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Study of the interaction of antiplasmodial strychnine derivatives with the glycine receptor

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Abstract

Strychnos icaja Baill. (Loganiaceae) is a liana found in Central Africa known to be an arrow and ordeal poison but also used by traditional medicine to treat malaria. Recently, many dimeric or trimeric indolomonoterpenic alkaloids with antiplasmodial properties have been isolated from its rootbark. ~~As~~ Since these alkaloids are derivatives of strychnine, it was important, in view of their potential use as antimalarial drugs, to assess their possible convulsant strychnine-like properties. In that regard, their interaction with the strychnine-sensitive glycine receptor was investigated by whole-cell patch clamp recordings on glycine-gated currents in mouse spinal cord neurons in culture and by [³H]strychnine competition assays on membranes from adult rat spinal cord. These experiments were carried out on sungucine (leading compound of the chemical class) and on the antiplasmodial strychnogucine B (dimeric) and strychnohexamine (trimeric). In comparison with strychnine, all compounds interact with a very poor efficacy and only at concentrations > 1 μM with both [³H]strychnine binding and glycine-gated currents. Furthermore, the effects of strychnine and protostrychnine, a monomeric alkaloid (without antiplasmodial activity) also isolated from *Strychnos icaja* and differing from strychnine only by a cycle opening, were compared in the same way. The weak interaction of protostrychnine confirms the importance of the G cycle ring structure in strychnine for its binding to the glycine receptor and its antagonist properties.

Keywords :

Strychnine; Glycine receptor; Patch-clamp; [³H]strychnine binding; Antimalarial drug

1 Introduction

Nowadays, malaria is still the major parasitic infectious disease in many tropical and subtropical regions. During the last years, the situation has worsened in many ways, mainly due to parasites becoming increasingly resistant to the currently most ~~currently~~-used drugs (Greenwood, 2004). In our search for new antimalarial agents, we have ~~been brought~~ ~~to~~initiated a study on *Strychnos icaia* Baill. (Loganiaceae), a species close to *Strychnos nuxvomica* which provides strychnine. This plant is an African liana mainly used as an ordeal poison, but also occasionally used in African traditional medicine to treat malaria (Neuwing, 1996). The roots of this species are rich in indolomonoterpenic alkaloids which belong to the strychnane group. Monomeric, dimeric and even trimeric alkaloids were isolated, examples of which are shown in **figure 1**. Among these, some bisindolic alkaloids, which all derive from sungucine (Kambu, Coune et al., 1979) and possess a particular 5'-23 linkage between the two constitutive moieties, show potent and selective antiplasmodial properties (Frederich, De Pauw et al., 2000; Frederich, De Pauw et al., 2001). In in vitro assays against chloroquino-resistant line of *Plasmodium falciparum*, their half-maximal inhibitory concentration_s (IC₅₀) ranges from 0.085 μ M for strychnogucine B to 10 μ M for sungucine. Strychnohexamine, the first naturally occurring trimeric indolomonoterpenic alkaloid recently isolated from this same plant also possesses antiplasmodial properties (Philippe, Prost et al., 2002). Moreover, *Strychnos icaia* contains a series of monomeric alkaloids, devoid of antiplasmodial properties, consisting of strychnine (whose toxicity is well-known) and its derivatives such as protostrychnine, hydroxystrychnine, icajine and vomicine. The convulsant effects of most of these monomeric derivatives have been described on mice many years ago (Sandberg and Kristianson, 1970).

