressed as a percentage of the third control period. Since spontaneous secretion decreases in time [Van Kerkhove et al., 1989], a series of experiments was done where the tubules were continually bathed in control solution and the time effect was assessed as a percentage of the third control period. Now the experimental values evaluating the effect of a compound or ion substitution could be time effect-corrected by dividing the experimental secretion percentage of collection period x by the control percentage of collection period x. Since on both experimental series a sizable standard deviation existed, this procedure clearly increased the total standard deviation. Thus comparisons between data, comparing the effect of DIDS in the presence and absence of cAMP, were done on the non-time corrected data since the experimental periods (fourth to sixth collection period) were assumed to have the same time effect anyway. In the series presented in chapter 5, where the effect of DNP in the presence and absence of probenicid was compared, the protocol used was : 3 periods of control, 3 periods of 2.10^{-4} mol/l DNP, 3 periods of control, 3 periods of 2.10^{-4} mol/l DNP plus 5.10^{-3} mol/l probenicid. The periods of exposure to DNP with and without probenicid were exchanged in half of the experiments to further compensate for the natural decrease in fluid secretion with time.

3 Electrical properties of the isolated, in vitro perfused Malpighian tubule of the ant, the Cl^--pathway

(adapted from Dijkstra S, Lohrmann E, Steels P, Greger R. (1994) Cell Physiol Biochem, 4:19-30)

3.1 Results

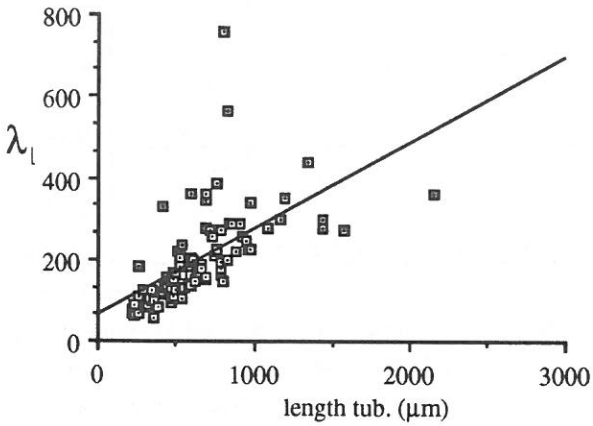
Electrical characterization

In a first series of 23 experiments extra care was taken with regard to electrical insulation (see materials & methods: theoretical considerations). The R_{te} and resulting I_{sc} calculated according to method 1 with control solution in both the bath and the lumen were: R_{te1} 164±18 (23) $\Omega.cm^2$, I_{sc1} 439±59 (23) $\mu A/cm^2$. According to method 2 the R_{te2} was 153±10 (23) $\Omega.cm^2$ and the I_{sc2} 397±30 (23) $\mu A/cm^2$. These values were not significantly different in a paired t-test. Since however, these extra insulation measures increased the difficulty of successful luminal perfusion, the importance of electrical insulation for the two calculation methods was tested. The values of length constants obtained from both the extra insulated series and from 77 experiments where these extra measures were not taken, were plotted against the length of the tubule under study, and the data fitted to a simple $y = a.x + b$ function (fig.3.1).

Values calculated according to method 1 were significantly dependent on the length of the tubule (fig.3.1A), whereas length constants calculated according to method two were independent of tubule lengths (fig.3.1B).

Values calculated according to method 2 in the series of tubules, where no extrra insulation measures were taken, were compared to those found under the better isolated circumstances. The values of this not extra insulated series were R_{te2} 178±8 (77) $\Omega.cm^2$ and the I_{sc2} 337±19 (77) $\mu A/cm^2$. Again, no statistically significant difference was found (unpaired t-test). Therefore the values of these experiments were pooled with those of the extra insulated series. In the rest of the study the extra insulation measures were not taken and method 2 was used to calculate all parameters. The pooled values (calculated according to method 2) characterizing the epithelium perfused with control solutions were: V_{te} +50.5±1.7 (100) mV, R_{te} 173±7 (100) $\Omega.cm^2$, length constant λ 270±7 (100) µm, I_{sc} 351±16 (100) $\mu A/cm^2$, V_{bl} -18.1±0.7 (70) mV and VDR 51±7 (18). The average distance of impalement from the tip of the perfusion pipette to the electrode was 78±9 µm (n=70) ranging from 4 to 445 µm.

A.



B.

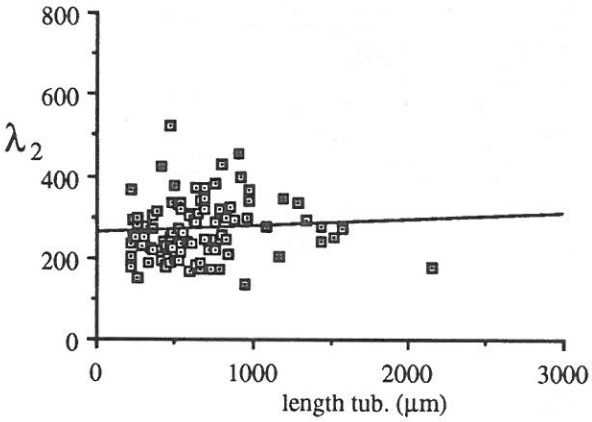


Fig.3.1 A.The length constant calculated according to method 1 (λ_1, see methods section), plotted against the length of the tubule under study. The linear regression was: $\lambda_1 = 0.210 \cdot$ length tub. + 63.25
The correlation between λ_1 and the tubule length was statistically significant ($P<0.001$, n=100).
B. The length constant calculated according to method 2 (λ_2, see methods section) is plotted as a function of the tubule length. Linear regression was: $\lambda_2 = 0.016 \cdot$ length tub. + 259
There is no statistically significant correlation between λ_2 and the tubule length ($P>0.4$, n=100)

Individual membrane resistances

In order to obtain numerical values for the individual membrane resistances, a change in R_{bl} was induced experimentally. To this end the bath K^+-concentration was lowered from 51 to 5 mmol/l in paired experiments. The results of 18 experiments are summarized in table 3.1. A significant decrease in VDR was seen, indicating an

Table 3.1 Effect of lowering bath K^+-concentration in the absence (A) and presence of Ba^{2+}(B).

		V_{te} (mV)	R_{te} ($\Omega.cm^2$)	I_{sc} ($\mu A/cm^2$)	V_{bl} (mV)	VDR	n
(A)	control	45±4	175±23	302±44	-13±2	51.1±7.3	18
	5K	21±3*	186±23	138±24*æ	-53±4*	10.1±1.2*	
(B)	control	40±4	210±16	213±29	-18±2	-	16
	$6BaCl_2$	33±3*	284±35*	151±29*	-23±3*	6.5±1.0	
	$5K6BaCl_2$	13±2*"	399±59*"	47±10*"æ	-64±5*"	1.2±0.2"	

Paired results of the effect of lowering the basolateral K^+concentration from 51 in control to 5 mmol/l. This both in the absence (A, 18 tubules) and in the presence of 6 mmol/l basolateral $BaCl_2$ (B, 16 tubules). *Indicates significantly different from control. "Indicates significantly different from control with 6 mmol/l $BaCl_2$. æNote: no symmetrical perfusion in this case, therefore the I_{sc} does not equal net secretion.

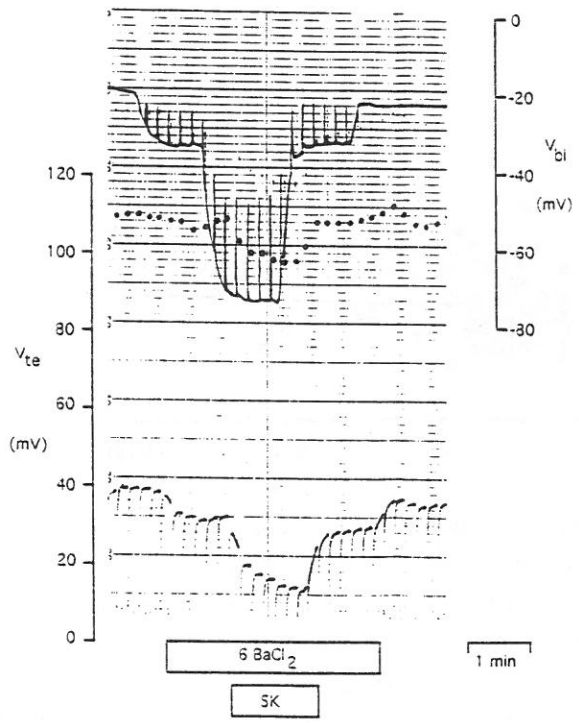


Fig.3.2 Typical recording of V_{te} and V_{bl}: effect of 6 mmol/l $BaCl_2$ in the bathing solution and of a drop in bath K^+-concentration from 51 to 5 mmol/l. The pen recording of V_{bl} lags 1 cm (half a minute) behind the V_{te}-recording. Luminally injected current pulses cause deflections in V_{te} (ΔV_{te}, see dots above V_{te} trace) and V_{bl} (ΔV_{bl}, see spikes on the V_{bl} trace). Other deflections visible are due to capacitive effects. From these ΔV_{te} the R_{te} is calculated via cable analysis. The appearance of ΔV_{bl} in the presence of $BaCl_2$ suggests an increase of R_{bl} over R_l.

elevation of R_{bl} or a decrease in R_l. As R_{te} was elevated, increase of VDR was caused by an increase of R_{bl} by lowered bath K^+-concentration. V_{bl} hyperpolarized significantly, causing a decrease in V_{te}, while R_{te} increased slightly, leading to a significant decrease in I_{sc}. It must be noted that the tubule is not symmetrically perfused during basolateral decrease of K^+ and therefore I_{sc} is not proportional to secretion in this case. The K^+-concentration step was repeated in the presence of 6 mmol/l $BaCl_2$ in the bath. Fig.3.2 shows an example of such an experiment. The occurence of deflections of V_{bl} due to the luminal current pulses in the presence of Ba^{2+} indicate a decrease of VDR. VDR decreased further upon lowering of the bath K^+-concentration. R_{te} raised significantly, both after the addition of Ba^{2+} and after lowering the bath K^+-concentration, indicating an increase in R_{bl} in both cases. The effects on the electrical properties for 16 tubules in paired experiments, are summarized in table 3.1. The VDR measurements in the absence of $BaCl_2$ in this series of experiments are missing because basolateral voltage deflections due to the transepithelial current pulses were too small to be detected in 15 out of 16 experiments. In one it was 35.

Ion-specificity of the shunt

When the bath solution containing 62 mmol/l Na^+ and 57 mmol/l Cl^- was changed to a high NaCl solution (127 mmol/l Na^+ and 122 mmol/l Cl^-), thereby creating a concentration gradient for both these ions directed towards the lumen, V_{te} decreased by 7.1±0.9 (16) mV, whereas the V_{bl} showed no significant change (ΔV_{bl} was -0.7±0.4 (10) mV). Replacement by a low NaCl solution (36 mmol/l Na^+ and 30 mmol/l Cl^-) led to an increase in V_{te} by 5.0±0.7 (12) mV, again without a significant change in V_{bl} (ΔV_{bl} was +1.0±0.4 (7) mV).

Cl^--channel blockers

Neither addition of 10^{-5} mol/l NPPB (n=5) nor 10^{-3} mol/l DIDS (n=6) to the luminal perfusate led to a significant increase in R_{te} or an inhibition of I_{sc} (table 3.2). The V_l was derived from V_{te} and V_{bl}. V_{te} decreased slightly, by 0.6 mV, upon addition of luminal NPPB but, although this is statistically significant, the decrease is smaller than the recorder registration precision and is therefore probably of no significance. Both V_{bl} and V_l, and V_{te} in the case of luminal DIDS addition, showed no changes. Neither for NPPB nor for DIDS a significant increase in VDR was found ($VDR_{experiment}$ /$VDR_{control}$ 0.96±0.00 (n=3) and 1.28±0.35 (n=3), respectively).

Table 3.2 Effect of luminally applied Cl^--channel blockers

	ΔV_{te} (mV)	R_{te} (%)	I_{sc} (%)	ΔV_{bl} (mV)	ΔV_l (mV)
NPPB	-0.6±0.2*	95±5	105±5	0.0±0.2	-0.4±0.3
DIDS	-0.7±0.6	83±7	116±11	-0.6±0.3	0.8±0.8

The results of 10^{-5} mol/l NPPB and 10^{-3} mol/l DIDS on resp. 5 and 6 tubules. R_{te} and I_{sc} in percentages of control. Apical membrane voltage V_l is calculated as V_{te}-V_{bl}.

* Indicates statistically significant as tested with two sided t-test, $P<0.05$.

3.2 Discussion

The luminal perfusion technique and cable analysis applied on Malpighian tubules of ants.

The two methods utilized to calculate R_{te} in this study use different measured parameters, thereby presenting an internal control on the quality of the measurement. Optimalisation of the electrical insulation and minimalization of damage to the apical membrane, finally led to an agreement between the values of R_{te} calculated according to either method. However, to achieve this we had to decrease the diameter of the collection pipette and move liquid Sylgard over the edge of the collection pipette, where the tubule enters the pipette. This because damage to the tubule and imperfect fitting of the collection pipette could produce electrical leaks there. Both measures to improve electrical insulation tended to constrict the tubule at the collection end, thereby hindering an effective perfusion and enlarging the pressure within the tubules, which could cause internal damage to the tubule. Therefore another series of experiments was done to find out whether electrical insulation measures could be lessened thus improving luminal perfusion, with the R_{te}'s and I_{sc}'s still approaching the values found in the more rigorously isolated tubules. This turned out to be so for the values found with the second method of calculation, using the optically measured radius instead of the voltage deflections measured at the collection end. This is most likely the reason why the R_{te} reported in the earlier study [Leyssens et al., 1992] is not significantly different from that presented here. The I_{sc} reported there however, is significantly lower than the value presented here, because the V_{te} found in the present study was about twice as high. This was observed despite the fact that the bath Cl^--concentration in the first study was elevated, which should have increased secretion [Van Kerkhove et al., 1989] and thereby I_{sc}. An important experimental difference however between this study and the earlier one is that in the earlier study often a current higher than 50 nA was injected (30-90 nA), which in our hands led to a decrease in V_{te}. Besides the difference in bath Cl^--concentration, there were other minor differences in the composition of the solution: in our study glucose was the only sugar present, in the first

there was also trehalose. In the present study citrate was the only organic anion offered basolaterally and gluconate luminally, in the first study citrate, succinate and fumarate were present both basolaterally and luminally.

The calculation according to method 2 assumes a tubule of infinite length and should therefore only be applied on tubules clearly exceeding the length constant, which in our case was 270±7 (n=100) µm. However, as demonstrated in fig.3.1, the length constant calculated according to method 2 was independent of the length in the range of tubule lengths used in this study, and hence the above theoretical restriction is not important for our observations. On the other hand, too long tubules tend to be badly perfused, probably due to the elasticity of the tubule. Consequently perfusion pressure has to be increased with the length of the tubule in order to guarantee perfusion. Too high perfusion pressure is not desirable because it damages the tubule near the perfusion pipette. The compromise found empirically was a length between 300 and 800 µm. A note of caution must be added concerning the reliability of the optical radius measurement. The Malpighian tubules of ants are clear, when of good quality, thereby permitting easy measurement of the radius. But in many tubules this radius varies irregularly along the length of the tubule. Additionally, fluid exchanges decrease the visibility of the luminal membrane (make it "fuzzy") near the perfusion pipette where the radius is most important. These considerations might well explain the relatively high variability in our measurements.

Analysis of membrane resistances.

In the system under study a very high VDR was found (51±7(18)). This in combination with the high absolute value found for the resistance of the apical membrane (see below), leads to a situation where the injected current seldom produces measurable deflections on the V_{bl} under control conditions. Specifically, in 18 of the 100 experiments (ie. 1 in 5 only) VDR's could be measured under control conditions. Enlarging the injected current is of little help, since in our hands V_{te} was observed to fall if I_0 was ≥ 50 nA. If the basolateral K^+-concentration is lowered, R_{bl} increases as a result of the lowered conductivity of the abundantly present K^+-channels. The slight basolateral hyperpolarization and transepithelial depolarization upon addition of Ba^{2+} have been described previously [Weltens et al., 1992] but were more pronounced in the earlier study. The basolateral hyperpolarization upon lowering bath K^+ in the presence of Ba^{2+}, suggests the Ba^{2+}-induced block of the K^+-channels was not complete at this concentration. R_l is not expected to be affected from spatial considerations and the shunt is assumed not to be specific for K^+ over Na^+ (with which the K^+ was replaced) and thus R_s is also expected not to be affected. With the aid of the simultaneous equation method presented in appendix 3.1, the R_{bl}, R_l and R_s can be estimated from R_{te} and VDR in both conditions. By lowering the basolateral K^+ from 51 to 5 mmol/l,

we found the R_{te} to increase in these 18 tubules from 176±23 to 186±23 $\Omega.cm^2$ and the VDR to decrease from 51±7 to 10±1 (table 3.1) resulting in the following values for the individual membrane resistances in control conditions: R_{bl} 4, R_l 214 and R_s 895 $\Omega.cm^2$. Unfortunately we cannot get an idea of the reliability of the results by simple mathematical error analysis, because external, non-mathematical criteria result in dependent, discontinuous functions of the distributions. For example, the prerequisite that R_{te}' is larger than R_{te}, chops off both the R_{te}-distributions at some fluctuating point depending on the absolute value of either. This results in a non-Gaussian distribution on which mathematical error analysis is not allowed. Filling in the extreme values in the formulae gives non-real solutions like negative resistances, for the same reason that these extremes do not fit in with the external criteria. To at least gain a qualitative insight, the same protocol was repeated under different circumstances. Basolaterally 6 mmol/l $BaCl_2$ was added, which has the two advantages of appreciably enlarging R_{bl}, enabling a more precise reading of VDR, and enlarging the difference between the two states, since Ba^{2+} competes with K^+ and will therefore block more effectively in a 5 mmol/l K^+-solution. Ba^{2+} has been shown to partially block the tight junction of *Necturus* gallbladder epithelium [Langer & Frömter, 1985] at 5 mmol/l $BaCl_2$. However, this effect is thought to arise from the screening of negative charges in the cation selective shunt [Langer & Frömter, 1985;Wright & Diamond, 1968]. We therefore expect negligible interference in the epithelium under study where the shunt is anion specific (see below).

In a series of paired experiments (n=16) an increase in R_{te} from 284±35 to 399±59 $\Omega.cm^2$ and a decrease in VDR from 6.5±1.0 to 1.2±0.2 (table 3.1) on decreasing basolateral K^+ in the presence of Ba^{2+} was found. This leads to the respective membrane resistances of: R_{bl} 50, R_l 320 and R_s 1230 $\Omega.cm^2$ in control conditions. R_{bl} is now about 10 times as large, confirming an effective Ba^{2+}-block. Values for both the apical and shunt resistance are appreciably higher (about 1.5 times as high) than the ones found in the absence of Ba^{2+}, which indicates that our values are still to be regarded as estimates. The fact that, despite an effective Ba^{2+}-block, the same change in V_{bl} (ΔV_{bl}) is observed upon lowering the bath K^+-concentration, is due to the fact that R_{bl} still remains by far the smallest resistance of the three resistances in the system. As can be deduced from the electrical circuit model (fig.1.5, introduction):

$\Delta V_{bl} = \Delta E_{bl} \cdot (R_s+R_l)/(R_l+R_s+R_{bl})$ where ΔE_{bl} represents the change in electromotive force over the basolateral membrane due to the K^+-concentration change. Thus with the estimates found for the individual resistances under control conditions $\Delta V_{bl} = 1109/1113 \cdot \Delta E_{bl} = 0.996 \Delta E_{bl}$ and in the presence of Ba^{2+} : $\Delta V_{bl} = 1550/1600 \cdot \Delta E_{bl} = 0.969 \Delta E_{bl}$. With a ΔE_{bl} of 40 mV, ΔV_{bl} under control conditions would be 39.8 mV and in the presence of Ba^{2+} 38.8 mV.

Until now this epithelium has always been assumed to be leaky, i.e. the resistance to flow of ions was thought to be less through the paracellular pathway than through the cellular pathway, irrespective of absolute magnitudes. This assumption seemed confirmed in the approximations of the individual membrane resistances published recently [Weltens et al., 1992] ie. R_{bl} 10, R_l 475 and R_s 228 $\Omega.cm^2$. However, here the transition of control solution to basolateral 6 mmol/l Ba^{2+} had been used to obtain the basolateral resistance elevation with the assumption that Ba^{2+} did not affect R_l or R_s. We were unable to repeat the same protocol, since in only 3 experiments where Ba^{2+} was added, a VDR could be measured under normal control conditions. As stated earlier, in many experiments no voltage deflection was visible on the basal membrane potential when current was applied in control conditions.
In conclusion, results presented here suggest that the Malpighian tubule of the ant is a rather tight epithelium. This will reduce the backflux of the actively transported K^+, reducing the loss of energy.