Indeed, the latter compounds, *i.e.* strychnine and some of its analogs, such as pseudostrychnine (Rajendra, Lynch et al., 1997), are better known as potent and highly

selective antagonists of the strychnine-sensitive glycine receptor which is the predominant carrier of fast inhibitory neurotransmission at synapses in the vertebrate spinal cord and brain stem. The glycine receptor belongs to the family of ligand-gated ion channels. Functional glycine receptors are pentameric protein complexes which consist either of homomers of α subunits or of heteromers of 2α and 3β subunits (Grudzinska, Schemm et al., 2005). Four α ($\alpha 1$ - $\alpha 4$) and one β subunit have been so far described (Legendre, 2001). In the spinal cord, a developmental switch in glycine receptor composition occurs that lead to the replacement of immature $\alpha 2$ homomers to mature $\alpha 1/\beta$ heteromers (Singer and Berger, 2000). This switch is also known to occur during *in vitro* maturation (Tapia and Aguayo, 1998; Withers and St John, 1997; Mangin, Nguyen et al., 2005). Its activation by glycine, its main agonist, induces the opening of an anion-selective channel, thereby allowing influx of Cl^- into the cytoplasm. The resulting hyperpolarization of the postsynaptic membrane stabilizes the resting potential of the cell and thus inhibits neuronal firing. Hence, a strong antagonism of glycine receptors can cause motor disturbance and increased muscle tone, what can lead to convulsions and, at high doses, even to death (Breitinger and Becker, 2002; Laube, Maksay et al., 2002).

Since the above-mentioned bisindolic and trisindolic alkaloids include in their structure moieties identical or very similar to strychnine, it could be hypothesized that they also could antagonize glycine-induced currents. This could lead to a convulsant effect, a potential deleterious side-effect that should be studied before using these compounds as antimalarial drugs. Cytotoxic activities on human cancer lines have been previously evaluated and the antiprotozoal selectivity index has indicated a good selectivity (Frederich, De Pauw et al., 2001), so that our major concern remains to check whether these alkaloids are devoid of strychnine-like properties.

As it has previously been demonstrated that the affinity of strychnine analogs to strychnine binding sites highly correlates with their convulsant and lethal pharmacological

activities (Mackerer, Kochman et al., 1977), we decided to investigate the interaction of sungucine, strychnogucine B and strychnohexamine with the glycine receptor. Moreover, in order to complete the establishment of a structure-activity relationship, we have also tested the monomeric protostrychnine, isolated concurrently, whose toxicity is unknown. First, we have carried out [^3H]strychnine displacement binding experiments on membranes from adult rat spinal cord. Then, to tackle the functional aspect of the interaction, we have performed whole-cell patch-clamp recordings on in vitro-cultured embryonic mouse spinal cord neurons.

2 Materials and methods

2.1 Tested drugs

The four tested alkaloids were isolated and purified in our laboratory from *Strychnos icaja* root bark as previously described (Frederich, De Pauw et al., 2000; Frederich, De Pauw et al., 2001; Philippe, De Mol P. et al., 2003; Philippe, Prost et al., 2002). Unwanted strychnine impurities in these alkaloids samples were searched by ~~means of an already described~~ high-pressure liquid chromatography (HPLC) method (Gadi Biala, Tits et al., 1996) that we have slightly modified. According to the obtained chromatograms (data not shown), sungucine, strychnohexamine, and protostrychnine samples were completely devoid of strychnine. The sample of strychnogucine B, however, appeared to contain strychnine impurities. That could result from a degradation of strychnogucine B ~~which~~that includes the strychnine moiety in its structure but, more likely, a ~~bit~~ small amount of strychnine, one of the most abundant alkaloids found in *Strychnos icaja*, was isolated at the same time as strychnogucine B. Since the latter was obtained from the plant in very small quantities, no further purification could be carried out. These traces of strychnine in the sample were taken in consideration in the next experiments.

2.2 Binding assays

2.2.1 Membrane preparation

All procedures for preparing membranes were carried out at 4 °C. Spinal cords from adult rats were dissected. Then, 3 g of fresh or frozen (-80 °C) tissue were homogenized in 10 volumes of phosphate buffered saline (PBS; 0.15 M NaCl in 0.01 M sodium phosphate buffer, pH 7.20) with an Ultra-Turrax homogenizer for 60 s. The homogenate was centrifuged for 10 min at 50,000 g. The supernatant was discarded and the pellet was resuspended by homogenization ~~as before~~. The homogenate was centrifuged a second time and the final pellet

was resuspended by rehomogenization in 5 volumes PBS buffer. Determination of protein content was performed according to the method described by Bradford (1976). The homogenate was kept frozen at -70°C until use.