The Cl^--pathway.
K^+ is secreted actively in the Malpighian tubules and Cl^- follows. It is not known whether it follows trans- or paracellularly. For the shunt to be responsible for the Cl^--transport, it has to be specific for Cl^- over Na^+ and K^+. Therefore the specificity of the shunt was investigated.
Since changes in the basolateral Na^+ and Cl^- concentrations had no effect on the V_{bl}, conductivities of any importance for these ions in the basolateral membrane can be excluded. Therefore, changes in the V_{te} can be ascribed to changed electromotive forces (E) over the shunt and the voltage changes over the respective resistances caused by the current resulting from the changed E over the shunt. To find the specificity of the shunt for Cl^- over Na^+, the effect on ΔE_s has to be known. However, only ΔV_{te}(bath solution) (i.e. the change in V_{te} as a result of the change in bath solution) can be measured which is proportional to ΔE_s according to $\Delta V_{te}(\text{bath solution}) = \Delta E_s .(R_l + R_{bl})/(R_l + R_{bl} + R_s)$ (appendix 3.2). For a very leaky epithelium R_s is much smaller than the sum of R_l and R_{bl} and therefore $\Delta V_{te}(\text{bath solution}) \sim \Delta E_s$. However, the tighter the epithelium, the lower the impact of ΔE_s on ΔV_{te}(bath solution), the more the real ΔE_s will differ from the measured ΔV_{te}(bath solution). Thus, equating ΔV_{te}(bath solution) to ΔE_s establishes a lower limit for ΔE_s.
With the formulae derived in appendix 3.2, a lower limit to the specificity of the shunt of Cl^- over Na^+ (P_{Cl^-}/P_{Na^+}) can then be calculated from the observed ΔV_{te}(bath solution) . Pooled results express a P_{Cl^-}/P_{Na^+} of at least 2.9±0.4 (28), since this is a lower limit, we can safely state that the shunt is anion specific. Due to the large basolateral K^+-conductance, it was not possible to establish P_{Cl^-}/P_{K^+} with a similar procedure.

With an anion specific shunt and electrochemical gradients allowing for a passive transport of Cl^- ([Van Kerkhove et al., 1989] and chapter 6), it is likely that most of the transport of Cl^- will take place paracellularly. However, a transcellular pathway cannot be excluded. For Cl^- to be transported through the cell, it needs both an entrance mechanism basolaterally and an exit apically. A significant basolateral Cl^--conductance seems unlikely as V_{bl} does not change when the bath Cl^--concentration is altered (since R_{bl} is very low compared to the other resistances in the system, ΔV_{bl} would be a good approximation of ΔE_{bl}).

Experiments with bumetanide have suggested a coupled basolateral uptake of Cl^- and K^+ [Verhulst et al., 1988] in accordance with furosemide and bumetanide experiments in *Rhodnius* [O'Donnell & Maddrell, 1984]. However, if a coupled mechanism of entry for Cl^- and K^+ were present, basolateral Cl^- changes might be expected to affect the intracellular K^+-concentration which would be expressed in a ΔV_{bl}.

To further investigate the apical Cl^--conductance, Cl^--channel blockers NPPB and DIDS were added to the lumen. This had no significant effect on either R_{te}, I_{sc} or V_l. So at least the presence of NPPB- or DIDS-sensitive Cl^--channels can be excluded in the perfused, unstimulated tubule.

Little is known about the Cl^--pathway in insect epithelia. However, basolateral Cl^--channels have been found in the Malpighian tubules of the desert beetle [Nicolson & Isaacson, 1987] and both apically and basolaterally in the salivary gland of the blowfly [Berridge et al., 1975]. In the Malpighian tubule of the yellow fever mosquito they are absent basolaterally [Sawyer & Beyenbach, 1985] but present in the luminal membrane [Wright & Beyenbach, 1987]. In stimulated condition they can also be found basolaterally in the Malpighian tubules of the mosquito [O'Donnell & Maddrell, 1984]. In *Locusta* Malpighian tubules they have been demonstrated to be absent basolaterally under control conditions [Baldrick et al., 1988].

In summary, the transcellular Cl^--pathway clearly requires further investigation.

In conclusion, in this study we have characterized the Malpighian tubule of the ant electrically. We have shown that the calculation of R_{te} appears valid according to the method utilizing the optically measured radius, even if electrical insulation is not optimal. The approximations found for the individual membrane resistances and the shunt indicate that this epithelium is rather tight. Further, evidence is given for the Cl^- specificity of the shunt.

Appendix 3.1

Assuming the simplified electrical circuit as depicted in fig. 1.5 (in the introduction) to represent the Malpighian tubule, we can write

$1/R_{te} = 1/R_S + 1/(R_l+R_{bl})$ (1)

$VDR = R_l / R_{bl}$ (2)

Changing the basolateral K^+-concentration changes R_{bl} into R_{bl}', R_{te} into R_{te}' and VDR into VDR'. Solving the simultaneous equations for R_{te} and VDR in the two different states leads to the formula with which the R_{bl} can be calculated:

$$R_{bl} = \frac{R_{te} \cdot R_{te}'\left(VDR - VDR'\right)}{VDR\left(1 + VDR'\right)\left(R_{te}' - R_{te}\right)}$$

The other membrane resistances can then be derived from equations (1) and (2).

Appendix 3.2

Under control conditions, where solutions are symmetrical, the electromotive force over the shunt (E_S) is 0. Changing the basolateral solution can, from spatial considerations, not affect the E over the apical membrane. Since no measurable potential changes over the basolateral membrane occurred, and R_{bl} is very small so that the change in V_{bl} caused by the change in basolateral solution approximately equals the change in E_{bl}, the change in E_{bl} must be negligible too. Therefore the change in E (ΔE) can be exclusively ascribed to the shunt.

From the electric circuit model (see introduction, fig.1.5) we derive

$\Delta V_{te}(\text{bath solution}) = \Delta E_S - \Delta I.R_S = \Delta I(R_l + R_{bl})$

where ΔI is the current resulting from the ΔE_S and ΔV_{te}(bath solution) is the change in V_{te} as caused by the change in bath solution. This can be rewritten as

$\Delta V_{te}(\text{bath solution}) = \Delta E_S .(R_l + R_{bl})/(R_l + R_{bl} + R_S)$

thus ΔV_{te}(bath solution) is only a minimal estimate of ΔE_S.

$\Delta E_S = E_{S2} - E_{S1}$ where E_{S2} represents the situation after the change in bath solution and E_{S1} the control state, which, due to symmetrical perfusion, equals zero, thus $\Delta E_S = E_{S2}$.

Goldmann: $E_s = 60 \log \frac{P_{Na^+}[Na^+]_{bl} + P_{Cl^-}[Cl^-]_{lumen}}{P_{Na^+}[Na^+]_{lumen} + P_{Cl^-}[Cl^-]_{bl}}$ P_x = permeability of ion x.

$$\text{thus } \Delta E_s = E_{s2} = 60 \log \frac{P_{Na^+}[Na^+]_{bl2} + P_{Cl^-}[Cl^-]_{lumen}}{P_{Na^+}[Na^+]_{lumen} + P_{Cl^-}[Cl^-]_{bl2}}$$

where the ionconcentrations represent the concentrations after the change in bath solution. Dividing top and bottom of the equation by P_{Na^+}:

$$\Delta E_s = 60 \log \frac{[Na^+]_{bl2} + {}^{P_{Cl^-}}\!/_{P_{Na^+}} [Cl^-]_{lumen}}{[Na^+]_{lumen} + {}^{P_{Cl^-}}\!/_{P_{Na^+}} [Cl^-]_{bl2}} \quad \Leftrightarrow$$

$$[Na^+]_{bl2} + {}^{P_{Cl^-}}\!/_{P_{Na^+}} [Cl^-]_{lumen} = 10^{\Delta E_s/60} \left([Na^+]_{lumen} + {}^{P_{Cl^-}}\!/_{P_{Na^+}} [Cl^-]_{bl2} \right) \quad \Leftrightarrow$$

$${}^{P_{Cl^-}}\!/_{P_{Na^+}} \left\{ [Cl^-]_{lumen} - 10^{\Delta E_s/60} \cdot [Cl^-]_{bl2} \right\} = 10^{\Delta E_s/60} \cdot [Na^+]_{lumen} - [Na^+]_{bl2} \quad \Leftrightarrow$$

$${}^{P_{Cl^-}}\!/_{P_{Na^+}} = \frac{10^{\Delta E_s/60} \cdot [Na^+]_{lumen} - [Na^+]_{bl2}}{[Cl^-]_{lumen} - 10^{\Delta E_s/60} \cdot [Cl^-]_{bl2}}$$

4 Characteristics of the luminal proton pump in Malpighian tubules of the ant.

(adapted from Dijkstra S, Lohrmann E, Van Kerkhove E, Greger R. Renal Physiol Biochem, in press)

4.1 Results

A. *Effects of luminal pH-changes*

The effect of low luminal pH on I_{sc} was measured by exchanging the control solution (pH 7.2) in the barrel of the perfusion pipette by a solution identical to the control solution but with lowered pH. After stabilization of the measured parameters, the perfusion fluid was exchanged again to the control pH-solution. An example of such an experiment is given in fig.4.1. Acid pH lowered V_{te}. The effects were fully reversible down to about pH 4, lower pH-values resulted in only a partial recovery. Down to pH 4, R_{te} rose steadily, at lower pH-values a sharp decrease occurred (fig.4.2). Neither V_{bl} nor VDR showed a significant change in any of the experiments. The effects of lowered luminal pH on the V_{te} and V_l (luminal membrane potential difference, calculated as

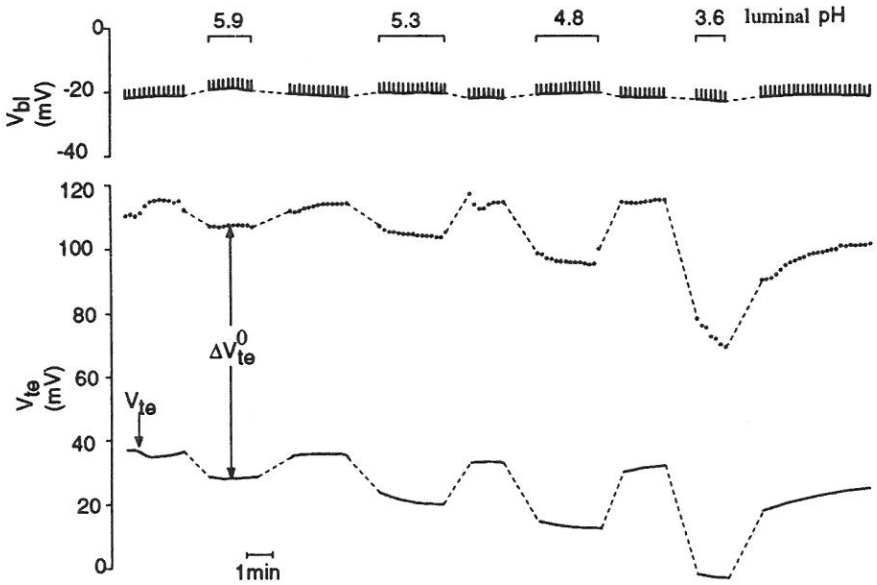


Fig. 4.1 The effect of decreasing luminal pH on V_{te} (solid line) and ΔV^0_{te} (the deflections of V_{te} as caused by the injected current, used to calculate R_{te}. The dotted line indicates the tops of the deflections). Further the effect on V_{bl} and on the the deflections of V_{bl} (used to calculate VDR). The dashed lines in the traces indicate where luminal fluid exchanges took place.

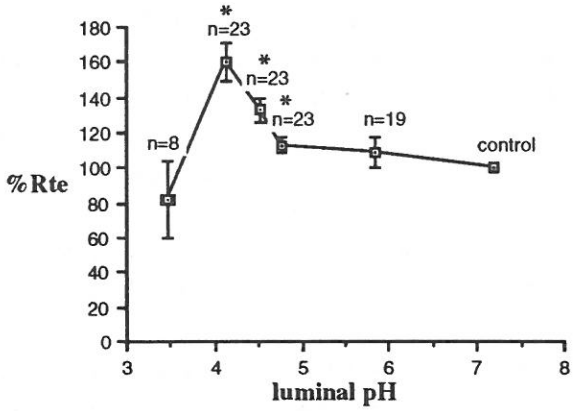


Fig.4.2 Summary of the effect of decreasing luminal pH on R_{te} given as percentage of control values.

* Indicates significantly different from control.

V_{te} - V_{bl}) parallelled those on the I_{sc}. This is summarized in fig.4.3 for V_l and I_{sc}. I_{sc} decreased when luminal pH was lowered. The decrease was significant for pH-values from 4.8 downwards. I_{sc} approached zero at a luminal pH of 3.5 where V_l was still 13±3 mV (n=5). Half-maximal inhibition was found at pH 4.5.

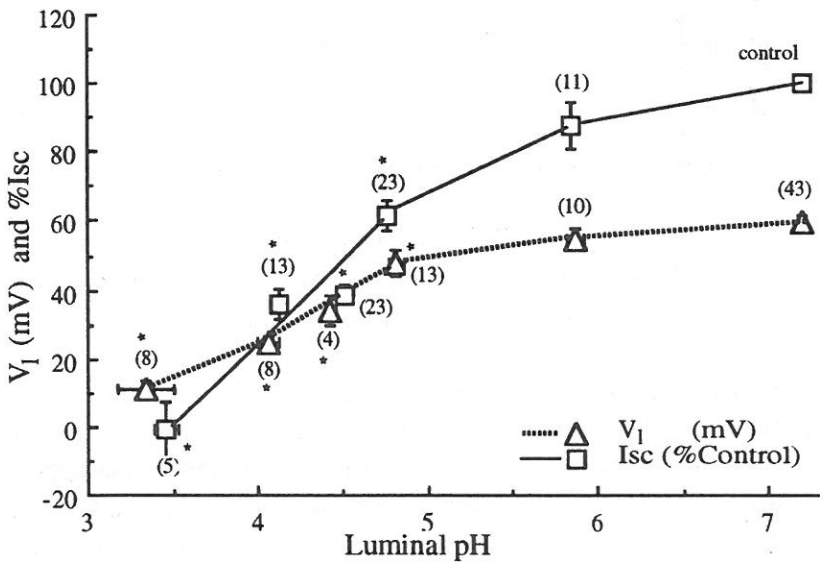


Fig.4.3 Effect of decreasing luminal pH on the I_{sc} as percentage of control and on V_l (in mV).

* Indicates significantly different from control.

B. Effects of inhibitors of the V-type ATPase, bafilomycin and N-ethylmaleimide

Bafilomycin A1 is a specific inhibitor of the vacuolar type H^+-ATPase [Bowman et al., 1988]. The effects on I_{sc} of luminal application of bafilomycin at different concentrations, are presented in fig.4.4. The concentrations of bafilomycin were determined spectrophotometrically. The inhibiting effect at 10^{-5} mol/l was irreversible. Half-maximal inhibition was reached at a luminal bafilomycin concentration of 10^{-5} mol/l. Higher concentrations were not tested. The decrease of I_{sc} could be fully ascribed to a decrease in V_{te} (by 20±5 mV, n=6), which in turn was due to an exlusive decrease in V_l (by 20±5 mV, n=5). Neither R_{te} nor V_{bl} changed significantly in any of the concentrations tested.

To ensure that the bafilomycin batch used had a normal bio-activity, experiments on membrane vesicles from dog kidneys were very kindly performed by Mrs. Sabotni in the laboratory of Prof. Burckhardt (MPI für Biophysik, Frankfurt am Main). These experiments proved that the used bafilomycin had the expected bio-activity (half-maximal inhibition at 10^{-9} mol/l).

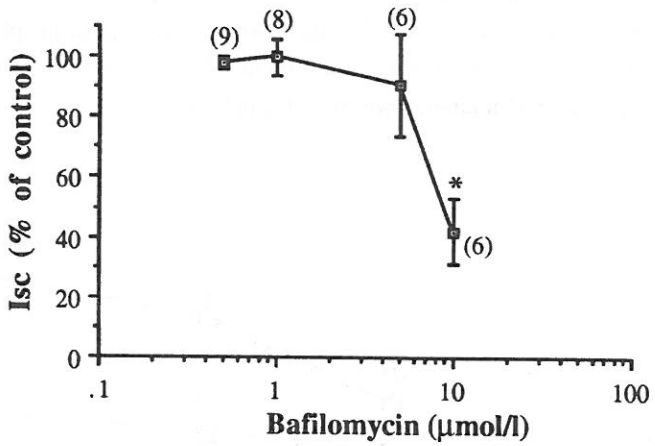


Fig.4.4 Effect of luminal Bafilomycin A_1 on the I_{sc} given as percentage of control.

* Indicates significantly different from control.

N-ethylmaleimide (NEM) was tested both luminally and from the bath side at a concentration of 5.10^{-4} mol/l. As shown in Table 4.1, its effect from the basolateral side was much more pronounced than after luminal application: upon luminal application I_{sc} decreased to 78± 7% of control, whereas upon basolateral application I_{sc} decreased to 21 ± 12% of control. The decrease in V_{te} was statistically significant only after basolateral application. It amounted to -8 ± 3mV (luminal application) and -26 ± 10mV (basolateral application) and could clearly be attributed to the depolarization of the

luminal membrane. Again, neither R_{te} nor V_{bl} changed significantly. These effects were irreversible.

Table 4.1 The effects of NEM, omeprazole and Schering compound

	ΔV_{te} (mV)	ΔV_{bl} (mV)	ΔV_l (mV)	$\%R_{te}$	$\%I_{sc}$
lum.NEM	-8±3 (4)	1±1 (4)	-6±4 (4)	85±14 (4)	78*±7 (4)
bath NEM	-26*±10(4)	0±1 (3)	-28±12(3)	153±60 (4)	21*±12 (4)
lum.ome	0±1 (5)	0±0.3 (5)	0±1 (5)	96±5 (5)	100±6 (5)
lum.ome pH_{low}	-2±2 (5)	-1±1 (3)	-4±2 (3)	138*±13(5)	71*±9 (5)
lum.S28080	-2±2 (9)	-1±1 (8)	-1±2 (8)	101±3 (9)	90*±4 (9)

Effects are presented as changes in potential differences upon inhibitor application. R_{te} and I_{sc} are given as percentage of control. Numbers in brackets are number of experiments. NEM was applied both luminally and basolaterally at 5.10^{-4} mol/l. Omeprazole (ome) was applied luminally both in pH 7.2 and acid activated with the lowered pH-values between 4.5 and 5.5 at a concentration of 10^{-4} mol/l. For the experiments with lowered luminal pH, values with the same low pH-solution in the lumen but without omeprazole, were taken as control. Schering compound 28080 was applied at 10^{-4} mol/l. *Indicates statistical significance tested with two-sided students t-test, $P<0.05$.

C. *Effects of inhibitors of the gastric H^+/K^+-ATPase, omeprazole and Schering compound 28080*

Omeprazole, an inhibitor of the gastric H^+/K^+-ATPase [Mattsson et al., 1991], was added to the luminal fluid at 10^{-4} mol/l. At pH 7.2 there was no significant effect on any of the measured parameters. If the pH of the luminal solution was first lowered to pH-values between 4.5 and 5.5, and omeprazole was added in the presence of low pH, a reversible increase in R_{te} and decrease of I_{sc} was measured. The results are summarized in table 4.1. The changes in R_{te} and I_{sc} were expressed in % of the control values in the presence of the low pH in the lumen, before and after application of omeprazole. No significant potential changes were seen.

The effect of luminal application of Schering compound 28080 (S28080) was also investigated, since it also targets on the gastric H^+/K^+-ATPase but does not need to be activated by an acid environment. As S28080 competes with K^+ and since we have 51 mmol/l K^+ in the luminal solution, we applied it at a rather high concentration of 10^{-4} mol/l. It had little or no effect on the parameters measured except for a small but significant reduction in I_{sc} (see table 4.1).

D. *Effects of ortho-vanadate, an inhibitor of P-type ATPases*

Ortho-vanadate was tested both luminally and basolaterally. Application time varied between 1.5 to 11 min when the drug was applied at the luminal side (10^{-3} mol/l, n=17). Basolaterally concentrations of 10^{-5} to 10^{-3} mol/l were tested. The concentration of 10^{-3} mol/l was applied between 2.5 to 14 min (short term application) in one series and for 30 min (long term application) in a further series. The effects of short term vanadate application (10^{-3} mol/l both luminally and basolaterally) are summarized in fig.4.5A. The effects of long term application from the bath (10^{-4} and 10^{-3} mol/l) are summarized in fig.4.5B. During short term application of 10^{-3} mol/l (fig.4.5A), the decrease in V_{te} was significantly larger upon basolateral than upon luminal addition. The effect on all other parameters was comparable. Neither after luminal nor after basolateral application of 10^{-3} mol/l a significant inhibition of secretion (reduction of I_{sc}) was observed within the first ten minutes, whereas after half an hour of basolateral application I_{sc} was

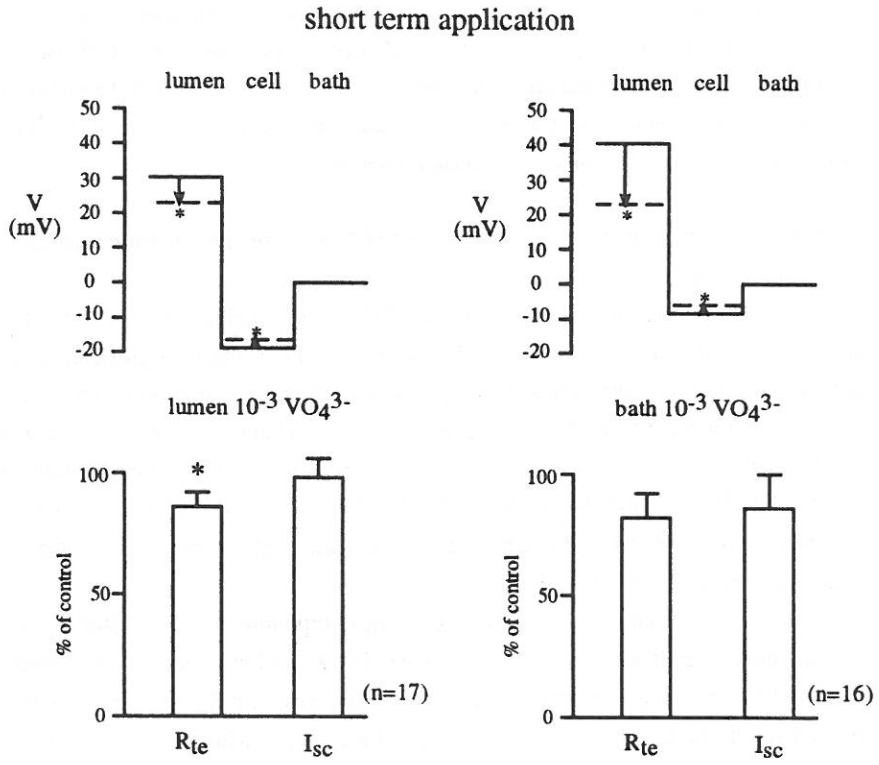


Fig. 4.5A Effect of short term application (up to 10 min) of vanadate both luminally and basolaterally at 10^{-3} mol/l. The continuous lines in the potential profiles represent the values in the control state, the dashed lines the values after vanadate application. * Indicates significantly different from control, in the case of basolateral application of 10^{-3} mol/l vanadate, ΔV_{bl} was significant in a one-sided t-test only.

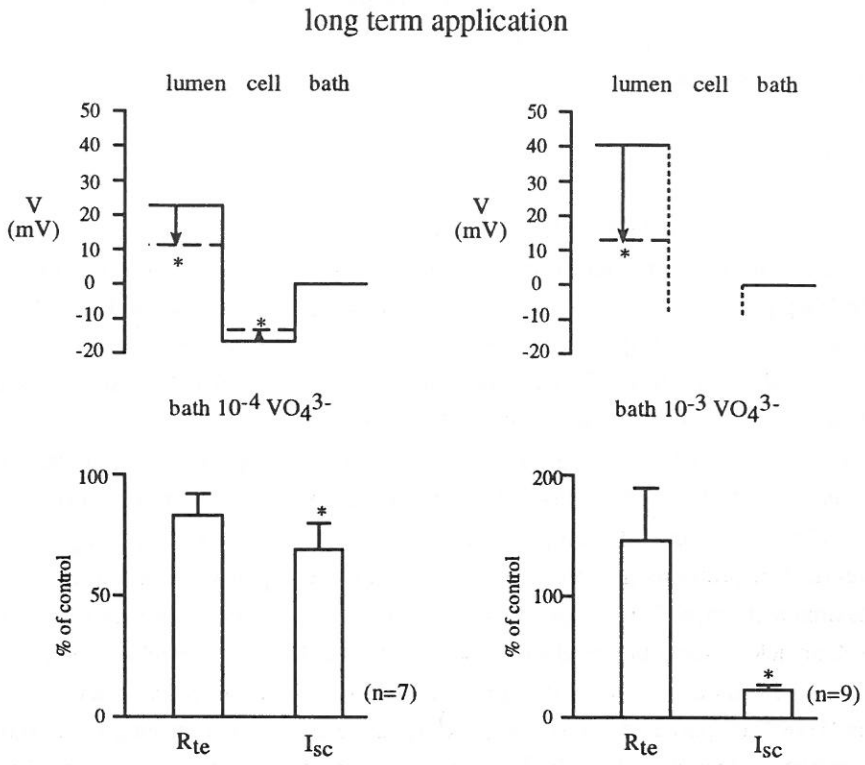


Fig.4.5B long term application (30 min) of vanadate basolaterally in the concentrations of 10^{-3} and 10^{-4} mol/l. The continuous lines in the potential profiles represent the values in the control state, the dashed lines the values after vanadate application. No V_{bl} was measured in the series of tubules where basolateral vanadate application at a concentration of 10^{-3} mol/l lasted 30 min. I_{sc} and R_{te} are given as percentages of control. * Indicates significantly different from control, in the case of basolateral application of 10^{-4} mol/l vanadate, ΔV_{bl} was significant in a one-sided t-test only.

reduced to 23% (fig.4.5B). A significant reduction in the R_{te} was found upon short term luminal application, but the lowering of R_{te} upon both short and long term basolateral application was not significant. In the individual experiments the V_{te} traces showed variable patterns ranging from a distinct S-shaped curve to a slow, linear decrease. With a bath concentration of 10^{-4} mol/l vanadate (n=7), no distinct effect was observed during the first few minutes. If the onset of a slower effect was detectable within the first 10 minutes, the application was prolonged until stable values were reached. Therefore "long term" application time varied between 10 to 27 minutes. During this time a significant decrease in I_{sc}, was found (fig.4.5B). In a series of 5 tubules where 10^{-5} mol/l was used basolaterally, the measured parameters showed no change within

the first 10 minutes, however, even within this short time V_l, caclulated as V_{te} - V_{bl}, decreased significantly (data not shown).
The effects of vanadate were poorly and only slowly reversible.

4.2 Discussion

A. *An acid luminal pH inhibits active transport*
The experiments where luminal pH was decreased, demonstrate that active transport in the Malpighian tubules of the ant was reversibly inhibited at low luminal pH. This suggest that active transport is mediated by a H^+-pump. The decrease in I_{sc} was largely due to a decrease in V_l. Between pH-values of 4.8 and about 4 a rise in R_{te} was observed and this also contributed to a further drop in I_{sc}.
Half-maximal inhibition of I_{sc} was achieved at pH 4.5, complete inhibition was found around pH 3.5. There, the V_l averaged 13±3 mV (n=5). If the lowered luminal pH did not affect intracellular pH, this pH could be assumed to be around 7.8 [Zhang et al., in press]. The protonmotive force of the respective pump would then amount to a maximum of around 270 mV (4.3 pH-units plus 13 mV). It is however highly likely that such an acid luminal pH, producing not fully reversible results, could also acidify the cell to a certain degree, since the apical H^+ extrusion pump should be inactivated and the H^+/K^+-antiporter, if it was also present, due to an increased driving force, should transport protons into the cell. Thus the proton gradient built up will in reality be smaller. So far, in earlier studies estimates for the protonmotive force of 130 mV [Leyssens et al., 1992] and 150 mV [Zhang et al., in press] have been reported. Conversely, from these values and subtracting the remaining V_l, we calculate that the minimal value for the cell pH would be 5.5-5.8 when luminal pH is lowered to 3.5.

A comparison between values for protonmotive forces found for the different types of electrogenic H^+-ATPases reveals that the E_1E_2-type can exhibit a wide range of protonmotive forces. For example, in the plasma membrane of *E.Coli* and *Salmonella typhi murium*, maximal electrochemical gradients for protons of respectively 130 and 180 mV [Padan et al., 1976; Tokuda & Kaback, 1977] are found. However, the stoichiometry of this type of pump has been shown to be 1 H^+ transported per ATP hydrolysed [Slayman, 1985] thus its protonmotive force can theoretically be 350 mV or more. An example of the F_1F_0-type H^+-ATPase is the H^+-pump in mitochondria of the rat liver which generates a large electrochemical gradient for H^+ of 230 mV, of which only 30 mV is translated into a ΔpH of 0.5 units [Mitchell & Moyle, 1969]. The F_1F_0-pump in chloroplasts has been shown to produce maximal pH-differences over the membrane of about 3.5 pH-units, with negligible membrane potential differences, resulting in a protonmotive force of 210 mV [Rottenberg et al., 1972].

The family of vacuolar type proton pumps generates a protonmotive force of about 120-180 mV or of 2-3 pH-units. In chromaffin granule ghosts for example the protonmotive force was found to be 133-150 mV [Phillips & Allison, 1978], in rat liver multivesicular bodies it was 118 mV [Van Dyke, 1988]. In plant tonoplasts protonmotive force is 125 mV [Thom & Komor, 1985]. The vacuolar type proton pump found in the urinary bladder of the turtle, an epithelium that is relatively impermeable to protons, generates a protonmotive force of about 180 mV [Al-Awqati et al., 1977]. Fungi do not have a V-type ATPase in the plasma membrane because they often live in acid environments and an external pH of 5 places the V-ATPase close to its thermodynamic limit for proton transport [Gluck, 1992]. From the above it is evident that it is impossible to base any positive conclusions concerning the family of ATPases to which the pump in the Malpighian tubules could belong, on the protonmotive force of the pump. Conversely, the observed responses of the Malpighian tubules to luminal pH-changes, do not disprove the hypothesis that active secretion is mediated by a vacuolar type pump.

The rise in resistance when luminal pH is decreased to values below pH 4.8 appears to be due primarily to an increase in the resistance of the shunt (R_S). Even though the closing of the basolateral K^+-channels is the first hypothesis that might come to mind, because of an acidification of the cell. An increase in R_{bl} explaining the observed rise in R_{te} is not very likely: neither V_{bl} nor VDR changed significantly. It is more difficult to exclude an increase in R_l (apical or luminal membrane resistance): R_l is very large compared to R_{bl} (VDR is around 50 in control conditions). As a consequence the deflections in the V_{bl} necessary to determine VDR are detected only rarely on current injection (1 out of 5 times in control conditions [Dijkstra et al., 1994]). Still, if the increase in R_{te} were caused by an increase in R_l only, due to the inhibition of the apical electrogenic pump, a rise of around 60 % in R_{te} as seen in fig.4.2, would cause a rise of more than 60% in the VDR. This should have been detected. This suggests that the rise in R_{te} is, at least in part, due to an increase in R_S or due to a combined increase of R_l and R_{bl}. The decrease in R_{te} at a luminal pH of around 3.5 units might indicate that the tight junctions become damaged at such low pH-values.

B. *Effects of vacuolar type ATPase inhibitors, bafilomycin and NEM*

In an attempt to characterize the elements partaking in active secretion more closely, a variety of inhibitors has been tested. Bafilomycin A1 has been presented as a specific inhibitor for the vacuolar type H^+-ATPase with an IC_{50} around 5.10^{-9} mol/l when applied to purified ATPase [Bowman et al., 1988; Mattsson et al., 1991]. Luminal bafilomycin application on Malpighian tubules resulted in a depolarization of the apical membrane and no effect on V_{bl}, indicating an inhibition of an electrogenic apical pump. Since the basolateral membrane resistance is so low [Weltens et al., 1992; Dijkstra et

al., 1994], the change in V_l is not reflected in a change in V_{bl} . The decrease in V_l resulted in a reduction of I_{sc}. As I_{sc} is a measure for active transport, this indicates that a V-type ATPase may indeed be involved in the active transport.

In general, membrane bound ATPases were found to be less sensitive to bafilomycin than the purified ATPase by a factor 10 to 100 [Bowman et al., 1988; Moriyama & Nelson, 1989]. The V-ATPase from oat roots in purified preparations was inhibited at 10^{-7} mol/l [Ward & Sze, 1992]. A K_i of 6.10^{-6} mol/l bafilomycin was reported for the inhibition of transepithelial fluid secretion in Malpighian tubules of *Aedes aegypti* [Pannabecker & Beyenbach, 1993]. The apical vacuolar H^+-ATPase in the isolated, larval Malpighian tubules of *Drosophila hydei*, showed half-maximal inhibition by bafilomycin at 10^{-6} mol/l [Bertram et al., 1991]. In cells of *Entamoeba histolytica* IC_{50} was also at 10^{-6} mol/l [Löhden-Bendinger & Bakker-Grunwald, 1990]. A concentration of 10^{-5} mol/l was used to inhibit the vacuolar H^+-ATPase in murine macrophages [Swallow et al., 1990].

The concentration of bafilomycin needed in this study to inhibit the H^+-ATPase was a factor 5000 higher than the reported IC_{50} value for purified ATPases, and thus near the IC_{50} for purified E_1E_2-ATPases [Bowman et al., 1988]. The fact that a high dose is needed cannot be explained by a loss of bio-activity of bafilomycin, because it proved to act with its normal IC_{50} in dog kidney cortex vesicles. Another explanation could be the fact that inhibition by bafilomycin has been shown to be due to stoichiometric binding near the catalytic site [Hanada et al., 1990]. Therefore its effectiveness in intact epithelia will depend on how well it can enter the cell. Also cellular uptake seemed to be more difficult when bafilomycin was applied from the lumen. When applied from the bath side the concentration needed to affect membrane potential was lower (10^{-6} mol/l) [Weltens et al. 1992].

In conclusion, considering the difficulties in getting bafilomycin into the cell and the much lower sensitivity of membrane bound as compared to purified enzymes, we consider the effective concentration of bafilomycin low enough to conclude that the pump may be of the vacuolar type.

Another inhibitor specific for vacuolar ATPases is NEM if used at low concentrations. Purified vacuolar ATPases are generally sensitive to 10^{-5} mol/l NEM. Studies done on purified phosphorylated ATPases exhibited sensitivity to NEM at $1\text{-}10 \cdot 10^{-4}$ mol/l [Forgac, 1989] but since NEM is expected to attack cytosolic sulfhydryl-groups near the ATP-binding site the concentrations needed in intact epithelia is expected to be higher. The concentration used here was 5.10^{-4} mol/l which resulted in a decrease of I_{sc} to 21 % upon basolateral application. Earlier studies on natural fluid secretion where the same concentration was used also showed a comparable inhibition [Weltens et al., 1992]. As with bafilomycin, this concentration is low enough to be specific for vacuolar ATPases

considering that the substance has to get into the cell first and that the effective concentration may therefore be a factor of magnitude lower. Studies on the isolated larval Malpighian tubules of *Drosophila* [Bertram et al., 1991] and vesicles of the midgut of *Manduca Sexta*, demonstrated 50 % inhibition at resp. 50.10^{-6} mol/l and $0.5.10^{-6}$ mol/l [Schweikl et al., 1989]. The much more pronounced effect observed when NEM is added from the bath side compared to the lumen side, suggests that the luminal membrane is less permeable. This is consistent with the fact that more bafilomycin was needed luminally than basolaterally to block secretion.

C. *Effects of H^+/K^+-ATPase-inhibitors: omeprazole and Schering compound 28080*

Omeprazole is known as a specific inhibitor of the gastric H^+/K^+-ATPase at concentrations of around 3.10^{-6} mol/l [Mattsson et al., 1991]. A much higher concentration was needed in the present study. By an acid induced transformation of the drug, it reacts with the luminally accessible sulfhydryl groups of the pump, thereby causing inhibition. However, with lower potency (10^{-4} mol/l) omeprazole has also been shown to inhibit the V-type ATPase in kidney- and bone-derived membrane vesicles-studies [Mattsson et al., 1991]. In the case of inhibition of a vacuolar type ATPase, cytosolic rather than luminal SH-groups were shown to interact with the omeprazole [Mattsson et al., 1991]. Therefore, the fact that 10^{-4} mol/l acid activated omeprazole caused only a 29 % inhibition of I_{sc} in our preparation, again is compatible with the suggestion that a vacuolar ATPase is involved in the secretion process.

S28080 is also a specific inhibitor of the H^+/K^+-pump but with a different mechanism of action. At present, we are not aware of any other type of pump than the H^+/K^+-ATPase being inhibited by S28080 at the concentration used. Froissart et al. (1992) have demonstrated that 10^{-4} mol/l S28080 does not inhibit the NEM- and bafilomycin-sensitive ATPase in mammalian kidney medullary thick ascending limb. S28080 does not need to be acid activated and inhibits by competing with K^+ [Wallmark et al., 1987]. In this study, the compound had only a very slight, but significant, effect on I_{sc} although none of the other measured parameters were significantly affected. These data are consistant with the view that the proton pump in Malpighian tubules is not the H^+/K^+-ATPase.

Note of the author: after acceptation of this paper by Renal Physiol Biochem, a paper has appeared demonstrating both omeprazole and S28080 also inhibit the vacuolar type ATPase of the turtle bladder. H^+-secretion was shown to decrease to 45±16 % upon mucosal addition of $3.5*10^{-4}$ mol/l omeprazole and $4*10^{-4}$ mol/l s28080 produced essentially complete inhibition [Graber & Devine, 1993].

D. *Effects of ortho-vanadate*

Vanadate, a phosphate analogue, inhibits P-type ATPase from the intracellular side, by forming a putatively stable intermediate vanadate-enzyme complex [Cantley et al., 1978]. It is thought to enter the cell via an anion exchange mechanism [Edward & Grantham, 1983]. It inhibits purified E_1E_2-ATPases at concentrations of 1-10 µmol/l, the most notable of these being the Na^+/K^+-ATPase. Vacuolar type ATPases are insensitive to concentrations up to at least 1 mmol/l (review: [Forgac, 1989]). So if a V-type H^+-ATPase drives active secretion in Malpighian tubules of *Formica*, vanadate was not expected to exert any effect on the electrical parameters thought to be important in this transport. The results showed that long term exposure to vanadate did have a significant inhibitory effect. Unfortunately vanadate has an effect on a variety of other cellular processes. It inhibits (and stimulates) a variety of other enzymes at different concentrations (review [Chasteen, 1983]). For instance, it stimulates adenylate cyclase at 0.01 and 0.1 mmol/l, it inhibits a variety of ribonucleases at $1\text{-}6.10^{-5}$ mol/l and Ca^{2+}-pumps at concentrations of $1\text{-}50.10^{-6}$ mol/l. Thus it may exert effects on different systems whereby the exact role of these components in our system are not fully understood as yet [De Decker et al., 1991; De Decker et al., 1993]. Vanadate has also been shown to inhibit isolated mitochondrial ATPase at a concentration of ~ 1.5 mmol/l [Schwartz et al., 1980]. Furthermore, in giant squid axons it inhibits a protein phosphatase thus influencing other enzyme activities [Altamiro et al., 1988] and it affects the gastric H^+/K^+-ATPase [Walderhaug et al., 1985]. The fact that vanadate in the present study had a strong effect only with some delay suggests an indirect mode of action. The decrease in R_{te} upon vanadate application (significant in the case of luminal application) seems to be unusual, but has been reported before in experiments on toad bladder [Beauwens et al., 1981]. Hypothetically, this decrease in R_{te} could be due to the negatively charged vanadate entering the shunt. The shunt was shown to be anion-specific [Dijkstra et al., 1994].

We cannot completely rule out the possibility that the apical H^+-pump, is sensitive to vanadate to some extent, although, in the face of all the evidence presented and indicating that this pump is most likely of the vacuolar type, we consider this possibility unlikely. It is worth noting in this context that the V-type ATPase in osteoclasts has recently been reported to be inhibited by vanadate with an IC_{50} of 10^{-4} mol/l [Chatterjee et al., 1992]. The cause is sought in the different subunit A of the catalytic domain which is found to have a mass of $63x10^3$ as opposed to the $70x10^3$ of the classical subunit A. The V-type ATPase of the *Manducta Sexta* midgut, the model for an insect V-type ATPase, has been established to express the classical $70x10^3$ subunit A [Schweikl et al., 1989].

As opposed to the results obtained with inhibitors specific for the vacuolar type ATPase, vanadate application resulted in a significant decrease of the V_{bl} already within the first ten minutes. So far, the Na^+/K^+-pump has always been named as the most likely target for the action of vanadate in the Malpighian tubules of insects [Bertram et al., 1991; Baldrick et al., 1988]. In some insects the existence of a Na^+/K^+-pump in the basolateral membrane could be demonstrated (*Locusta* : [Baldrick et al., 1988]; *Rhodnius* : [Maddrell & Overton, 1988]; *Drosophila* : [Wessing et al., 1987]).

Second note of the author: just before the final revision of this thesis, M. Garayoa (university of Pamplona) presented immunological results illustrating binding of an antibody against the α-subunit of the avian Na^+/K^+-pump, which has been shown to crossreact with *Drosophila* Na^+/K^+-ATPase, to the basolateral membrane of the Malpighian tubules of *Formica polyctena*.

In conclusion, the present data indicate the presence of a proton pump in the luminal membrane of ant Malpighian tubules. This pump is blocked by acidic luminal pH and it behaves qualitatively like the V-type H^+-ATPase although its sensitivity towards inhibitors is less pronounced than might have been predicted. These quantitative differences might, to a large extent, be explained by the impermeability of the luminal membrane for inhibitors and by the need to add them to the basolateral pole. Furthermore, the effects of vanadate on the basolateral membrane potential, suggest some active element other than the luminal proton pump also plays a role in the secretion process. One most likely candidate is the Na^+/K^+-ATPase.