2.2.2 *Binding assays*

All experimental points were done in triplicate. The binding assay was performed in a final volume of 1 ml of PBS buffer. The radioligand [^3H]strychnine (23.7 Ci/mmol, NEN) was diluted in PBS and used at a final concentration of 2-4 nM. The membrane preparation was resuspended in PBS to give a final protein concentration in the binding assay of 0.3-1 mg/ml. Non-specific binding was defined using 10^{-4} M strychnine.

Commentaire [JMR1] : Ajouter ville et pays.

After addition of the tested alkaloids, the radiolabelled ligand and finally the membrane suspension to the incubation tubes, the samples were thoroughly mixed and incubated at 4°C for 30 min. Protein content of each tube was accurately evaluated according to Bradford (1976). After incubation, the samples were poured directly onto Whatman GF/B glass fiber filters with vacuum applied and immediately washed with 3 x 4 ml cold buffer. Filters were, then, transferred into vials containing 10 ml scintillation fluid (Ecoscint-a, National diagnostics, Atlanta, GA, USA). Radioactivity on the filters was determined by conventional liquid scintillation counting using a Beckman LS3801 counter.

2.3 *Electrophysiology*

2.3.1 *Cell culture*

Spinal cord neurons in culture were obtained from 14-days-old mouse embryos using an adaptation of a method fully described by Withers and St John (1997). Briefly, pregnant mice obtained from our breeding rodent facility were killed following National Institute of Health animal welfare guidelines. All animal husbandry and handling conditions were in

accord with the Belgian regulation (“Arrêté Royal du 14 novembre 1993 relatif à la protection des animaux d’expérience”). ~~Embryo-~~The spinal cords were carefully dissected and freed of meninges and attached dorsal root ganglia. This was followed by a wash with their culture medium which consisted of Dulbecco’s modified minimum essential medium (Invitrogen, Gent, Belgium) supplemented with glucose (6 g/l, final concentration), 5% (V/V) fetal calf serum (FCS, Invitrogen), 10% (V/V) horse serum (HS, Invitrogen) and ~~the~~ N1 supplement (insulin 5 µg/ml; transferrin 5 µg/ml; progesterone 20 nM; putrescine 100 µM, selenium 30 nM) (Bottenstein and Sato, 1979). Cell dissociation was achieved by up and down aspirations through the large tip of a 5 ml plastic pipette put on the bottom of a conical glass tube. The resulting cell suspension was filtered through a 40 µm nylon sieve. Fifty microliters of the cell suspension was seeded on polyornithine (0.1 mg/ml in distilled water) coated glass coverslips (10 mm diameter) in the centre of 35 mm plastic Petri dishes (NUNC, Roskilde, Denmark) at a concentration of 1.25×10^6 cells per ml. The medium was renewed once weekly and cells were used for electrophysiological recordings after 7-14 days-in-vitro (DIV).

2.3.2 Whole-cell recordings and drugs

Coverslips containing cultured spinal cord neurons were transferred to the stage of an ~~an~~ ~~inverted~~ Zeiss interferential contrast microscope. They were maintained at room temperature (20-25 °C) in a recording chamber which was continuously perfused with a physiological saline solution containing (in mM): NaCl, 116 ; D-glucose, 11.1 ; KCl, 5.4 ; CaCl₂.2H₂O, 1.8 ; MgCl₂.6H₂O, 2.0 ; HEPES, 10.0; pH 7.2. Glycine was purchased from UCB (Brussels; Belgium). Strychnine and SR-95531 (gabazine) were purchased from Sigma (USA). All the drugs were applied by means of a local microperfusion system (SPS-8, List Medical). Borosilicate patch-clamp recording electrodes (5-10 MΩ) were made using a Flaming-Brown microelectrode puller (P97, Sutter Instrument Co). Micropipettes were filled with an

intracellular-like solution containing (in mM): KCl, 130.0; CaCl₂·2H₂O, 1.0; D-glucose, 11.1; EGTA, 10.0; Na₂-ATP, 2.5; Mg-ATP, 2.5; HEPES, 10.0, pH 7.4. Standard whole-cell recordings (Hamill et al., 1981) were performed with a Bio-Logic RK400 patch-clamp. Series resistances (10-20 MΩ) were electronically compensated (80 to 85%) and current traces were filtered at 3 kHz, acquired and digitized at 0.5 kHz, and stored on a personal computer system. Control of drug application and data acquisition were achieved using an ITC-16 acquisition board (Instrutech Corporation) and the TIDA for Windows software (HEKA Elektronik Lombrecht/Pföhl, Germany).

2.4 Data analysis

For binding experiments, “n” represented the number of experiments (usually 4) resulting from 3 replicates for a given drug concentration and a given experiment. Counting results were corrected for the protein content of the samples and, after subtracting non-specific binding, subsequently normalized to binding without any added drugs (100%). Drug concentration-response profiles were fitted either to ‘one site competition’ equation of the form :

$$Y = Bottom + \frac{Top - Bottom}{1 + 10^{X - \log IC_{50}}}$$

or to ‘two sites competition’ equation of the form:

$$Y = Bottom + Fraction1 \times \frac{Top - Bottom}{1 + 10^{X - \log IC_{50}1}} + (1 - Fraction1) \times \frac{Top - Bottom}{1 + 10^{X - \log IC_{50}2}}$$

where Y = percent of strychnine specific binding, X = log (drug concentration), IC_{50} is the half-maximal inhibitory drug concentration and Fraction1 is the fraction of all sites that have affinity 1 ($IC_{50}1$).

For whole-cell recordings, “n” represented the number of recorded cells. Peak currents in the different experimental conditions were measured and subsequently normalized to the

preceding and the following responses (100%) in control conditions. Drug concentration-response profiles were fitted to the following equation:

$$\frac{I}{I_{ctrl}} = \frac{1}{1 + \frac{IC_{50}}{[drug]^{n_H}}}$$

where I and I_{ctrl} respectively represent the glycine-induced current at a given concentration of the drug and the control current induced by glycine in the absence of any drug. IC_{50} is the half-maximal inhibitory drug concentration and n_H is the Hill slope.

For all experiments, a statistical analysis was performed using either unpaired two-tailed Student's t -test between control and experimental conditions or one-way analysis of variance (ANOVA-1) followed by a Dunnett's multiple comparison post-tests when significance was reached or least sum-of-squares F -test to compare fits of concentration-response curves (GraphPad Prism software, version 4.00, San Diego, CA).

Except when stated otherwise, the results are expressed as mean and standard error of the mean (SEM).

3 Results

3.1 Binding assays

The interaction of protostrychnine, sungucine, strychnogucine B and strychnohexamine with the strychnine-sensitive glycine receptor was first studied by [^3H]strychnine binding displacement experiments. ~~As opposed to [^3H]glycine which also binds to strychnine-insensitive sites, i.e. N-methyl-D-aspartate (NMDA) receptors, [^3H]strychnine specifically binds to glycine receptors.~~ The interaction of glycine and strychnine at glycine receptors involves overlapping, but conformationally distinct, recognitions sites (O'Connor, Phelan et al., 1996). The competitive nature of strychnine inhibition of agonist action at glycine receptors may rely on its steric inhibition of agonist binding (Rajendra, Lynch et al., 1997). Since our aim was to study strychnine-related alkaloids, cold strychnine was preferred to glycine for the determination of non-specific binding to address both glycine-sensitive and – insensitive sites (Cimino, Marini et al., 1996).