5 Effects of dinitrophenol on active transport processes and cell membranes in the Malpighian tubule of *Formica.*

(adapted from Dijkstra S, Lohrmann E, Van Kerkhove E, Steels P, Greger R, Pflügers Arch, submitted)

5.1 Results

DNP, a well-known inhibitor of ATP-production, was tested on the luminally perfused Malpighian tubules of the ant. The drug was added from the bath side in two different concentrations: 2.10^{-4} mol/l, the 'low' concentration, and 10^{-3} mol/l, the 'high' concentration. The effect of the high concentration was also tested when perfused into the lumen of the tubule. In the Malpighian tubules of the ant the relative apical resistance is so high and the basolateral resistance so low (VDR equals 51 under control conditions [Dijkstra et al., 1994]), that the deflections of V_{bl} on current injection, allowing an estimate of the VDR to be made, were seldom seen (1 out of 5 impalements). To circumvent this problem 6 mmol/l $BaCl_2$ was added to the bath in the series where the effect of 2.10^{-4} mol/l DNP was tested, both in control conditions and when DNP was present. This markedly increased the basolateral resistance by reducing the high basolateral K^+-conductivity [Dijkstra et al., 1994; Weltens et al., 1992]. The absolute transepithelial resistance in this series increased from 215±13 to 485±164 (n=4) $\Omega.cm^2$ in the presence of Ba^{2+}, and inhibited I_{sc} from 226±50 to 95±26 $\mu A/cm^2$. Original traces for all three situations are presented in fig.5.1. The different effects on potential differences are summarized in fig.5.2 and the effects on R_{te}, I_{sc} and VDR are presented in fig.5.3. In general, DNP depolarized both the V_{te} and V_{bl}. The immediate drop in V_{te} upon DNP addition to the bath as seen both in fig.5.1A upon the addition of the lower concentration of DNP and fig.5.1B upon addition of the higher concentration, was generally more marked in the presence of the higher concentration: V_{te} dropped from 46±5 to 2±1 mV (n=16) as opposed to the lower concentration where V_{te} dropped from 37±7 to 8±1 mV (n=4, fig.5.2). Whereas luminal application of DNP at 10^{-3} mol/l (fig.5.1B) had no clear effect on either V_{te} or V_{bl} in the experiment shown. In general, luminal application of DNP at 10^{-3} mol/l resulted in a significant depolarization from 52±7 to 40±8 mV (n=6). V_{bl} depolarized upon the addition of both the lower and higher concentration of DNP to the bath (fig.5.1A and 5.1B) with 8 and 12 mV respectively. In general the basolateral depolarization was in the same order of magnitude for low and high basolateral DNP concentrations (from -19±5 to -11±4, n=4, and -16±2 to -7±3 mV, n=8, respectively), but here it must be kept in mind that 6 mmol/l $BaCl_2$ was present in the bath when the lower concentration of DNP was tested. This increased R_{bl} and thus the portion of the apical depolarization that is translated onto the basolateral membrane. In fig.5.1B upon luminal application of 10^{-3} mol/l DNP, no depolarization of V_{bl} was

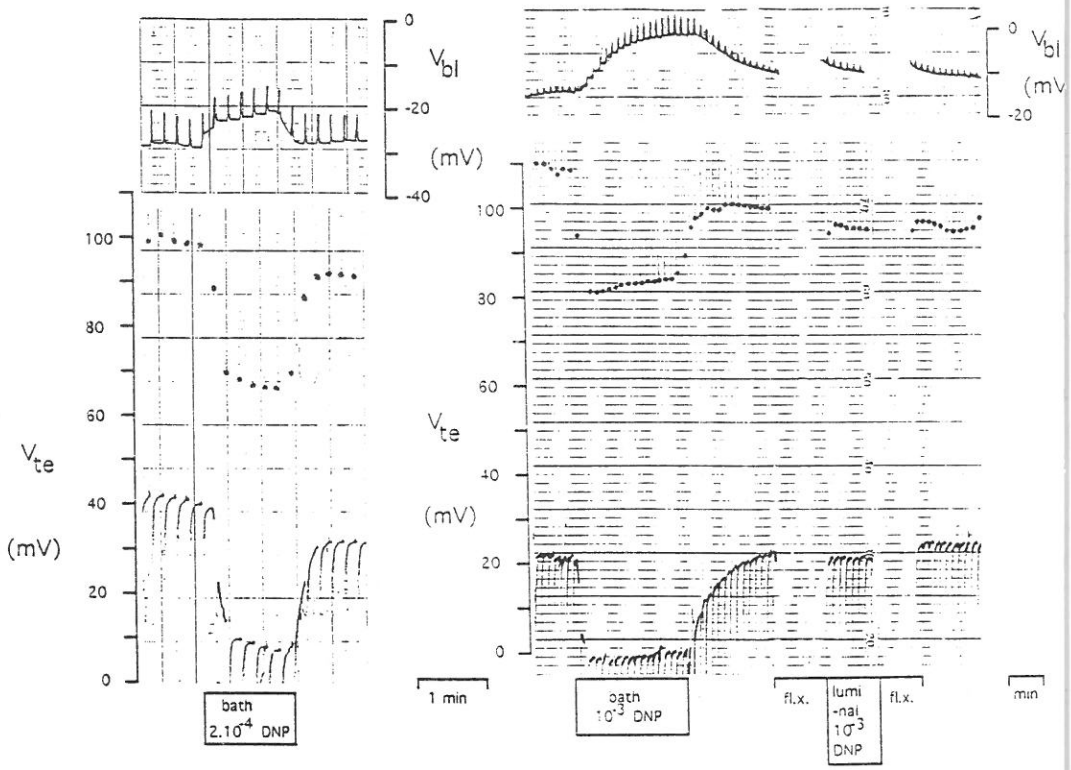


Fig.5.1 A.The effect of 2.10^{-4} mol/l dinitrophenol (DNP) added basolaterally in the presence of 6 mmol/l $BaCl_2$. B. The addition of 10^{-3} mol/l DNP to the bath and to the lumen. The downward deflections on the V_{te}-trace are artifact capacities. Short voltage changes due to current injection are seen as dots (see V_{te} trace) or deflections (V_{bl} trace). Via cable analysis they are used to calculate R_{te} and the voltage divider ratio (VDR). During fluid exchanges (fl.x.) the measured values were unstable and these parts of the traces have been left out for clarity.

evident. In general the basolateral depolarization upon luminal application of DNP (10^{-3} mol/l) was not significant (from -12±2 to -9±2 mV, n=6). The most striking difference between the effect of the low (fig.5.1A) and high DNP concentration (fig.5.1B) in the bath, is the behaviour of the VDR. Application of the lower concentration of DNP caused no change in the measured deflections of V_{bl} from which the VDR was calculated. Whereas upon application of the higher concentration the deflections of V_{bl} clearly increased, indicating a decrease in the apical over basolateral resistance. On average, 10^{-3} mol/l DNP in the bath significantly decreased VDR (to 37±12 (n=6) % of control value), whereas 2.10^{-4} mol/l DNP did not (VDR was 109±28 (n=4) % of control value, fig.5.3). Luminal application of the higher concentration decreased the VDR, but

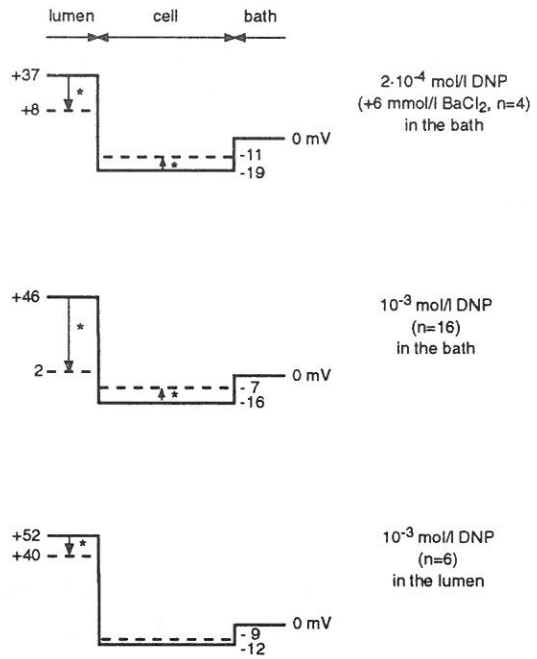


Fig.5.2 The effects of the addition of DNP in different concentrations to both the bath and the lumen, on the membrane potentials. With the addition of 2.10^{-4} mol/l DNP to the bath 6 mol/l $BaCl_2$ was present both in control and during DNP-addition. * Indicates statistically significant changes.

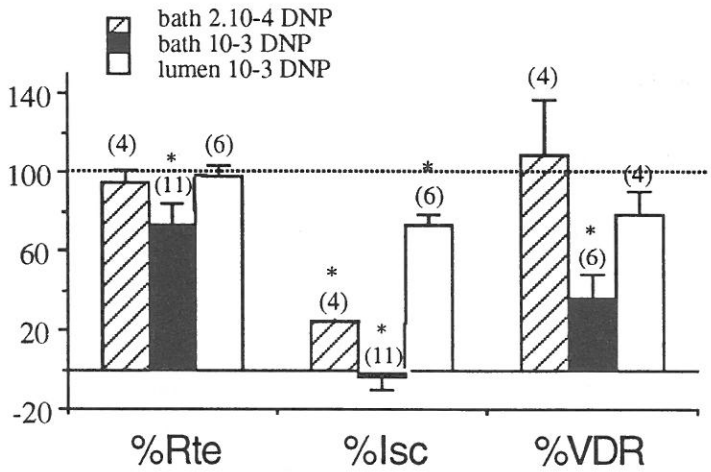


Fig.5.3 The effects of the addition of 2.10^{-4} mol/l DNP to the bath already containing 6 mmol/l $BaCl_2$, and the addition of 10^{-3} mol/l DNP to either the bath or the lumen. The transepithelial resistance (R_{te}), the equivalent short circuit current (I_{sc}) and the voltage divider ratio (VDR) are given as percentages of control values. * Indicates statistically significant difference from 100%. The I_{sc} after addition of 10^{-3} mol/l DNP to the bath is not significantly different from 0.

not significantly, to 79±12 (n=4) % of control value (P=0.054). With the decrease in VDR after basolateral application of a high concentration of DNP, a concomitant decrease of R_{te} was observed: to 74±11 (n=11) % of control value. Whereas when either the low concentration was applied to the bath or the high concentration to the lumen, R_{te} did not change significantly (respectively to 95±7 (n=4) and 99±5 (n=6) % of control value). Despite the decrease in R_{te}, the I_{sc} dropped dramatically under high DNP and was not significantly different from 0, namely -3±7 (n=11) % of control value. Both the low concentration in the bath and the high concentration in the lumen also led to a significant decrease in I_{sc} to respectively 25±3 (n=4) and 74±5 (n=6) % of control values.

Prompted by the different effectiveness of basolateral as opposed to luminal application of DNP, fluid secretion experiments investigating the possible uptake mechanism of DNP were conducted. Secretion was compared in a paired fashion between periods with 2.10^{-4} mol/l DNP present in the bath and periods in which in addition 5.10^{-3} mol/l probenecid were present in the bath. Probenecid is a competitive inhibitor of an anion carrier in Malpighian tubules of insects [Bresler et al., 1990]. No significant difference was found between the effects of DNP whether probenecid was present or not. The secretion rates found in the three collection periods with only DNP present were respectively 14±8, 2±5 and 3±6 % of control, whereas the rates found in the presence of both DNP and probenecid were 33±26, 6±9 and 3±5 % of control for the three consecutive collection periods (n=8).

5.2 Discussion

1. Effect of DNP on active processes: I_{SC}.

In general, DNP is expected to enter the cell and inhibit the ATP-production in the mitochondria acting as a protonophore on the inner mitochondrial membrane [Loomis & Lippmann, 1948; Mitchell, 1961]. ATP depletion results in an inhibition of all active processes. The decrease of I_{SC} to a value not significantly different from 0 % of control value under application of 10^{-3} mol/l DNP from the bath, confirms this assumption. Accordingly 2.10^{-4} mol/l DNP inhibited spontaneous fluid secretion to values not significantly different from 0 % of control value within the first collection period (10 min). However, the membrane resistance measurements obtained in this paper suggest that an additional protonophoric action may take place at the cell membranes. If this is indeed the case it is possible to explain why the cellular K^+ concentration, $[K^+]_C$, decreases in the presence of DNP as was found by Leyssens et al. [Leyssens et al., in press (b)].

2. The effect of DNP on the electrical properties of the membranes

The reduction in R_{te} and VDR caused by 10^{-3} mol/l DNP in the bath suggests that the apical membrane resistance has dropped significantly. In the presence of DNP a new conductance for H^+ may have been created across this membrane by its protonophoric action as described by McLaughlin and Dilger [McLaughlin & Dilger, 1980]. The mechanism by which this occurs will be discussed below (see also Appendix 5.1). In principal there is no reason why the protonophoric action of DNP should be confined to the mitochondrial membrane only. In fact, DNP has been shown to cause a proton "shunt" across the toad bladder epithelium [Beauwens & Al-Awqati, 1976].

Since in control conditions in non-perfused Malpighian tubules an alkaline intracellular pH of 7.81±0.04 (n=18) was measured [Zhang et al., in press] and the range of luminal values included a pH of 7.20, i.e. the pH of the luminal perfusion fluid used in this study, the distribution of protons over the membrane would indeed favour a DNP-protonophoric action at the apical membrane. The same protonophoric action is expected to occur at the basolateral membrane, but because of the high K^+-conductivity present in the basolateral membrane, the new conductance created by DNP would have to be of a considerable magnitude before a decrease in R_{bl} became detectable.

The absence of a significant decrease in R_{te} when 2.10^{-4} mol/l DNP is applied does not exclude a protonophoric effect on the cell membrane at this concentration. An effect on the basolateral membrane would most probably not be detected (see above) and an effect on the apical membrane, producing currents in the nanomolar range, may also be too small to be measurable.

As mentioned in the introduction, the effect of DNP on R_{te} has also been measured on the Malpighian tubules of the mosquito *Aedes aegypti* [Pannabecker et al., 1992] where it was found to increase both R_{te} and VDR, as opposed to the effects found in this study. The increase in R_{te} was hypothesized to result from a blocking of all the pumps as well as a decrease of membrane conductances for Na^+ and K^+ basolaterally and Cl^- apically. This last suggestion resulted from the apparent lack of ionic diffusion potentials during DNP application and the rapid on/off effects. However, in our preparation V_{bl} was still sensitive to a change in the bath K^+-concentration [Leyssens et al., in press (b)]. We are, at present, unable to explain the contradiction in the results found on such relatively similar epithelia.

DNP has been shown to directly inhibit Cl^--permeability of erythrocytes with an IC_{50} of 8.10^{-5} mol/l [Motais et al., 1978]. It has however also been reported to increase rather than decrease a K^+-conductance in cortical neurons of cats, presumably mediated by a rise in intracellular free Ca^{2+} as caused by a slowing of mitochondrial activity [Godfraind et al., 1971]. Preliminary attempts to raise intracellular Ca^{2+} in the Malpighian tubules of the ant by applying the Ca^{2+}-ionophore A23187, had only a small stimulatory effect on secretion rates [DeDecker, 1993].

3. The effect of DNP on the ion concentrations in the cell

In the light of the present findings, i.e. that DNP can affect the cellular membranes and create an extra conductance, it becomes possible to give at least a partial explanation for the decrease in cellular K^+ concentration, $[K^+]_{cell}$, and the concomitant depolarization of the basolateral membrane in the presence of DNP. $[K^+]_{cell}$ decreased from 104 to 80 mmol/l in the presence of 2.10^{-4} mol/l DNP in the bath [Leyssens et al., in press (b)]. In the models formalizing the protonophore effect of DNP across a membrane or a planar lipid bilayer [McLaughlin & Dilger, 1980] it was assumed in most cases that the compartments on both sides of the membrane were large and well buffered so that the pH in the bulk solution and in the unstirred layers would not be affected. Also other conductances present in those membranes were not considered. We therefore applied the model for the specific case of the Malpighian tubule cell (see appendix 5.1) which has a very high K^+ conductance in the basolateral membrane and a finite cellular volume (although it was assumed that the buffering in the cell is sufficient, so that no pH gradient was created between the bulk solution and the unstirred layers near the membrane). When DNP is applied at the basolateral side, as suggested earlier, H^+ ions may be shuttled across the membrane. A complete cycle would consist of 2 DNP molecules entering the cell and returning to the bath side as DNP and DNP^- leaving a proton inside the cell (see Appendix 5.1). This would however depolarize the membrane. As a reaction and due to the dominant K^+ conductance, K^+ would leave the cell to restore the membrane potential difference according to its Nernst potential. The overall result would be that one K^+ exchanges for one H^+. Provided that the uncharged DNP is in equilibrium across the membrane the process will continue until the Nernst potential for protons equals the Nernst potential for K^+. From the experimental results ([Leyssens et al., in press (b)] and present results) it is clear that V_{bl} depolarizes following the decrease in $[K^+]_{cell}$ and that the cell acidifies (from pH 7.62 to 7.09) so that E_{H+} approached V_{bl} and thus E_{K+} (the difference between V_{bl} and E_{H+} is reduced from 46 to 12 mV). H^+_{cell}/H^+_{bath} was 81nmol/l / 56 nmol/l which was comparable to K^+_{cell}/K^+_{bath} as 80 mmol/l / 51 mmol/l after treatment with 10^{-4} mol/l DNP [Leyssens et al., in press (b)], as would be expected if DNP acts as a electrogenic protonophore on the basolateral cell membrane (see appendix 5.1).

4. Is DNP taken up via an anion-carrier?

The more marked effect of DNP when added to the bath could have been due to an anion-carrier in the basolateral membrane facilitating DNP entrance into the cell. This possibility was tested by studying the effect of the inhibitor probenecid on fluid secretion. Concentrations reported in the literature vary, but since K_i's of around 3.10^{-4} mol/l were found for different Malpighian tubules [Bresler et al., 1990], a concentration of 5.10^{-3} mol/l was chosen. No significant change in the inhibitory effect of DNP was

seen, whether probenecid was present in the bath or not. However, since the electrical effects took place within a minute of application of DNP and the first measurement of spontaneous fluid secretion can take place only after ten minutes because the absolute values secreted are so low (in the order of 100 pl/min. under control conditions), this test was not completely conclusive.

5. Differences in the effect of DNP when applied at the bath or at the luminal side
If an anion-carrier is not present in the basolateral membrane the differences found between luminal and basolateral application of 10^{-3} mol/l DNP are most likely due to differences in the properties of the respective membranes. DNP seems to enter the cell less readily when applied luminally than from the bath side, as measured by the difference in reduction in I_{SC}. Differences in the properties of the respective membranes, such as the different mucopolysaccharides obscuring passage to the membrane, lipid composition, dielectric constants and electro-static double layer potentials, will have a large influence on the permeabilities of the membranes for DNP. Since one of the functions of the Malpighian tubule is to clear the haemolymph of possibly dangerous substances, some form of luminal barrier preventing reabsorption of these substances would seem usefull.
Such a difference in sensitivity depending on where the drug is applied, has been noticed before in the rectal tubule complex of the *Tenebrio molitor* where injection into the tubule lumen of 1.10^{-3} mol/l caused a rapid decline in V_{te}, but basolateral application produced no significant effect within the first 15 minutes [O'Donnell & Machin, 1991].

In conclusion we suggest that DNP exhibits an additional protonophoric effect on the cell membrane, besides its well known inhibition of ATP-production. When concentrations as high as 10^{-3} mol/l are added to the bath, this effect is large enough to cause a reduction of R_{te}. Furthermore, DNP-entry into the cell is more efficient when the drug is applied from the bath than from the luminal side in the epithelium under study.

Appendix 5.1

A model for the protonophoric action of DNP across the cell membrane adapted from McLaughlin & Dilger (1980) is shown in fig.5.4. It shows the initial situation expected to be present when DNP is applied to the basolateral side. Transiently the following may happen: 1) DNP enters the cell and, as it is highly lipophylic, it rapidly equilibrates across the basolateral membrane. 2) DNP will dissociate into H^+ and DNP^- and as $[H^+]_{cell}$ is very low, in the beginning $[DNP^-]$ may be higher in the cytosol than in the

bath. 3) DNP and DNP^- strongly adsorb to the membrane-solution interface: their high concentration at the interface will promote the formation of a dimer which is much more soluble in the membrane than the monomer anion, because its size results in a better distribution of the charge. 4) The higher concentration on the cytosolic-solution membrane interface and the existing electrical potential difference across the membrane (cell negative) will cause a net movement of the negatively charged dimer across the membrane to the bath side. 5) The increase in dimer concentration at the bath side will result in the dissociation into the charged and uncharged species. The increase in monomeric DNP-anion will cause the uptake of a proton and form the neutral species which diffuses back to the other side of the membrane. A complete cycle thus consists of 2 DNP molecules entering the cell and returning to the bath side as DNP and DNP^-.

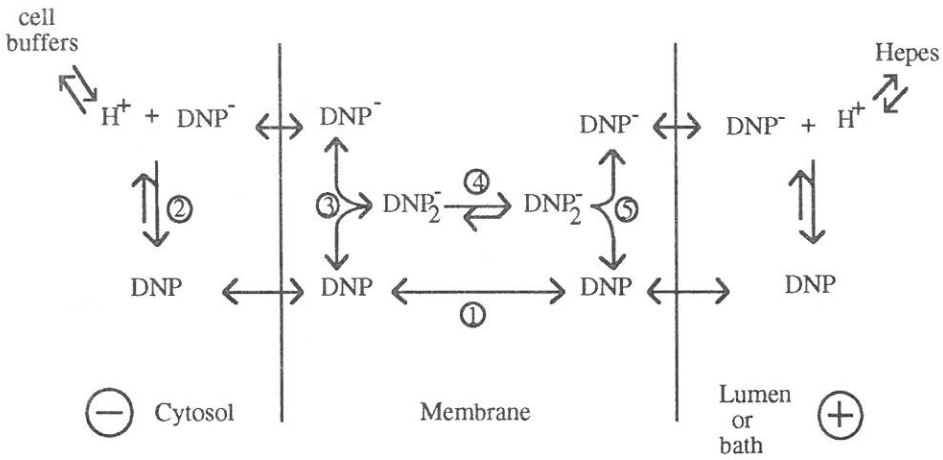


Fig.5.4 Model for the DNP protonophore effect across the membrane. Figure adapted from McLaughlin & Dilger (1980). See text for explanation.

In the model it is assumed that the permeability of the membrane to the uncharged DNP molecule is orders of magnitude larger than the permeability of the aqueous unstirred layers [McLaughlin & Dilger, 1980], which implies that the concentration of DNP at the two membrane solution interfaces will be identical. All reactions are assumed to be at equilibrium and the adsorption coefficients to be the same on either side of the membrane.

As the cells have a limited volume and since the uptake of protons may change the intracellular pH depending on the buffer capacity of the cells, it is then easy to show that the proton ions should redistribute across the membrane according to the actual membrane potential difference present which may be determined by other electromotive forces than the proton transfer itself:

In the case of the basolateral membrane the dimer is expected to distribute inside the membrane according to V_{bl}.

$RT/F*\ln\{[DNP_2^-]_{cell}/[DNP_2^-]_{bath}\} = V_{bl}$

Taking into account all equilibria we find that

$V_{bl} = RT/F*\ln\{[DNP]_{cell}^2*[H^+]_{bath}/[DNP]_{bath}^2*[H^+]_{cell}\}$

Assuming that $[DNP]_{cell}=[DNP]_{bath}$ (see above)

the equation simplifies to

$V_{bl} = RT/F*\ln\{[H^+]_{bath}/[H^+]_{cell}\}$

6 Chloride-selective measurements on the Malpighian tubule of the ant.

(adapted from Dijkstra S, Leyssens A, Van Kerkhove E, Steels P, J Exp Biol, submitted)

6.1 Results

1. Control conditions

With a bath Ringer's control solution containing 51 mmol/l K^+ and 143 Cl^-, intracellular Cl^- (Cl_i) was found to be 50±2 mmol/l, the associated electrical potential difference over the basolateral membrane (V_{bl}) was -24±1 mV (n=20). Thus the electrochemical gradient for Cl^- (V_{bl}-$E(Cl)_{bl}$) was slightly but significantly inwardly directed (+4±1 mV). In the lumen of a different series of tubules the Cl^--concentration (Cl_l) was found to be 244±19 mmol/l, the transepithelial potential difference (V_{te}) was 39±3 mV (n=20). The resulting electrochemical gradient for Cl^- over the epithelium (V_{te}-$E(Cl)_{te}$) was +27±3 mV lumen directed. Calculating the potential difference over the luminal membrane (V_l) as V_{te} - V_{bl}, the electrochemical gradient over the luminal membrane (V_l-$E(Cl)_l$) calculated with the unpaired average values, was also lumen directed with a magnitude of +23 mV. To obtain an idea of other substances present in the cell that might interfere with the Cl^- ligand, in three experiments all Cl^- in the bath was replaced by SO_4^{2-}, in these cases Cl_i went down to 3±2 mmol/l within 10 minutes. Thus we can assume interference of other anions present in the cell with Cl_i-measurements to be negligibly small. Since the decrease in K^+-activity of the Ringer's solution caused by the replacement of Cl^- by SO_4^{2-} had not been compensated for, the V_{bl} hyperpolarized from -21±1 mV to -27±1 mV. V_{te} was not measured.

2. Bath Cl^--changes

The effect of a tenfold decrease in the bath Cl^--concentration (from 143 mmol/l to 14 mmol/l) was tested on fluid secretion (fig.6.1A) and electrical potential differences as well as intracellular and intraluminal Cl^-- and K^+-concentrations (fig.6.2). As the ionic strength of the solution decreases by the substitution of Cl^- by SO_4^{2-}, the K^+ decreases as well. Therefore the chemical K^+-concentration in the control solution was lowered to 37 mmol/l as the K^+-activity, as measured with a single-barrelled K^+-selective electrode, was then comparable to the K^+-activity in the SO_4^{2-}-containing solution. As shown in fig.6.1A, after half an hour in low Cl^- medium, secretion was still sustained on a level 48±11 (n=8) % of control. Secretion was also measured when all Cl^- was replaced by SO_4^{2-} (and bath K^+-activity kept constant) as shown in fig.6.1B. Under these circumstances secretion immediately dropped to 30±5 (n=6) % of control value and stabilized at this value. The secretion found after half an hour of Cl^--free incubation was not significantly different from that found after half an hour of low Cl^--solution

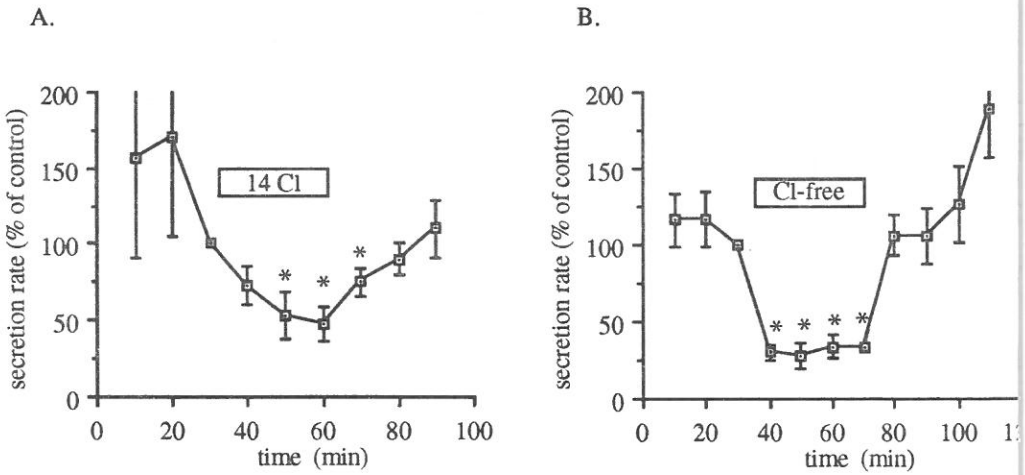


Fig.6.1 Time corrected secretion rates measured during A. 30 minutes of low Cl^--Ringer (37 mmol/l K^+, 14 mmol/l Cl^-, n = 8); B. 40 minutes of nominally Cl^--free Ringer (33 mmol/l K^+, n = 6). * Indicates significantly different from control.

(P=0.345, unpaired two-tailed student t-test on the non-time corrected data of both series). As shown in fig.6.2, a decrease in bath Cl^- hardly affected basolateral and transepithelial potentials. The small hyperpolarization detected with the Cl^- selective measurements is of the order of the experimental error and in the opposite direction to the expected change if an appreciable Cl^--conductivity were present. Further, the decrease in bath Cl^- concentration clearly caused a significant decrease in both cellular and luminal Cl^-, but did not affect either cellular or luminal K^+. Worth remarking is also the large negative basolateral electrochemical gradient for Cl^- in low Cl^- bath and the fact that the transepithelial electrochemical gradient under these circumstances is no longer significantly different from 0. The estimated lumen directed V_l-$E(Cl)_l$ increased from +23 mV to +49 mV.

3. Bath K^+-changes

The effect of a tenfold decrease in bath K^+-concentration (from 51 to 5 mmol/l) was also tested. Earlier experiments in our laboratory in 51K57Cl and 5K57Cl-solutions showed the respective fluid secretions to be 104±7 (n=24) and 72±7 (n=24) pl/min [Zhang et al., in press]. In these solutions the lower Cl^--concentrations had been achieved by replacement with citrate. Electrophysiological data obtained after decreasing the bath K^+-concentration tenfold in 143 mmol/l Cl^--Ringer's solution, are summarized in fig.6.3. For the sake of completeness, data of K_i and K_l-measurements already published [Leyssens et al., in press (a)], have been added.

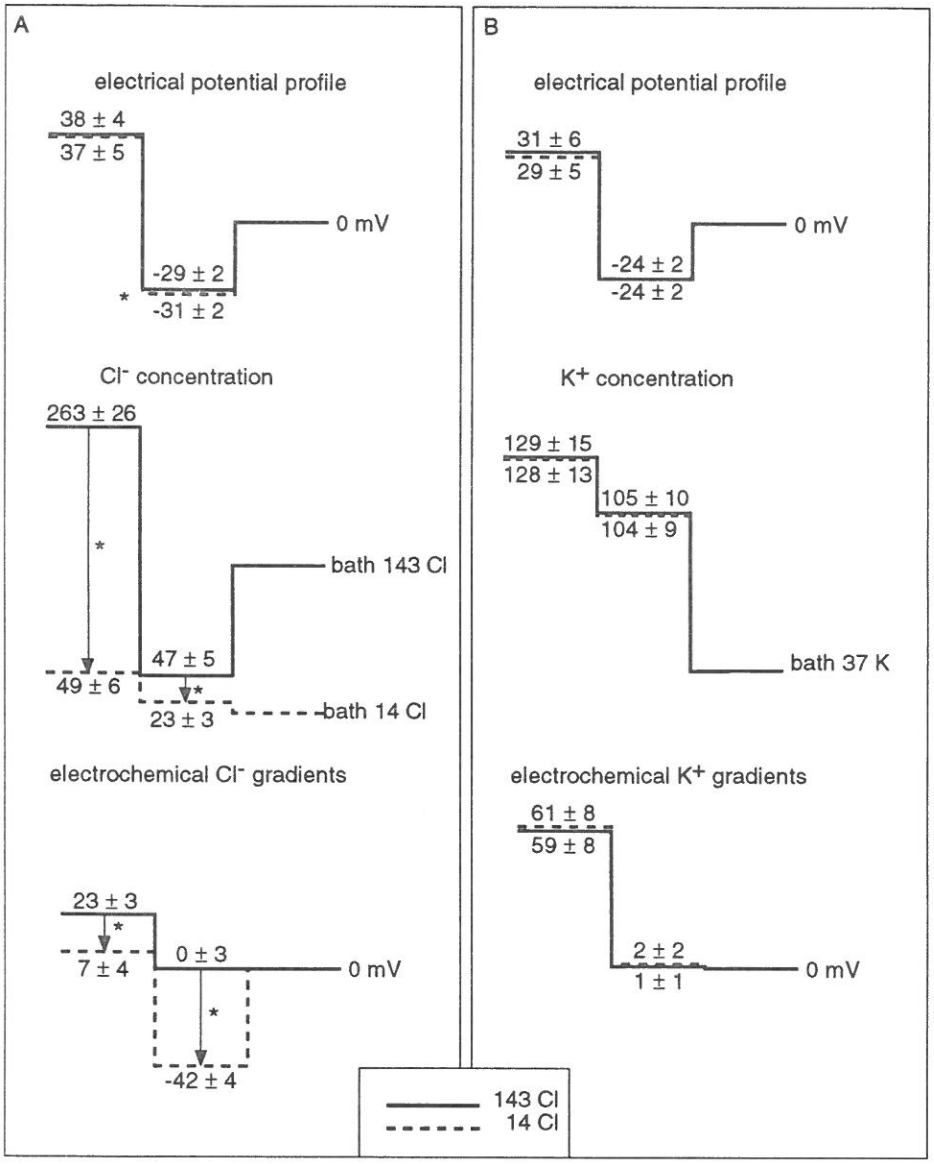


Fig.6.2. Summary of the effect of a tenfold decrease in bath Cl^--concentration. Solutions have been compensated for the decrease in K^+-activity due to replacement of Cl^- with SO_4^{2-}, chemical composition respectively 37 mmol/l K^+, 143 mmol/l Cl^- and 51 mmol/l K^+, 14 mmol/l Cl^- A. Electrical potential differences, Cl^--concentrations and electrochemical Cl^--gradients as measured with double-barrelled Cl^--selective electrodes. Transepithelial (n=9) and basolateral measurements (n=6) were performed on different tubules. B. Electrical potential differences, K^+-concentrations and electrochemical K^+-gradients as measured with double-barrelled K^+-selective electrodes.Transepithelial (n=9) and basolateral measurements (n=9) were performed on the same tubule on 7 occassions. * Indicates significantly different from control.

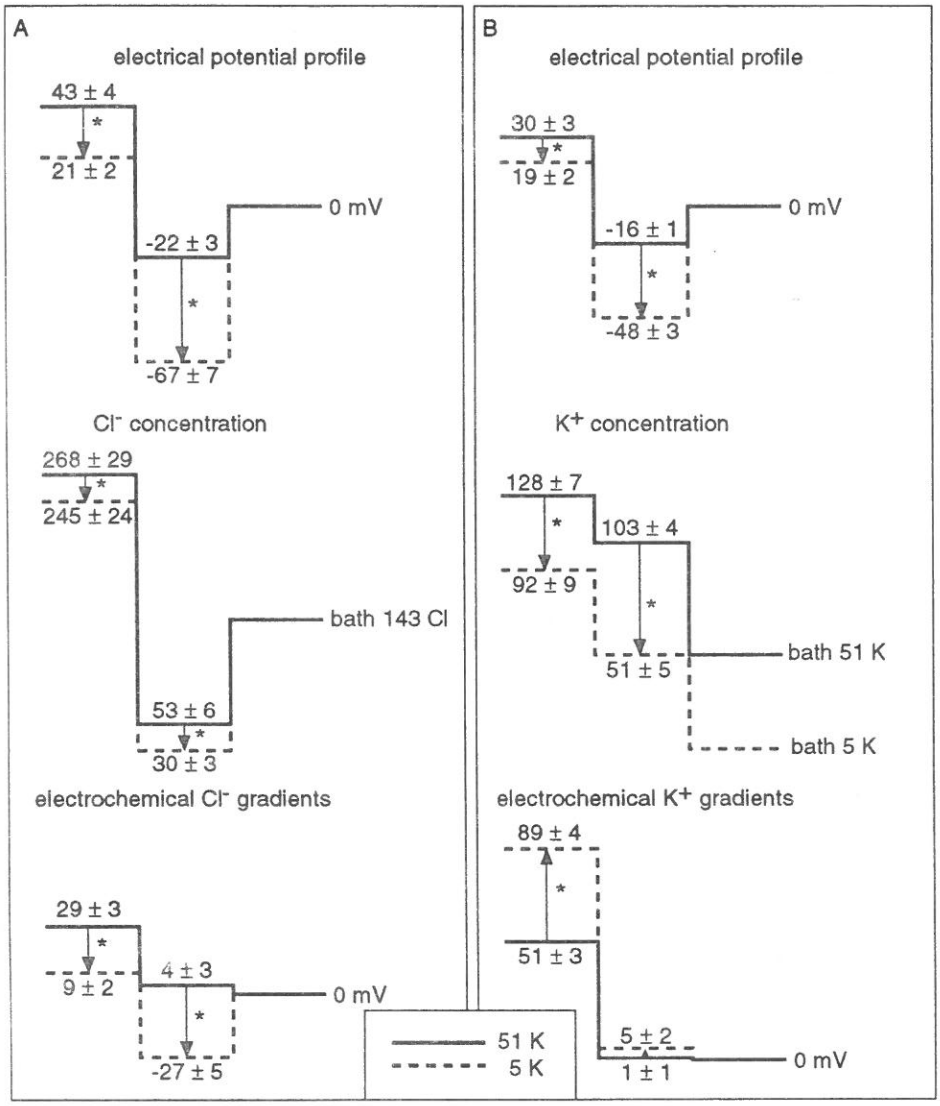


Fig.6.3 Summary of the effect of a tenfold decrease in bath K^+-concentration, from 51 mmol/l K^+, 143 mmol/l Cl^- to 5 mmol/l K^+, 143 mmol/l Cl^-. A. Electrical potential differences, Cl^--concentrations and electrochemical Cl^--gradients as measured with double-barrelled Cl^--selective electrodes. Transepithelial (n=10) and basolateral measurements (n=6) were performed on different tubules. B. Electrical potential differences, K^+-concentrations and electrochemical K^+-gradients as measured with double-barrelled K^+-selective electrodes. This unpaired data is taken from [Leyssens et al., in press a.]. In 51 mmol/l K^+ n = 30 for transepithelial measurements, n = 63 for basolateral measurements. In 5 mmol/l K^+ n = 18 for transepithelial measurements, n = 35 for basolateral measurements. * Indicates significantly different from control.

It is clear that a decrease in bath K^+-concentration hyperpolarizes V_{bl} and decreases both Cl_i and K_i significantly. It also lowers V_{te} and Cl_l and K_l. Again, the basolateral electrochemical gradient for Cl^- is outwardly directed as was the case in low Cl^- solutions. Transepithelially the gradient is strongly reduced but still significantly lumen directed, luminally the estimated V_l-$E(Cl)_l$ is slightly increased from +25 to +36 mV.

4. DIDS

10^{-4} mol/l DIDS , an inhibitor of Cl^-/HCO_3^- exchange systems or some Cl^- channels when used in high enough concentrations, did not affect the unstimulated fluid secretion rate significantly; 10^{-3} mol/l DIDS did cause a small, significant drop of the fluid secretion to 76±10 % of control (fig.6 4).

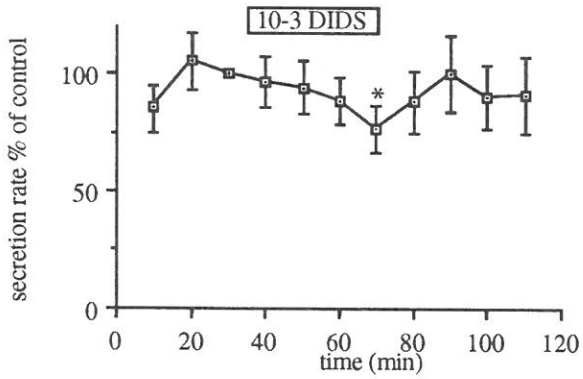


Fig.6.4 Effect on secretion rate of 40 minutes 10^{-3} mol/l DIDS-application (n=15).
* Indicates significantly different from control.

The effect was reversible. To test whether DIDS interfered with transport mechanisms that are activated after stimulation, the effect was also tested in the presence of 10^{-5} mol/l CPT-cAMP. When 10^{-5} mol/l CPT-cAMP was added to the control solution, mean fluid secretion rate (absolute value) in the third collection period was 267±106 (n=6) pl/min as opposed to the series without cAMP where fluid secretion was 186±28 (n=15) pl/min (despite the fact that this meant a 44 % increase in fluid secretion, the increase was not significant due to the large standard deviations). In the presence of 10^{-5} mol/l CPT-cAMP, the inhibitory effect of 10^{-3} mol/l DIDS was slightly less: secretion decreased to 83±8 % of control. The fact that secretion was slightly less affected in the presence of cAMP then in the absence of cAMP, would indicate cAMP stimulates secretion via a DIDS-insensitive pathway. However, due to the large variabilities in the fluid secretions the difference was not statistically significant and no firm conclusions can be reached. DIDS did not affect V_{bl} or V_{te} within the first 5 minutes of application (V_{bl} was -13±1

mV in control and -12±1 mV during DIDS application, n=8. V_{te} was 35±8 mV in control and 33±7 mV during DIDS application, n=6).

5. CPT-cAMP

The effect of 10^{-4} mol/l CPT-cAMP, a dose that also stimulates fluid secretion [De Decker, 1993], was investigated on Cl_i, Cl_l and electrical potential differences. Fig.6.5 presents a representative cellular measurement.

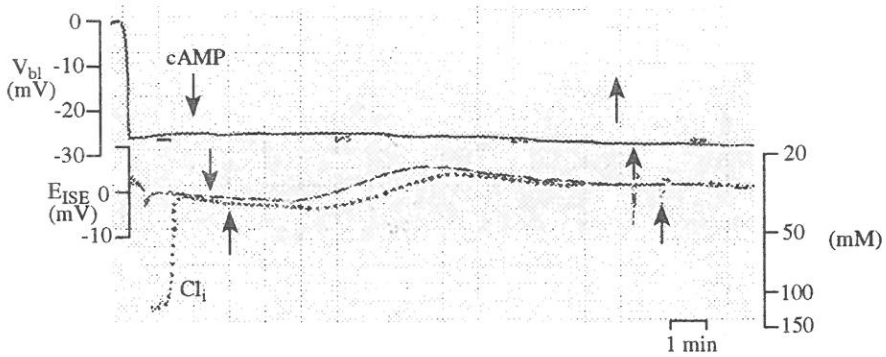


Fig.6.5 Cellular impalement with double-barrelled Cl^--selective electrode. Sudden change in V_{bl} and Cl_i-trace at the beginning of the trace indicate cell impalement. First arrows indicate addition of 10^{-4} mol/l CPT-cAMP, second arrows indicate removal of cAMP. The pen recording E_{ISE} (signal of the ion-selective barrel) lags half a minute behind the V_{bl}-recording. The pen recording Cl_i lags 1 minute behind the V_{bl}-recording.

It is clear that V_{bl} is not affected by the addition of cAMP. In the 5 tubules tested V_{bl} was -25±1 mV in control and -25±2 mV during the application of CPT-cAMP. The effect on Cl_i was biphasic. Averaged over the 5 tubules, the Cl_i first decreased to a minimum in all 5 tubules within 6-7 minutes of cAMP-application from 47±4 mmol/l to 37±7 mmol/l. The mean decrease was only significant at the 10 % level (P=0.08). Cl_i then increased again to reach a steady state at 43±6 mmol/l. This increase was significant (P=0.04). During the whole procedure the electrochemical gradient for Cl^- over the basolateral membrane did not change (+3±2 mV before cAMP-application, +2±4 mV as Cl_i was at its minimum and +6±4 when Cl_i had risen and stabilised again). In the experiment shown in fig.6.5 no reversibility was observed within the experimental time. In the other experiments partial reversibility was noted (max. experimental time after cAMP removal was 36 minutes).