Figure 2 displays the competition binding curves of the four tested alkaloids and of unlabelled strychnine used as a reference. Unlabelled strychnine potently displaced [^3H]strychnine binding to adult rat spinal cord membranes with an IC_{50} of 84.6 ± 17.7 nM (mean \pm standard deviation; $n = 4$ experiments; **figure 2A**). The experimental data could slightly better be fitted with a two-site competition curve ($P = 0.0378$, least sum-of-squares F -test) yielding an IC_{50} of 63.3 ± 23.3 nM for $79.6 \pm 8.1\%$ of the binding (site 1) and of 5.5 ± 2.2 μM for the remaining (site 2). Interestingly, the fraction of site 1 nicely correlated with the inhibition of [^3H]strychnine binding by 10 mM glycine ($74.2 \pm 7.2\%$; $n = 4$ experiments).

As shown in **figures 2A to 2C**, the four tested alkaloids were very weak at displacing [^3H]strychnine: none of them showed a significant effect up to 1 μM and their inhibition at 10 μM was rather modest: $33.5 \pm 14.0\%$ for protostrychnine, $13.4 \pm 8.11\%$ for sungucine, $34.7 \pm 17.4\%$ for strychnogucine B and $52.0 \pm 7.4\%$ for strychnohexamine.

3.2 Patch-clamp recordings

Our binding experiments suggest that the four tested strychnine-related alkaloids very weakly interact, if at all, with the strychnine binding to glycine receptors. This does not rule out an interaction of those compounds with glycine-triggered functional responses. Therefore, we compared the effects of the four alkaloids with the one of strychnine on glycine-induced currents evoked in ~~cultured~~ spinal cord neurones cultured for 7-14 days *in vitro* and recorded using the patch-clamp technique in the whole-cell configuration and in the voltage-clamp mode. At this stage of *in vitro* maturation, glycine receptors are mainly constituted by $\alpha 1/\beta$ heteromers (Tapia and Aguayo, 1998; Withers and St John, 1997; Mangin, Nguyen et al., 2005) and, thus, correspond to the mature form of the receptor that was studied in our reported binding experiments.

In our recording conditions, ~~the~~ spinal cord neurones ~~cultured for 7-14 days *in vitro*~~ responded to bath application of glycine by inward currents (see **figure 3A**) which reversed around 0 mV, a value close to the Nernst equilibrium potential for chloride (0.6 mV; data not shown). Those glycine currents were unaffected by 10 μ M gabazine, showing that there was no cross-activation of type A GABA receptors, and poorly inhibited by picrotoxin, suggesting that they were mainly carried by heteromeric glycine receptors, as already described for mature spinal cord neurone glycine receptors (heteromeres of 2 $\alpha 1$ subunits for 3 β subunits) (Grudzinska et al., 2005), showing that they were mainly carried by heteromeric glycine receptors (Mangin, Nguyen et al., 2005; Tapia and Aguayo, 1998).

Figures 3 to 5 show the inhibition profiles of the four tested alkaloids compared to strychnine on currents induced by 100 μ M glycine (a concentration close to its EC_{50} (Rajendra, Lynch et al., 1997)) in cultured spinal cord neurones. Strychnine (**figure 3**) inhibited glycine-evoked currents with an IC_{50} of 196.9 ± 18.1 nM ($n = 5-62$) and a Hill

coefficient of 1.1 ± 0.1 . As in the binding experiments, the strychnine-related alkaloids only showed weak interactions with GlyR-mediated responses. The maximal inhibition of glycine currents was $19.9 \pm 1.8\%$ ($n = 7-14$) for protostrychnine (**figure 3**), $13.6 \pm 0.9\%$ ($n = 7-27$) for sungucine (**figures 4A and 4B**), $32.9 \pm 12.0\%$ ($n = 11-19$) for strychnogucine B (**figures 4C and 4D**) and 10.0 ± 8.4 ($n = 9$) for strychnohexamine (**figure 5**). Due to its low availability, strychnohexamine could only be tested at $1 \mu\text{M}$.

4 Discussion

The present study reports that strychnogucine B and strychnohexamine, new alkaloids isolated from *Strychnos icaja* which are able to inhibit the growth of malaria parasites, interact, as sungucine, with a very poor efficacy and only at concentrations $> 1 \mu\text{M}$ with both [^3H]strychnine binding on rat spinal cord membranes and glycine responses in whole-cell patch- and voltage-clamped cultured mouse spinal neurones. Although we cannot completely rule out a pro-convulsant effect, which would be associated to the use of these drugs close to strychnine as antimalarials, the data reported here are, to our knowledge, the first attempts to evaluate their acute toxicity.