The effect on V_{te} was also biphasic as demonstrated by the experiment shown in fig.6.6. After nearly 10 minutes a plateau value in V_{te} is reached. In three out of 5 experiments, cAMP-application was ended during this plateau, after 12 minutes of cAMP-application.

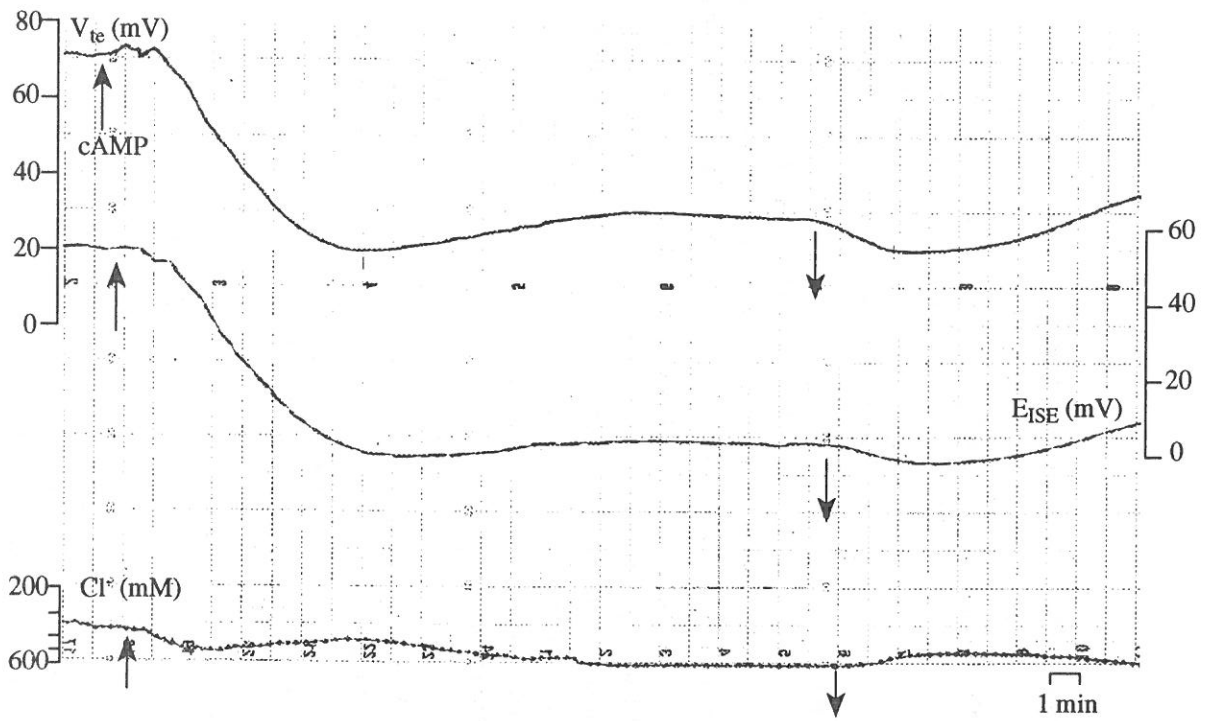


Fig.6.6 Luminal impalement with double-barrelled Cl^--selective electrode. Upward arrows indicate addition of 10^{-4} mol/l CPT-cAMP, downward arrows indicate removal of cAMP. The pen recording E_{ise} (signal of the ion-selective barrel) lags half a minute behind the V_{te}-recording. The pen recording Cl_l lags 1 minute behind the V_{bl}-recording.

V_{te} had now decreased from 45±9 mV to 16±1 mV and Cl_l had significantly increased fom 281±25 mmol/l to 329±31 mmol/l (n=5).V_{te}-$E(Cl)_{te}$ had decreased from +29±8 mV in control conditions to -4±2 mV (not significantly different from 0 on 10% level, P=0.06). In the two experiments where total cAMP-application time exceeded 20 minutes, a slow rise in V_{te} was registered, 9 mV in the experiment shown, 7 mV in the other. In both cases Cl_l continued to increase. Interestingly, despite the changes in V_{te} and Cl_l, in both cases V_{te}-$E(Cl)_{te}$ did not decrease any more during this time. In the first 10 minutes V_{te}-$E(Cl)_{te}$ had decreased from +45 and +48 mV to -5 and -7 mV in the two experiments, whilst after a further 10 and 14 minutes, V_{te}-$E(Cl)_{te}$ was still at -5 and -8 mV respectively. Pooling the results at the moment of cAMP-removal, Cl_l had increased to 383±62 mmol/l, V_{te} had decreased to 20±3 mV and V_{te}-$E(Cl)_{te}$ had decreased to -4±2 mV (n=5), which was not significantly different from zero at a 10% level (P=0.08). Surprisingly, calculating V_l and V_l-$E(Cl)_l$ from the averaged, unpaired data we find V_l decreased from 70 to 45 mV and the electrochemical gradient for Cl^- over the luminal membrane decreased from +25 mV lumen directed in control conditions to -10 mV cell inwardly directed after cAMP application. However, this -10 mV is probably not significantly different from 0 since the results were taken from unpaired measurements. If we estimate the range for V_l-$E(Cl)_l$ from the mean values ± standard error we find V_l-$E(Cl)_l$ ranges between +2 to -22 mV. It is however evident that the driving force for Cl^- across the luminal membrane has been reduced to a small value in the presence of cAMP. This could be the result of an increase in the conductivity for Cl^- bringing the Nernst equilibrium for Cl^- over the luminal membrane ($E(Cl)_l$) close to V_l. An increased net lumen directed Cl^- flux through a Cl^--channel would only be possible if some gradient was still present and if the Cl^--conductance had undergone a relatively substantial increase. CAMP application is then expected to result in a significant transepithelial resistance decrease and a decrease in the voltage divider ratio since the apical membrane is the major determinant of the transcellular resistance [Dijsktra et al., in press]. Preliminary results investigating this hypothesis however, give no indication of a decrease in resistance upon cAMP application (unpublished observations).

The removal of CPT-cAMP resulted in a decrease in V_{te} in 3 out of 4 experiments (5^{th} impalement lost) and a concomitant (but not significant) decrease in Cl_l. V_{te} then increased again but full reversibility was not observed within the experimental time (max. 20 minutes).

Discussion

The data presented in this study allow us to draw some general conclusions concerning the secretion of ions under control conditions in vitro . First, the Cl_i and Cl_l found in this study compare well to the few values reported so far for other similar insect tissues (table 6.1).

Table 6.1 Comparison of Cl_i and Cl_l-values in other insect tissues.

Cl_i (mmol/l)	Cl_l (mmol/l)	Cl_{bl} (mmol/l)	method[1]	tissue, insect [2]	reference
50	244	143	ISM	MT, *Formica*	this study
51	203	142	ISM	MT, *Locusta*	Morgan & Mordue, 1983
31	140	160	EMP	MT, *Rhodnius*	Gupta et al., 1976
26	42	40	ISM	larval MT, *Drosophila*	Wessing et al., 1987
-	161	157	EMP	MT, *Aedes*	Williams & Beyenbach, 1983
-	1037	157	ISM	MT, *Tenebrio*	O'Donnell & Machin, 1991
39	168	158	both [3]	SG, *Calliphora*	Gupta et al., 1978

[1] results obained with ion-selective microelectrodes (ISM) or electron microprobe analysis (EMP). [2] MT Malpighian tubule, SG salivary gland. [3] Cl_l with ISM, Cl_i with EMP

Second, the Cl_l found in this study (244 mmol/l) is comparable to the value found with the electron probe technique (200 mmol/l) [Van Kerkhove et al., 1989] but significantly higher than luminal free K^+ concentration measured with ion-selective electrodes in an earlier study (128 mmol/l) [Leyssens et al., in press (a).]. This indicates that some other cation, most likely Na^+, must also be actively transported in considerable quantities. Third, secretion was still sustained at 30 % of control level when all Cl^- in the bath was replaced by SO_4^{2-}, thus Cl^- is not the only anion that can be transported efficiently. Similar experiments in Malpighian tubules of *Locusta* resulted in a decrease to ± 20 % of control [Morgan & Mordue, 1981] and in *Aedes* to 0 % [Pannabecker et al., 1993]). Fourth, when bathed in control solution, the electrochemical gradient for Cl^- across the shunt was directed from the bath to the lumen, it was very small but significantly inwardly directed across the basolateral membrane and significant cell outwardly directed across the apical membrane, thus allowing passive Cl^--transport both trans- and paracellularly. This indicates that the cells maintain a driving force for Cl^- in steady state. In other words, the conductances over both the shunt and the transcellular pathway are too low to let Cl^- ions follow as fast as the electrical potential differences are built up. A thought that is supported by the high absolute values (of the order of 0.5 to 1 $k\Omega.cm^2$) found for both the shunt and apical membrane resistances [Dijkstra et al., 1994]. This then implies that it is the transport of Cl^- that is a rate limiting step in the secretion

process. The physiological role of Cl^--conductances as a possible site for regulation is then self evident. Since the shunt has been shown to be Cl^- selective [Dijkstra et al., 1994] and has a finite resistance, at least part of the Cl^- transport will be through the shunt. However, the fact that a lowering of bath K^+ from 51 to 5 mmol/l, decreasing the electrochemical driving force for Cl^- over the shunt from 29 to 9 mV, only resulted in a lowering of fluid secretion to 69% of control-value and the fact that lowering the bath Cl^- reduced the driving force for Cl^- over the shunt to a value not significantly different from 0 but secretion was still sustained at 30 % of control-value, implies a significant part of Cl^- transport may well take place through the cell. The possible cellular transport mechanisms were further investigated.

Transcellular Cl^--transport mechanisms

In general the possible Cl^- transporters are 1. Cl^- channels, 2. some form of cation/Cl^- cotransport and 3. some form of Cl^-/HCO_3^- or Cl^-/OH^--antiporter. Since upon lowering of the bath Cl^- concentration, Cl_i decreased significantly whereas both V_{bl} and K_i (the main determinant of V_{bl}, see [Leyssens et al., in press (a)]) remained constant, we can conclude Cl^- entry over the basolateral membrane is by some form of electroneutral transport. Additionally, basolateral Cl^- entry is against its electrochemical gradient both in low K^+ and in low Cl^- Ringer's solutions again suggesting the need for some form of (secondary) active entrance mechanism. This absence of a significant basolateral Cl^- conductance in control conditions would be in agreement with findings on *Locusta, Rhodnius, Drosophila* and *Aedes* [Baldrick et al., 1988;O'Donell & Maddrell, 1984;Wessing et al., 1987;Sawyer & Beyenbach, 1985] and in contrast to those on *Onymacris* and *Glossina* [Nicolson & Isaacson, 1987;Nicolson, 1993]. Upon a tenfold lowering of the bath K^+ concentration, a decrease in Cl_i was observed that was comparable to the decrease found when bath Cl^- was decreased tenfold (to 57 and 49 % of control value respectively), this would be expected if some form of KCl-cotransport were present. If some form of Cl^-/HCO_3^- or Cl^-/OH^--antiporter were present, a much smaller effect would be expected since earlier measurements from our laboratory have shown the intracellular acidification upon a tenfold decrease in bath K^+ is so small that the ratio $(OH^-)_{cell}/(OH^-)_{bath}$ decreases only with 17% [Zhang et al., in press]. Furthermore, application of 10^{-3} mol/l DIDS to the bath resulted in a significant inhibitory effect after 40 minutes of application only, implying a possible Cl^-/HCO_3^- or Cl^-/OH^--antiporter does not play a major role in secretion in the (H_2CO_3-free)-control solution. To distinguish between a KCl- and a NaK2Cl-cotransporter, the equilibrium values for Cl_i when the tubules are bathed in respectively control solution (51 mmol/l K^+, 143 mmol/l Cl^- and 62 mmol/l Na^+) or lowered K^+ solution (5 mmol/l K^+, 143 mmol/l Cl^- and 103 mmol/l Na^+) can be calculated. This under the assumption that the sum of all cellular K^+ and Na^+ will stay constant and equal K_i found in Na^+-free conditions (i.e. 123 mmol/l

[Leyssens et al., in press (a)]). Under control conditions the equilibrium value for Cl_i calculated for a KCl-cotransporter would be 71 mmol/l and for a NaK2Cl-cotransporter 177 mmol/l, both well above the Cl_i found, thus presenting a driving force for Cl^- into the cell. However, under low K^+ conditions, the calculated equilibrium Cl_i for a KCl-cotransporter would be 14 mmol/l, significantly lower (P=0.002) than the Cl_i observed, thus driving Cl^- out of the cell, whereas the equilibrium Cl_i calculated for a NaK2Cl-cotransporter under these conditions would be 55 mmol/l thus still providing a driving force for basolateral Cl^- uptake. Under these conditions the transepithelial gradient was shown to decrease from 29 to 9 mV whereas secretion only decreased to 69% of control value, thus we can assume more Cl^- is transported transcellularly for which Cl^--transport into the cell rather than out of it, is a prerequisite. Performing the same calculations with the data gathered in low Cl^--solution, we find the equlibrium Cl_i calculated for a NaK2Cl-cotransporter under these conditions would be 17 mmol/l, which is not significantly different from the 23 mmol/l reported in this study (P=0.163). The equlibrium Cl_i calculated for a KCl-cotransporter under these conditions would be 5 mmol/l which would again provide a significant (P=0.003) driving force for Cl^- out of the cell rather than in the cell. In the light of these findings and calculations, it can be concluded that in the presence of low K^+ or low Cl^- in the bath a NaK2Cl-cotransporter is the main mechanism of basolateral Cl^- uptake. Evidence was found by Leyssens (1993) that such a mechanism may indeed be functional in low K^+ concentrations in the bath: removal of Na^+ and addition of 10^{-5} mol/l bumetanide both reduced fluid secretion in low K^+-solution. This finding is of high physiological relevance since in the haemolymph the K^+ was found to be around 20 mmol/l and the Na^+ is at least 5 times more concentrated than K^+ [Van Kerkhove et al., 1989]. In 51 mmol/l K^+-solution, experiments showed that fluid secretion is still sustained when all Na^+ is replaced by N-methyl-D-glucamine (NMDG) [Leyssens, 1993] suggesting that under these conditions secretion can be sustained by a KCl-cotransporter. Which cotransporter, when both K^+ and Na^+ are present in control conditions, plays a more important role in vitro cannot be decided on the basis of the evidence presented here. The evidence presented by Leyssens (1993) would suggest the KCl-cotransporter to be the main entrance mechanism under control conditions (i.e. in 51 mmol/l K^+). That NaK2Cl-cotransport activity is a site for regulation by several (endogenous) factors amongst which ionic gradients and Cl_i is well recognized (for review:[Palfrey & O'Donnell, 1992]).

Concerning the apical exit mechanism, we can again take into account the afore mentioned three possible Cl^--transport mechanisms. However, since a large inwardly directed concentration gradient for both K^+ and Cl^- is present, possible KCl-cotransporters can be ruled out. Since the lumen of the Malpighian tubule of *Formica* has been reported to be acid as compared to the cell [Zhang et al., in press] the same applies to a form of Cl^-/HCO_3^--exchange. Thus apical Cl^- channels are the most likely pathway for all

transcellular Cl^- transport. This could explain the slight inhibition of fluid secretion found with 1 mmol/l of DIDS in the bath. However, in an earlier study where electrical potential differences and resistances were measured, luminally applied DIDS had no significant effect on these parameters [Dijkstra et al., 1994]. So far apical Cl^- channels have only been demonstrated in *Aedes* [Wright & Beyenbach, 1987]. Since across the apical membrane Cl^- is much further away from equilibrium than across the basolateral membrane (with the gradient directed towards the lumen) the apical membrane seems the site where a scarcity of Cl^- conductance is the most prominent, the rate limiting step, the most likely site of regulation.

CPT-cAMP stimulation

So far we have considered secretion under control conditions. To test whether indeed the Cl^- pathway is a site of regulation of the rate of secretion, fluid secretion stimulation with CPT-cAMP was investigated. The biphasic responses in V_{te}, Cl_i and V_{te}-$E(Cl)_{te}$ would suggest cAMP has at least two different effects. Again both the trans- and paracellular pathway have to be taken into consideration. Tight junction Cl^- conductances in goldfish intestinal mucosa have been shown to increase upon 10^{-3} mol/l 8-Br-cAMP application [Bakker & Groot, 1989]. An increase in the conductance of the Cl^- specific shunt [Dijkstra et al., 1994], would bring V_{te} closer to the diffusion potential of Cl^- over the epithelium thus depolarizing V_{te}. After 10 minutes of cAMP application, V_{te} had fallen to 20 mV and V_{te}-$E(Cl)_{te}$ had fallen to a value not significantly different from 0. This possible effect of cAMP would have to be further investigated with transepithelial resistance and biionic replacement measurements. Nevertheless, the fact that V_{te}-$E(Cl)_{te}$ had fallen markedly, suggesting a Cl^- conductance was increased somewhere, but the transepithelial resistance appeared not to change within the first 3 minutes of cAMP application in preliminary experiments, would tie in nicely with an increased Cl^--specificity of the shunt, rather than an absolute decrease of shunt or apical resistance. The biphasic effect on V_{te} and the fact that significant Cl_i changes were registered, combined with experiments published elsewhere [De Decker, 1993], where the stimulatory effect of 10^{-3} mol/l 8-Br-cAMP on fluid secretion could be completely abolished by addition of 10^{-5} mol/l bumetanide, indicate cAMP also has an effect on the transcellular Cl^- pathway. In the light of the bumetanide results, a most likely candidate for this increased electroneutral (no changes in V_{bl} were registered) basolateral Cl^- entrance would be the NaK2Cl-cotransporter. Activation of a basolateral NaK2Cl-cotransporter as a result of cAMP stimulation has been postulated in the Malpighian tubules of *Manduca sexta, Aedes aegypti* and, as a result of 5-HT stimulation, in *Rhodnius prolixus* [Audsley et al., 1993, Hegarty et al., 1991, O'Donnell & Maddrell, 1984] on the basis of bumetanide sensitivity of the stimulation and (in some cases) removal of Na^+, K^+ and Cl^- from the bath medium. In *Locusta* the Malpighian tubules have been shown to fail to respond to cAMP

stimulation in the absence of Na^+ from the bath [Morgan & Mordue, 1981]. Direct stimulation of activity of such cotransporters by cAMP has been observed before (review [Palfrey & O'Donnell, 1992]). Stimulation by cAMP has been shown to increase the Na^+/K^+-ratio in the secreted fluid from 1:2 in control conditions to 1:1 in the Malpighian tubules of the house cricket [Kim & Spring, 1992] and from 1:1 in control to 10:1 in *Aedes aegypti* [Williams & Beyenbach, 1983]. As calculated before, the equilibrium Cl_i for a NaK2Cl-cotransporter under control conditions would be 177 mmol/l as opposed to the equilibrium Cl_i of 71 mmol/l calculated for a KCl-cotransporter. Thus, an increased importance of Cl^- uptake via a NaK2Cl-cotransporter as a result of cAMP stimulation, would indeed increase the basal Cl^- entrance and thus increase Cl_i as is observed in the second phase of cAMP-addition. V_l and the luminal over intracellular Cl^--gradient were estimated from measurements performed on different tubules, so the figures have to be interpreted with caution. Nevertheless, it is clear that the electrochemical gradient for Cl^- across the apical membrane becomes very small, Cl^- is close to equilibrium. This might mean an increase in Cl^--conductance occurred in the presence of cAMP. This has to be further investigated. Preliminary results did however not show a clear reduction in transepithelial resistance (the voltage divider ratio could only be measured in two tubules). If the increase in Cl^--conductance is such that Cl^- is passively distributed over the apical membrane, but the conductance is not large enough for the small driving force to account for all Cl^--extrusion, other luminal mechanisms have to be present responsible for an increased transfer of Cl^- through the cell. Luminal pH during cAMP application has not yet been measured. Under control circumstances the lumen is acid with respect to the cell [Zhang et al., in press] as is indeed neccesary for the secondary active K^+-extrusion via an electroneutral H^+/K^+-antiporter as described by Leyssens et al. (in press (b)). All other forms of secondary active Cl^- transport that have been described in the literature so far, as far as we are aware, would cause Cl^- to enter the cell rather than drive it out (luminally high K^+ and Na^+ concentration). Then some new form of secondary active transport, or even a "Cl-pump" should be hypothesized in order to explain the results.

In conclusion, in this study we have investigated the Cl^- pathway for the fluid secretion of the Malpighian tubule of *Formica*. That at least a part of the transported Cl^- is transported passively over the shunt has already been demonstrated in earlier work [Dijkstra et al., 1994]. The evidence presented in this study suggests the existence of a NaK2Cl-cotransporter both at low basolateral K^+ (the more physiological circumstance) and at low basolateral Cl^- concentrations. Apically Cl^- exit most probably occurs via Cl^- conductive channels. Cl^- transport has been postulated to be the rate limiting step in the KCl-transport and thus the most likely site of regulation of transport. Stimulation of fluid secretion by CPT-cAMP has been shown to be due to more than one effect of cAMP. Circumstantial evidence points to an increase in the Cl^- specificity of the shunt in the first phase of

cAMP-stimulation, though further experiments have to be undertaken. This appears to be followed by a gradual increase in basolateral NaK2Cl-cotransporter activity. The apical Cl^- exit pathway and the role of possible Cl^- channels or carriers in this has to be further investigated.