Two types of assays were used in the present study to address the putative analogy between bisindolic and trisindolic alkaloids and strychnine, both at the biochemical or structural level (binding) and at the functional level (patch-clamp). For sungucine and strychnogucine B, both types of experiments allowed us to draw similar conclusions, *i.e.* a poor efficacy of these compounds in antagonizing strychnine binding or glycine-gated currents and the high concentration ($> 1 \mu\text{M}$) needed to achieve this effect. Nevertheless, at first sight, strychnogucine B, which is the most promising antimalarial derived from *Strychnos icaja*, seems to interact with the glycine receptor with a greater efficacy than sungucine. However, this effect could likely be attributed to contaminating strychnine in our preparation. Indeed, a small peak corresponding to strychnine could be observed by HPLC in the strychnogucine B sample (data not shown). ~~The amount of strychnine impurities was estimated as not exceeding 1% (w/w), as appreciated from binding competition curves as well as from glycine current inhibition curves.~~ If the effects of strychnogucine B on [^3H]strychnine binding and on glycine-induced currents were to be attributed to contaminating strychnine, this would imply a 0.5 % strychnine contamination, as inferred from binding curves, and 1 %

from glycine current inhibition curves. This does not exceed the maximal contamination estimated for HPLC measures and, hence, could suggest that our observed effects of strychnogucine B could be attributed to contaminating strychnine. Anyhow, this would at least imply ~~still less that the~~ strychnine-like effects of strychnogucine B itself are far over-estimated.

As opposed to the dimeric compounds, no clear correlation was found between binding and electrophysiology results in the case of strychnohexamine. Two hypotheses could help explaining this discrepancy. First, the different experimental conditions of binding and electrophysiology could account for the differences. ~~First~~Second, since we have observed that ~20% of the strychnine binding was insensitive to glycine (Cimino, Marini et al., 1996), strychnohexamine could have a higher affinity for these glycine-insensitive sites, hence explaining its absence of effect on responses mediated by the glycine receptor. One could also speculate ~~A second possibility is~~ that strychnohexamine well interacts with the strychnine site of the glycine receptor, but does not hinder glycine binding and the subsequent conformational change leading to channel opening, ~~thus acting as a 'null' antagonist. Such 'null' antagonists are known at other ligand-gated channels related to glycine receptors, e.g. flumazenil at the benzodiazepine site of type A GABA receptors (Millan, 2003).~~ Further experiments (strychnine binding competition by strychnohexamine in the presence of glycine and strychnine antagonism of glycine-gated currents in the presence of strychnohexamine) are needed to fully address ~~these issues~~this issue.

Measuring the interaction between alkaloids derived from *Strychnos icaia* and the glycine receptor has also allowed us to study the influence of some chemical modifications in the structure of strychnine on the interaction with the glycine receptor. First, 'dimerization' seems to strongly reduce the intensity of the interaction. Indeed, strychnogucine B, the lower part of

which being a moiety strychnine bound to a moiety isotrychnine II, was at least a thousand times less potent in interacting with the glycine receptor, what could be the consequence of a too large sterical hindrance.

On the other hand, protostrychnine, which has been only recently found in this plant, but was already also previously described in *Strychnos nux vomica* (Baser, Bisset et al., 1979), possesses, in comparison with strychnine, an opening of the ether bond belonging to the G cycle (see **figure 1**). No data about the strychnine-like properties of protostrychnine were available, but the results obtained in this work had been previously somewhat hypothesized. Indeed, isostrychnine I, an alkaloid structurally close to protostrychnine (the hydroxyle in 17 is replaced in isostrychnine I by an insaturation in 16-17), had been assayed in binding experiments on membrane preparations obtained from the central nervous system of the rat (Mackerer, Kochman et al., 1977) and of the pigeon (LeFort, Henke et al., 1978) and was quite ineffective in both studies. Moreover, in the seventies, Swedish researchers had already hypothesized that ether oxygen between the carbons 17 and 18 may be involved in the fit to the receptor (Sandberg and Kristianson, 1970). Our *in vitro* assays thus confirm the importance of the ether bound of the G cycle in strychnine derivatives structures for their binding to the glycine receptor and for their antagonistic effect on glycine responses.