Summary

The main purpose of this electrophysiological study was to learn more about the transport mechanism of salt in the Malpighian tubules of the ant. This salt, secreted into the lumen of the tubules, is assumed to enable the water transport over the Malpighian tubules by creating local osmotic gradients thus forming the primary urine [Phillips, 1981]. In most insects secretion is driven by the active transport of K^+, whereby the active step is the extrusion over the apical membrane. The work presented in this thesis has concentrated on four sub-domains of the total transport question:

1. electrophysiological characterization of the Malpighian tubule at the epithelial and cellular level;
2. characterization of the luminal pump;
3. investigation of the Cl^--pathway;
4. some tentative investigation of the regulation of secretion by the second messenger cAMP.

Two techniques were used in approaching these questions, firstly the luminal perfusion technique of isolated tubules allowing simultaneous measurement of the transepithelial and basolateral potential differences, the transepithelial resistance and the ratio of the apical over basolateral resistance, i.e. the voltage divider ratio. This method has the additional advantage that the intraluminal environment is controlled. Secondly, non-perfused tubules were impaled with double barrelled Cl^--selective micro-electrodes allowing simultaneous measurement of electrical potentials and electrochemical gradients for Cl^-. The results have been presented schematically in fig.I.

Electrophysiological characterization of the Malpighian tubule at the epithelial and cellular level

The luminal perfusion technique of isolated tubules was optimized to meet the demands posed by the Malpighian tubules of the ant. Estimates for the individual membrane resistances were derived. This led to the conclusion that the luminal membrane was the major determinant of the transcellular resistance: $R_l/R_{bl} = 51$. Further it was found that the resistance over the paracellular pathway is about 4 times as high as the transcellular resistance and thus that the Malpighian tubule is a rather tight epithelium, as opposed to prior assumptions. By using Cl^--selective microelectrodes in non-perfused tubules, intracellular and intraluminal Cl^--concentrations (Cl_i and Cl_l) were established. In control conditions Cl_l was found to be significantly higher than the luminal K^+-concentration established in an earlier study [Leyssens et al., in press (a)], thus some other cation, most likely Na^+, must also be transported in significant amounts.

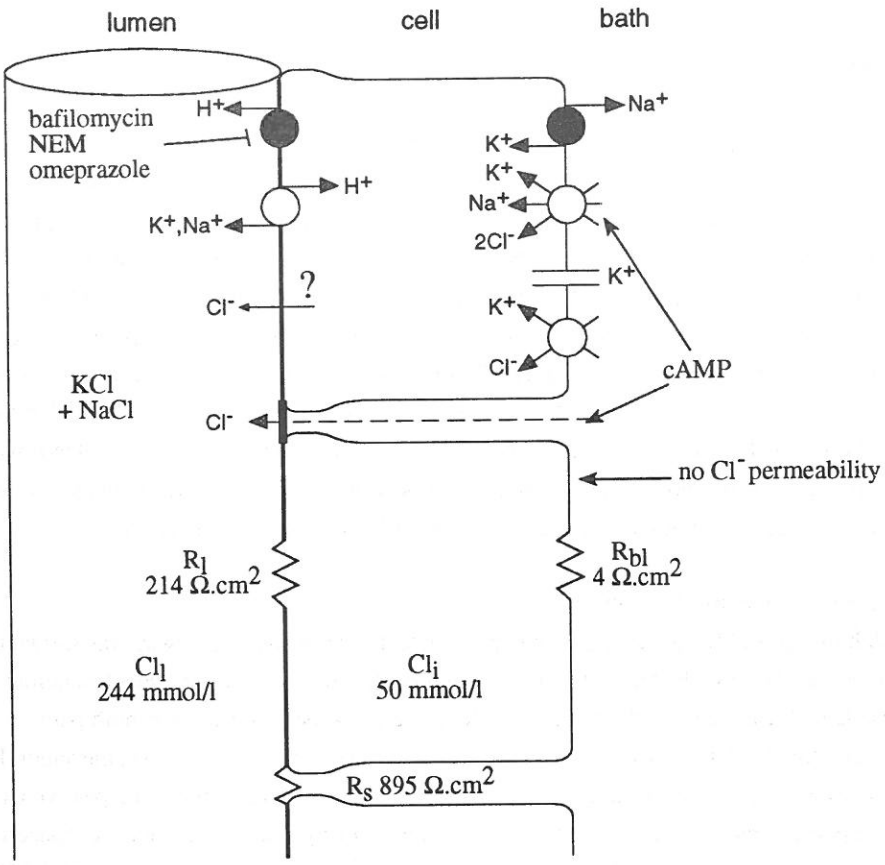


Fig.I Schematic summary of the results presented in this thesis. The Cl_i and Cl_l were measured in tubules bathed in 51 mmol/l K^+ and 143 mmol/l Cl^--solution. In short the following was established:

* anion specificity of the shunt
* presence of an apical H^+-pump, most likely of the vacuolar type
* $R_s > R_{cell}$, i.e. the epithelium is rather tight
* estimations of the individual resistances
* indications for the presence of a basolateral Na^+/K^+-ATPase
* luminal membrane generally less permeable to a variety of inhibitors than the basolateral membrane
* DNP has an additional protonophoric effect on the cell membrane
* considerable quantities of NaCl also secreted
* other anions than Cl^- (specifically SO_4^{2-}) also transported efficiently
* Cl^--transport in a continuous state of non-equilibrium -> a rate-limiting step in secretion
* a significant basolateral Cl^--conductance is absent
* basolaterally a NaK2Cl-cotransporter is active in low bath Cl^- and low K^+ conditions
* CPT-cAMP stimulation is biphasic. Possibly an increase in the Cl^--specificity of the shunt and a stimulation of the basolateral NaK2Cl-cotransporter

Characterization of the luminal pump

A dose-response curve relating both the calculated short circuit current I_{SC} (and therewith secretion) and the potential difference over the luminal membrane (V_l) to acidification of the lumen, demonstrated a reversible half-maximal inhibition at pH 4.5, indicating the active apical transport mechanism was an electrogenic proton pump. The K^+-extrusion is mediated by an electroneutral H^+/K^+-antiporter [Leyssens et al., in press (b)]. The proton pump was shown to be sensitive to bafilomycin A_1 and NEM (N-ethylmaleimide), two inhibitors of the V-type ATPase, and insensitive to high concentrations of vanadate, a P-type ATPase blocker. The inhibitors omeprazole and Schering compound 28080, supposedly specific for the gastric H^+/K^+-ATPase, also exhibited inhibitory effects on I_{SC}, but more and more evidence is emerging that these inhibitors also affect V-type ATPases [Mattsson et al., 1991; Graber & Devine, 1993]. In an earlier study DNP had been used to learn more about the pump [Leyssens et al., in press (b)]. In this study the protonophoric effect of DNP on the cell membranes was further investigated.

Investigation of the Cl^--pathway

With the aid of biionic replacement experiments the transcellular pathway was shown to be anion specific. Further, in control solution the direction of the electrochemical gradients for Cl^- across all barriers was found to allow passive transport both para- and transcellularly. Thus a portion of the transported Cl^- will certainly move via the shunt. In control conditions the Cl^--distribution was never near equilibrium, indicating passive Cl^--transport is a rate-limiting step, and therefore a most likely site of regulation. As concerns the transcellular pathway: by calculating driving forces for Cl^--movement, a NaK2Cl-cotransporter has been shown to be responsible for basolateral Cl^--entry both in low K^+-(the more physiological situation) and in low Cl^--solution. The presence of a KCl-cotransporter in control conditions has already been demonstrated in earlier work [Leyssens, 1993]. With the measurement of electrochemical Cl^--gradients, the absence of a basolateral Cl^--conductance both in control and in cAMP-stimulated conditions was established. The apical exit mechanism for Cl^- is less clear. The most likely candidate would be Cl^--channels but their presence could not be positively demonstrated. Luminally perfused blockers of some types of Cl^--channels, DIDS and NPPB, had no effect on electrical potential differences or resistances in control conditions. Incubation with a high concentration of DIDS did cause a small but significant inhibition of fluid secretion after 40 minutes.

Some tentative investigation of the regulation of secretion by the second messenger cAMP

If Cl^--transport over the shunt and the apical membrane were indeed a site of regulation, then it was hoped addition of a stimulatory dose of cAMP would enable us to demonstrate apical Cl^--channels. CPT-cAMP (a cell membrane permeable form of cAMP) had a

biphasic effect on both Cl_i and V_{te}, no effect on V_{bl} and caused a steady increase in Cl_l. The results were interpreted as an increase in the Cl^--selectivity of the shunt, followed by a stimulation of the basal NaK2Cl-cotransporter. The apical exit mechanism for Cl^- during stimulation requires further research.

Samenvatting

Deze elektrofysiologische studie was verricht om meer te weten te komen over het transport mechanisme van zouten in de buisjes van Malpighi van mieren. Het wordt algemeen verondersteld dat dit zout, in het lumen afgescheiden door de buisjes van Malpighi, het watertransport over de buisjes van Malpighi mogelijk maakt door de vorming van plaatselijke osmotische gradiënten waardoor het water volgt en aldus de primaire urine wordt gevormd [Philips, 1981]. In de meeste insecten is het actieve transport van K^+ de drijvende kracht voor de secretie, waarbij de actieve stap de extrusie over het apikale membraan is. Het hier voorgestelde werk is geconcentreerd op vier deelvragen van het totale transport probleem:

1. het elektrofysiologisch karakteriseren van het buisje van Malpighi op een epitheliaal en cellulair niveau;
2. het karakteriseren van de luminale pomp;
3. onderzoek naar de weg die het Cl^- volgt;
4. verkennend onderzoek naar de regulatie van secretie onder invloed van de intracellulaire boodschapper cAMP.

Twee technieken zijn gebruikt. Ten eerste de luminale perfusie techniek van geïsoleerde kanaaltjes, waarbij men tegelijkertijd de transepitheliale en basolaterale potentialen kan meten, de transepitheliale weerstand en de verhouding van de luminale en basolaterale membraanweerstanden. Deze techniek heeft voorts het voordeel dat men het intraluminale milieu ook beheerst. Ten tweede is geprikt met dubbellopige Cl^--selektieve mikro-elektroden. Hierbij meet men tegelijkertijd de elektrische potentiaal en de elektrochemische gradiënt voor Cl^-.
De resultaten zijn schematisch verwerkt in fig.I.

Het elektrofysiologisch karakteriseren van het buisje van Malpighi op een epitheliaal en cellulair niveau
De luminale perfusie techniek van geïsoleerde kanaaltjes werd aangepast om te voldoen aan de specifieke eisen verbonden aan het buisje van Malpighi van de mier. Schattingen van de individuele membraanweerstanden werden uit metingen afgeleid. Dit leidde tot de conclusie dat het luminale membraan de bepalende factor is van de transcellulaire weerstand: $R_l/R_{bl} = 51$. Bovendien werd aangetoond dat de weerstand van de paracellulaire weg ongeveer 4 keer zo groot is als de transcellulaire weerstand. Dientengevolge is het buisje van Malpighi een nogal 'dicht' epitheel in tegenstelling tot wat voorheen aangenomen werd. Door het gebruik van Cl^--gevoelige micro-elektroden op niet-geperfuseerde buisjes, werden intracellulaire en intraluminale Cl^--concentraties gemeten. In controle omstandigheden bleek de intraluminale Cl^--concentratie significant

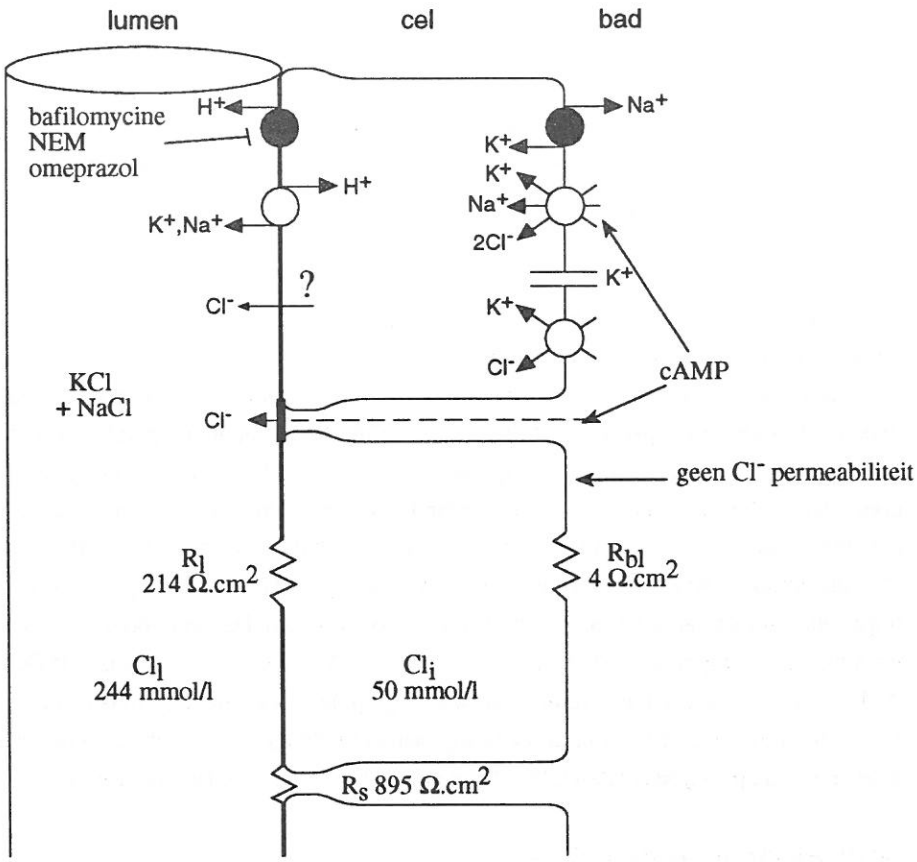


Fig.I Schematische samenvatting van de resultaten die in dit proefschrift gepresenteerd zijn. Cl_i en Cl_l werden gemeten in 51 mmol/ K^+ en 143 mmol/l Cl^--oplossingen. In het kort werd aangetoond:

* anionen specificiteit van de shunt
* aanwezigheid van een apikale H^+-pomp, waarschijnlijk van het vakuolaire type
* $R_s > R_{cel}$, i.e. het epitheel is nogal 'dicht'
* schattingen van de weerstanden: R_{bl} 4, R_l 214 en R_s 895 $\Omega.cm^2$
* aanwijzingen voor de aanwezigheid van een basolaterale Na^+/K^+-ATPase
* luminale membraan is in het algemeen veel minder toegankelijk en permeabel voor verschillende inhibitoren dan de basolaterale
* DNP (dinitrofenol) heeft extra protonofoor effekt op de celmembraan
* ook beduidende hoeveelheden NaCl gesekreteerd
* andere anionen dan Cl^- (met name SO_4^{2-}) ook efficiënt getransporteerd
* Cl^--transport continu niet in evenwichtstoestand -> een snelheidsbepalende stap
* een significante basolaterale Cl^--conductantie is afwezig
* basolateraal is een NaK2Cl-cotransporter actief in laag Cl^-- en laag K^+-oplossingen

* CPT-cAMP stimuleert bifasisch. Mogelijk via een verhoging van de Cl^--specificiteit van de shunt en een stimulatie van de basolaterale NaK2Cl-cotransporter

hoger te zijn dan de, in een eerdere studie gemeten [Leyssens et al., in press (a)], K^+-concentratie. Bijgevolg moet een ander kation, hoogst waarschijnlijk Na^+, ook in significante hoeveelheden getransporteerd worden.

Het karakteriseren van de luminale pomp

Een dosis-respons curve, die de relatie legde tussen enerzijds de berekende kortsluitstroom (en hiermee secretie) en het potentiaalverschil over de luminale membraan en anderzijds de mate van aanzuring van het lumen, vertoonde een reversibele half maximale inhibitie bij pH 4.5. Dit maakte duidelijk dat het actieve apikale transport mechanisme een elektrogene protonenpomp is. De K^+-extrusie wordt tot stand gebracht door een elektroneutrale H^+/K^+-antiporter [Leyssens et al., in press (b)]. Er werd gevonden dat de protonenpomp gevoelig was voor bafilomycine A_1 en NEM (N-ethylmaleimide), twee specifieke inhibitoren van V-type ATPasen, en ongevoelig voor hoge concentraties vanadaat, een inhibitor van P-type ATPasen. De inhibitoren omeprazol en Schering Compound 28080, naar verluidt specifiek voor de gastrische H^+/K^+-ATPase, vertoonden ook een remmende werking op de berekende kortsluitstroom. De laatste tijd komt echter meer en meer bewijs aan het licht dat deze inhibitoren ook een effect hebben op V-type ATPasen [Mattsson et al., 1991; Graber & Devine, 1993].

Onderzoek naar de weg die het Cl^- volgt

Met behulp van biïonische vervangings-experimenten werd aangetoond dat de shunt anionen specifiek is. Bovendien werd gevonden dat in controle omstandigheden de richting van de elektrochemische gradiënten over alle barrières dusdanig gericht was dat passief transport zowel para- als transcellulair mogelijk is. Dientengevolge zal een deel van het getransporteerde Cl^- via de shunt gaan. In controle omstandigheden was de Cl^--verdeling nooit in evenwicht wat erop wijst dat passief Cl^--transport een snelheidsbepalende stap is in het secretie proces en dus een zeer waarschijnlijk aangrijpspunt voor regulatie mechanismen. Met betrekking tot de transcellulaire weg: door het berekenen van de drijfkrachten voor Cl^--transport kon worden vastgesteld dat in laag K^+-houdende oplossingen (de meer fysiologische situatie) en in laag Cl^--houdende oplossingen, een NaK2Cl-cotransporter verantwoordelijk is voor basolaterale Cl^--opname in de cel. De aanwezigheid van een KCl-cotransporter in controle omstandigheden is in eerder werk al aangetoond [Leyssens et al., 1993]. Door het meten van elektrochemische gradiënten voor Cl^- is, zowel in controle als in cAMP gestimuleerde omstandigheden, de afwezigheid van een relevante basolaterale Cl^--conductantie vastgesteld. Hoe Cl^- apikaal de cel verlaat is minder duidelijk. De meest waarschijnlijke weg is via Cl^--kanalen, maar

hun aanwezigheid kon niet eenduidig worden vastgesteld. Luminaal toegevoegde inhibitoren van sommige soorten Cl^--kanalen, DIDS en NPPB, hadden geen effect op de elektrische potentiaalverschillen of de gemeten weerstanden in controle omstandigheden. Een universele Cl^--kanaal inhibitor bestaat (nog) niet. Wel had het aan de badvloeistof toedienen van een hoge concentratie DIDS, na 40 minuten een kleine maar significante inhibitie van de secretie tot gevolg.

Verkennend onderzoek naar de regulatie van secretie onder invloed van de intracellulaire boodschapper cAMP

Indien het Cl^--transport via de shunt en de luminale membraan inderdaad een aangrijpspunt voor regulatie zijn, dan werd gehoopt dat door het toevoegen van een stimulerende dosis cAMP, apikale Cl^--kanalen konden worden aangetoond. CPT-cAMP (een celmembraan permeabele vorm van cAMP) had een bifasisch effect op zowel de intracellulaire Cl^--concentratie als het transepitheliale potentiaalverschil. Er was geen effect op het basolaterale potentiaalverschil en de intraluminale Cl^--concentratie vertoonde een gestage stijging. Deze resultaten werden uitgelegd als een verhoging van de Cl^--specificiteit van de shunt, gevolg door een stimulering van de basolaterale NaK2Cl-cotransporter. De apikale weg voor Cl^- om de cel weer te verlaten behoeft nog verder onderzoek.

Appendix A

List of abbreviations used

CPT-cAMP	8-(4-chlorophenylthio)-adenosine 3'-5'-cyclic monophosphate
DIDS	4,4'-diisothiocyano-2,2'-stilbenedisulfonate
DMSO	dimethylsulfoxide
DNP	2,4-dinitrophenol
$E(Cl)_x$	Nernst potential for Cl^- over (x=bl) the basolateral membrane; (x=l) the apical or luminal membrane; (x=te) the epithelium
EMF	electromotive force
I_0	injected current [nA]
I_{sc}	equivalent short circuit current [$\mu A/cm^2$]
λ	length constant [μm]
MT	Malpighian tubule(s)
NEM	N-ethylmaleimide
NPPB	5-nitro-2-(3-phenylpropylamino)-benzoate
P_x	permeability of ion x
R_{bl}	electrical resistance of the basolateral membrane [Ω]
R_l	electrical resistance of the luminal or apical membrane [Ω]
R_s	electrical resistance of the shunt [Ω]
R_{te}	electrical resistance of the epithelium [$\Omega.cm^2$]
R_{tex}	R_{te} calculated according to method x (see derivation cable equations)
S28080	Schering Compound 28080
V_{bl}	electrical potential difference measured over the basolateral membrane [mV]
VDR	voltage divider ratio, i.e. R_l/R_{bl}
V_l	electrical potential difference measured over the luminal or apical membrane [mV]
V_{te}	electrical potential difference measured over the epithelium [mV]
ΔV_{te}	the voltage deflections caused by the current injection in the lumen, measured at position x from the tip of the perfusion pipette [mV]
ΔV_{te}^0	the voltage deflections caused by the current injection in the lumen, measured at the perfusion end [mV]
ΔV_{te}^L	idem measured at the collection end [mV]

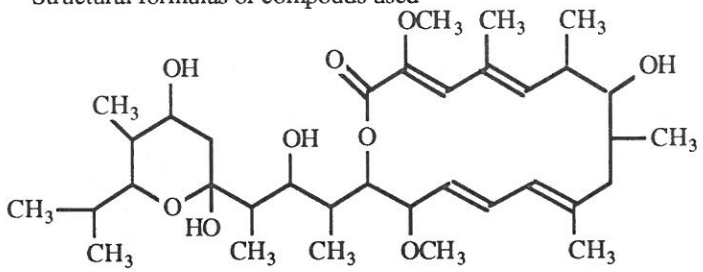
Appendix B.

English and dutch names of mentioned insects

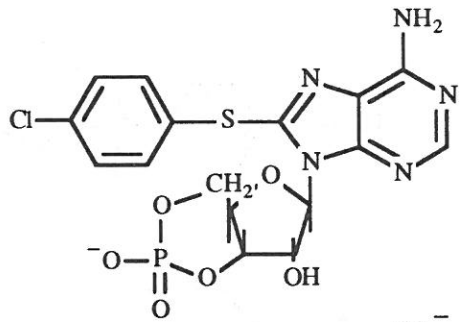
Latin	English	Dutch
Acheta domesticus	house cricket	huissprinkhaan
Aedes aegypti	yellow fever mosquito	gele koorts mug
Calliphora	blowfly	aasvlieg
Drosophila hydei	fruitfly	fruitvlieg
Formica polyctena	small forest ant	kleine bosmier
Formica rufa	red forest ant	rode bosmier
Manduca Sexta	tobacco hornworm	tabaksrups
Glossina	tsetse fly	tsetse vlieg
Locusta migratoria	locust	treksprinkhaan
Onymacris	desert beetle	woestijnkever
Rhodnius prolixus	blood sucking bug	roofwants
Tenebrio molitor	mealworm	meeltor

Appendix C. Structural formulas of compouds used

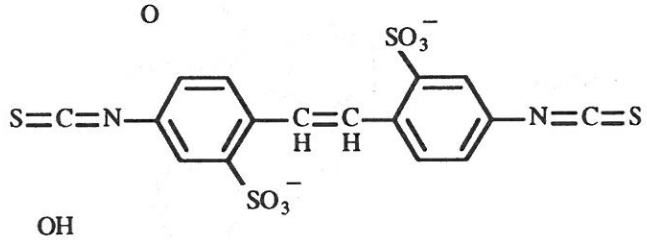
1. bafilomycin A_1



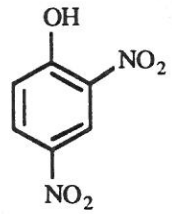
2. CPT-cAMP



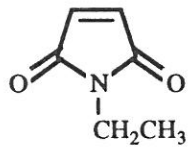
3. DIDS



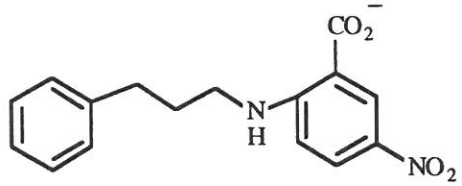
4. DNP



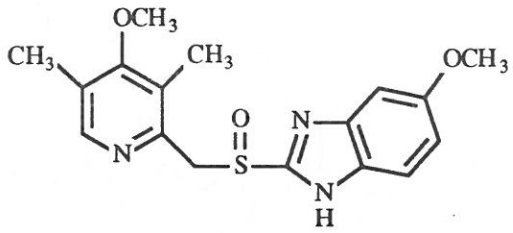
5. NEM



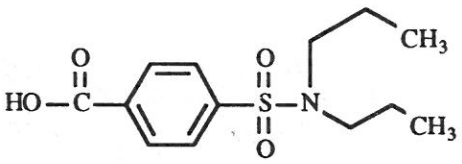
6. NPPB



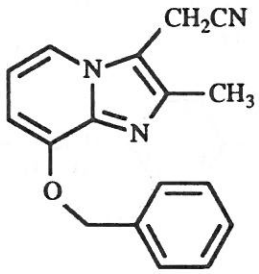
7. omeprazole



8. probenecid



9. S28080



Literature

Al-Awqati Q, Mueller A, Steinmetz PR (1977) Transport of H^+ against electrochemical gradients in turtle urinary bladder. Am J Physiol 233:F502-F508

Al-Awqati (1986) Proton-translocating ATPases. Ann Rev Cell Biol 2:179-199

Altamiro AA, Breitwieser GE, Russell JM (1988) Vanadate and fluoride effects on Na^+-K^+-Cl^- cotransport in squid axon. Am J Physiol 254:C582-C586

Alvarez-Leefmans FJ, Giraldez F, Russell JM (1990) Methods for measuring chloride transport across nerve, muscle and glial cells. In Alvarez-Leefmans FJ, Russell JM (eds): Chloride channels and carriers in nerve, muscle and glial cells. Plenum Press, New York, pp. 3-66

Anstee JH, Bowler K (1979) Ouabain-sensitivity of insect epithelial tissues. Comp Biochem Physiol 62A:763-769

Audsley N, Coast GM, Schooley DA (1993) The effects of *Manduca sexta* diuretic hormone on fluid transport by the Malpighian tubules and cryptonephric complex of *Manduca sexta* . J Exp Biol 178:231-243

Bakker R, Groot JA (1989) Further evidence for the regulation of the tight junction ion selectivity by cAMP in goldfish intestinal mucosa. J Membr Biol 111:25-35

Baldrick P, Hyde D, Anstee JH (1988) Microelectrode studies on Malpighian tubule cells of *Locusta migratoria* : effects of external ions and inhibitors. J Insect Physiol 34:963-975

Beauwens R, Al-Awqati Q (1976) Active H^+-transport in the turtle urinary bladder. J Gen Physiol 68:421-439

Beauwens R, Crabbé J, Rentmeesters M (1981) Effects of vanadate on the functional properties of the isolated toad bladder. J Physiol 310:293-305

Bernard F (1968) Les fourmis (*Hymenoptera Formicidae*). Masson et cie. Éditeurs Paris

Berridge MJ, Lindley BD, Prince WT (1975) Membrane permeability changes during stimulation of isolated salivary glands of *Calliphora* by 5-HT. J Physiol Lond 244:549-567

Bertram G, Schleithoff L, Zimmermann P, Wessing A (1991) Bafilomycin A_1 is a potent inhibitor of urine formation by Malpighian tubules of *Drosophila hydei* : is a vacuolar-type ATPase involved in ion and fluid secretion? J Insect Physiol 37(3):201-209

Bowman EJ, Siebers A, Altendorf K (1988) Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells. Proc Natl Acad Sci USA 85:7972-7976

Bresler VM, Belyaeva EA, Mozhayeva MG (1990) A comparative study on the system of active transport of organic acids in Malpighian tubules of insects. J Insect Physiol 36: 259-270

Burg MB (1972) Perfusion of isolated renal tubules. Yale J Biol Med 45:321-326

Cantley LC, Resh MD, Guidoth G (1978) Vanadate inhibits the red cell (Na^+,K^+)ATPase from the cytoplasmic side. Nature London 272:552-554

Chasteen ND (1983) The biochemistry of vanadium. Structure and Bonding 53:105-138

Chatterjee D, Chakraborty M, Leit M, Neff L, Jamsakellolumpu S, Fuchs R, Bartkiewicz, Hernando N, Baron R (1992) The osteoclast proton pump differs in its pharmacology and catalytic subunits from other vacuolar H^+-ATPases. J Exp Biol 172:193-204

Cherrett JM (1982) The economic importance of leafcutter ants. In Breeds MD, Michener CD, Evans HE (eds): The biology of social insects (Proceedings of the Ninth Congress of the International Union for the study of Social Insects, Boulder, Colorado, Westview Press, Boulder, pp. 114-118

De Decker N, Van Kerkhove E, Steels P (1991) Effects of second messengers on transport parameters of isolated Malpighian tubules of *Formica*. Arch Int Physiol Biochim 99(1):P9

De Decker N, Steels P, Van Kerkhove E (1993) Possible signal transducing mechanisms in the regulation of fluid secretion of Malpighian tubules (*Formica*). Arch Int Physiol Biochim 101(2):P2

De Decker N (1993) Regulation of fluid secretion in Malpighian tubules of *Formica polyctena* by exo- and endogenous factors. Ph D-thesis, dept of physiology LUC, Diepenbeek, Belgium

Dijkstra S, Lohrmann E, Steels P, Greger R (1994) Electrical properties of the isolated, in vitro perfused Malpighian tubule of the ant, the Cl^- pathway. Cell Physiol Biochem 4:19-30

Dijkstra S, Lohrmann E, Van Kerkhove E, Greger R (in press) Characteristics of the luminal proton pump in Malpighian tubules of the ant. Renal Physiol Biochem

Dröse S, Bindseil KU, Bowman EJ, Siebers A, Zeeck A, Altendorf K (1993) Inhibitory effect of modified bafilomycins and concanamycins on P- and V-type adenosinetriphosphates. Biochem 32:3902-3906

Edward RM, Grantham JJ (1983) Effect of vanadate on fluid absorption and PAH secretion in isolated proximal tubules. Am J Physiol 244:F367-F375

Forgac M (1989) Stucture and function of vacuolar class of ATP-driven proton pumps. Physiol Rev 69:765-796

Froissart M, Borensztein P, Houillier P, Leviel F, Poggioli J, Marty E, Bichara M, Paillard M (1992) Plasma membrane Na^+-H^+ antiporter and H^+-ATPase in the medullary thick ascending limb of rat kidney. Am J Physiol 262:C963-C970

Garayoa M, Villaro AC, Montuenga L, Sesma P (1992) Malpighian tubules of *Formica polyctena* (Hymenoptera): light and electron microscopic study. J Morphol 214:159-171

Gluck S (1992) V-ATPases of the plasma membrane. J Exp Biol 172:29-37

Godfraind JM, Kawamura H, Krnjevic K, Pumain R (1971) Actions of dinitrophenol and some other metabolic inhibitors on cortical neurons. J Physiol 215:199-222

Gößwald K (1989) Die Waldameise. Vol 1, Aula-Verlag, Wiesbaden

Graber ML, Devine P (1993) Omeprazole and SCH 28080 inhibit acid secretion by the turtle urinary bladder. Renal Physiol Biochem 16:257-267

Greger R, Hampel W (1981) A modified system for in vitro perfusion of isolated renal tubules. Pflügers Arch 389:175-176

Gupta B, Berridge MJ, Hall TA, Moreton RB (1978) Electron microprobe and ion-selective micro-electrode studies of fluid secretion in the salivary glands of *Calliphora* . J Exp Biol 72:261-284

Gupta B, Hall TA, Maddrell SHP, Moreton R (1976) Distribution of ions in a fluid-transporting epithelium determined by electron-probe X-ray microanalysis. Nature 264:284-287

Hanada H, Moriyama Y, Maeda M, Futai M (1990) Kinetic studies of chromaffin granule H^+-ATPase and effects of bafilomycin. Biochem Biophys Res Commun 170(2):873-878

Harvey WR, Cioffi M, Wolfersberger MG (1983) Chemiosmotic potassium ion pump of insect epithelia. Am J Physiol 244: R163-R175

Hegarty JL, Zhang B, Pannabecker DH, Petzel DH, Baustian MD, Beyenbach KW (1991) Dibutyryl cAMP activates bumetanide-sensitive electrolyte transport in Malpighian tubules. Am J Physiol 261:C521-C529

Hölldobler B, Wilson EO (1990) The Ants. Springer-Verlag Berlin Heidelberg

Kim IS, Spring JH (1992) Excretion in the house cricket (*Acheta domesticus*): relative contribution of distal and mid-tubule to diuresis. J Insect Physiol 38:373-381

Kondo Y, Bührer T, Seiler K, Frömter E, Simon W (1989) A new double-barrelled, ionophore based microelectrode for chloride ions. Pflügers Arch 414:663-668

Langer P, Frömter E (1985) Partial inhibition of tight junction conductance of *Necturus* gallbladder epithelium by barium. Plügers Arch 405:R30

Leyssens A, Steels P, Lohrmann E, Weltens R, Van Kerkhove E (1992) Intrinsic regulation of K^+ transport in Malpighian tubules (*Formica*): electrophysiological events. J Insect Physiol 38:431-446

Leyssens A (1993) Cellular mechanisms of K^+ transport in Malpighian tubules of *Formica* . Ph D-thesis, dept of physiology LUC, Diepenbeek, Belgium

Leyssens A, Van Kerkhove E, Zhang SL, Weltens R, Steels P (in press (a)) Measurement of intracellular and luminal K^+ concentrations in a Malpighian tubule (*Formica*). Estimate of basal and luminal electrochemical K^+ gradients. J Insect Physiol

Leyssens A, Zhang SL, Van Kerkhove E, Steels P (in press (b)) 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. J Insect Physiol

Löhden-Bendinger U, Bakker-Grunwald T (1990) Evidence for a vacuolar-type proton ATPase in *Entamoeba histolytica* . Z Naturf 45c:229-232

Loomis WF, Lippmann F (1948) Reversible inhibition of the coupling between phosphorylation and oxidation. J Biol Chem 172: 807-808

Maddrell SHP (1977) Insect Malpighian tubules. In Gupta BL, Moreton RB, Oschman JL, Wall BL (eds): Transport of Ions and Water in Animals. New York, Academic, pp 541-569

Maddrell SHP, Overton JA (1988) Stimulation of sodium transport and fluid secretion by ouabain in an insect Malpighian tubule. J Exp Biol 137:265-276

Mattsson JP, Vaananen K, Wallmark B, Lorentzon P (1991) Omeprazole and bafilomycin, two proton pump inhibitors: differentiation of their effects on gastric, kidney and bone H^+-translocating ATPases. Biochim Biophys Acta 1065(2):261-268

Maunsbach AB, Christensen EI (1992) Functional ultrastructure of the proximal tubule. Handbook of physiology, section 8, Renal physiology vol I. Windhager EE (ed) Oxford University Press

McLaughlin SGA, Dilger JP (1980) Transport of protons across membranes by weak acids. Physiol Rev 60(3):825-863

Mitchell P (1961) Coupling of phosphorylation to electron and hydrogen transfer by a chemiosmotic type of mechanism. Nature London 191:144-148

Mitchell P, Moyle J (1969) Estimation of membrane potential and pH difference across the cristae membrane of rat liver mitochondria. Eur J Biochem 7:471-484

Morgan PJ, Mordue W (1981) Stimulated fluid secretion is sodium dependent in the Malpighian tubules of *Locusta migratoria* . J Insect Physiol 27:271-279

Morgan PJ, Mordue W (1983) Electrochemical gradients across *Locusta* Malpighian tubules. J Comp Physiol 151:175-183

Moriyama Y, Nelson N (1989) H^+-translocating ATPases in Golgi Apparatus. J Biol Chem 264:18,445-18,450

Motais R, Sola F, Cousin JL (1978) Uncouplers of oxidative phosphorylation, a structure-activity study of their inhibitory effect on passive chloride permeability. Biochim et Biophys Acta 510:20-207

Nicolson S (1993) The ionic basis of fluid secretion in insect Malpighian tubules: advances in the last ten years. J Insect Physiol, 39:451-458

Nicolson SW, Isaacson LC (1987) Transepithelial and intracellular potentials in isolated Malpighian tubules of tenebrionid beetle. Am J Physiol 252:F645-F653

O'Donnell MJ, Maddrell SHP (1984) Secretion by the Malpighian tubules of *Rhodnius proxilus* Stal: electrical events. J Exp Biol 110:275-290

O'Donnell MJ, Machin J (1991) Ion activities and electrochemical gradients in the mealworm rectal complex. J Exp Biol 155: 375-402

Padan E, Zilberstein D, Rottenberg H (1976) The proton electrochemical gradient in *Escherichia coli* cells. Eur J Biochem 63:533-541

Palfrey HC, O'Donnell ME (1992) Characteristics and regulation of the Na/K/2Cl cotransporter. Cell Physiol Biochem 2:293-307

Pannabecker T L, Aneshansley DJ, Beyenbach KW (1992) Unique physiological effects of dinitrophenol in Malpighian tubules. Am J Physiol 263:R609-R614

Pannabecker TL, Hayes TK, Beyenbach KW (1993) Regulation of epithelial shunt conductance by the peptide leucokinin. J Membrane Biol 132:63-76

Pannabecker TL, Beyenbach KW (1993) Time-dependent mechanisms of action of bafilomycin in Malpighian tubules. FASEB J 7:A580

Phillips JH, Allison YP (1978) Proton translocation by the bovine chromaffin granule membrane. Biochem J 170: 661-672

Phillips (1981) Comparative physiology of insect renal function. Am J Physiol 241:R241-R257

Ramsay JA (1953) Active transport of potassium by the Malpighian tubules of insects. J Exp Biol 30:358-369

Rottenberg H, Grunwald T, Avron M (1972) Determination of ΔpH in chloroplasts. Eur J Biochem 25:54-63

Sackin H (1987) Electrophysiology and transport in the isolated perfused salamander proximal tubule. Ph D-thesis, Yale University

Sawyer DB, Beyenbach KW (1985) Dibutyryl-cAMP increases basolateral sodium conductance of mosquito Malpighian tubules. Am J Physiol 248:R339-345

Schwartz A, Adams RJ, Grupp I, Holroyde MJ, Millard RW, Solaro RJ, Wallick ET (1980) Effects of vanadate on myocardial function. Basic Res Cardiol 75:441-451

Schweikl H, Klein U, Schindlbeck M, Wieczorek H (1989) A vacuolar-type ATPase, partially purified from potassium transporting plasma membranes of tobacco hornworm midgut. J Biol Chem 264 no19:11136-11142

Slayman CL (1985) Plasma membrane proton pumps in plants and fungi. Bioscience 35:34-37

Swallow CJ, Grinstein S, Rotstein OD (1990) A vacuolar type H^+-ATPase regulates cytoplasmic pH in murine macrophages. J Biol Chem 13:7645-7654

Thom M, Komor E (1985) Electrogenic proton translocation by the ATPase of sugarcane vacuoles. Plant Physiol 77:329-334

Thomas RC (1978) Ion-sensitive intracellular microelectrodes. How to make and use them. Treherne JE, Rubery PH (eds) Academic Press Inc London LTD

Tokuda H, Kaback HR (1977) Sodium-dependent methyl 1-thio-ß-D-galactopyranoside transport in membrane vesicles isolated from *Salmonella typhimurium* . Biochem 16:2130-2136

Van Dyke RW (1988) Proton pump-generated electrochemical gradients in rat liver multivesicular bodies. J Biol Chem 263(6):2603-2611

Van Kerkhove E, Weltens R, Rionel N, De Decker N (1989) Haemolymph composition of *Formica (Hymenoptera)* and urine formation by the short isolated Malpighian tubules: electrochemical gradients for ion transport. J Insect Physiol 35:991-1003

Verhulst H, Van Kerkhove E, De Decker N (1988) KCl secretion by Malpighian tubules of *Formica* : evidence for the presence of a KCl cotransport system. Arch Int Physiol Biochim 96(2), P8

Walderhaug MO, Post RL, Saccomani G, Leonard RT, Briskin DP (1985) Structural relatedness of three ion-transport adenosine triphosphates around their active sites of phosphorylation. J Biol Chem 260:3852-3859

Wallmark B, Briving C, Fryklund J, Munson K, Jackson R, Mendlein J, Rabon E, Sachs G (1987) Inhibition of gastric H^+,K^+-ATPase and acid secretion by SCH28080, a substituted pyridyl(1,2a)imidazole. J Biol Chem 262:2077-2984

Ward JM, Sze H (1992) Subunit composition and organization of the vacuolar H^+-ATPase from oat roots. Plant Physiol 99(1):170-179

Weltens R, Pirotte P (1987) KCl-transport in Malpighian tubules: a fast method to prepare double-barrelled K^+-sensitive microelectrodes. Arch. Int. Physiol. Biochim. 95(4):P72-P73

Weltens R, Leyssens A, Zhang SL, Lohrmann E, Steels P, Van Kerkhove E (1992) Unmasking of the apical electrogenic H-pump in isolated Malpighian tubules (*Formica polyctena*) by the use of barium. Cell Physiol Biochem 2:101-116

Wessing A, Hevert F, Rönnau K (1987) Ion transport and intracellular activity of ions in Malpighian tubules of *Drosophila hydei* . Zool Beitr NF 30:297-314

Wieczorek H, Weerth S, Schindlbeck M, Klein U (1989) A vacuolar-type proton pump in a vesicle fraction enriched with potassium transporting plasma membranes of tobacco hornworm midgut. J Biol Chem 264 no19:11143-11148

Williams JC, Beyenbach KW (1983) Differential effects of secretagogues on Na and K secretion in the Malpighian tubule of *Aedes aegypti* (L.). J Comp Physiol 149:511-517

Wright EM, Diamond JM (1968) Effects of pH and polyvalent cations on the selective permeability of gall-bladder epithelium to monovalent ions. Biochem Biophys Acta 163:57

Wright JM, Beyenbach KW (1987) Chloride channels in apical membranes of mosquito Malpighian tubules. Fed Proc 46:A347

Zhang SL, Leyssens A, Weltens R, Van Driessche W, Van Kerkhove E, Steels P (in press) Electrophysiological evidence for the presence of an apical H^+-ATPase in Malpighian tubules of *Formica polyctena* : intracellular and luminal pH measurements. Pflügers Arch