In conclusion, our data provide the first evidence that promising antimalarial alkaloid agents derived from *Strychnos icaja* are devoid of strychnine-related properties, at least in vitro. Further studies will imply in vivo assays aimed at confirming the absence of pro-convulsant or toxic effects of these compounds together with examining their in vivo antimalarial potency.

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Figure legends

Figure 1 – Structures of tested strychnine derivatives

Chemical structures of strychnine (with classical numerotation) and tested alkaloids

Figure 2 – Interaction of strychnine derivatives with strychnine specific binding

Effect of increasing concentrations of strychnine (A;●), protostrychnine (A;○), sungucine (B;●), strychnogucine B (B;○) and strychnohexamine (C;●) on [³H]strychnine specific binding to spinal cord membranes from adult rats. Results (expressed in percent of [³H]strychnine specific binding in control buffer) are mean and SEM (n = 4) of four independent bindings for each condition (triplicates for each condition in a single binding). For strychnine, data were fitted both with one site (solid line) and with two sites (dotted line) competition curve (see Materials and Methods).

Figure 3 – Effect of protostrychnine on glycine-induced currents in cultured spinal cord neurons

- A The same cultured spinal cord neuron was voltage-clamped at a holding potential of -70 mV using the whole-cell patch-clamp technique (see Materials and Methods) and currents induced by a 10 s perfusion of 100 μM glycine alone (Control) or in combination with 1 μM strychnine or protostrychnine were recorded. Strychnine and protostrychnine were perfused for 15 s before being co-applied with glycine. A 60 s period was allowed for the washout of drugs.
- B Currents evoked by 100 μM glycine in cultured spinal cord neurons were recorded in the presence of increasing strychnine (black filled circles) and protostrychnine (empty

circles) concentrations. Results are expressed as percentage of glycine-induced current peak amplitudes in the absence of drugs (mean \pm SEM, n = 5-62 for strychnine and 7-14 for protostrychnine).

Figure 4 – Effect of sungucine and strychnogucine B on glycine-induced currents in cultured spinal cord neurons

- A,C Cultured spinal cord neurons were voltage-clamped at a holding potential of -70 mV using the whole-cell patch-clamp technique (see Materials and Methods) and currents induced by a 10 s perfusion of 100 μ M glycine alone (Control) or in combination with 0.1 μ M or 10 μ M sungucine (A) or strychnogucine B (C) were recorded. Sungucine and strychnogucine B were perfused for 15 s before being co-applied with glycine. A 60 s period was allowed for the washout of drugs.
- B,D Currents evoked by 100 μ M glycine in cultured spinal cord neurons were recorded in the presence of increasing sungucine (B) and strychnogucine B (D) concentrations. Results are expressed as percentage of glycine-induced current peak amplitudes in the absence of drugs (mean \pm SEM, n = 7-27 for sungucine and 11-19 for strychnogucine B).

Figure 5 – Effect of strychnohexamine on glycine-induced currents in cultured spinal cord neurons

- A The same cultured spinal cord neuron was voltage-clamped at a holding potential of -70 mV using the whole-cell patch-clamp technique (see Materials and Methods) and currents induced by a 10 s perfusion of 100 μ M glycine alone (Control) or in combination with 1 μ M strychnine or strychnohexamine were recorded. Strychnine

and strychnohexamine were perfused for 15 s before being co-applied with glycine. A 60 s period was allowed for the washout of drugs.

- B Currents evoked by 100 μ M glycine in cultured spinal cord neurons were recorded in the presence of 1 μ M strychnohexamine. Results are expressed as percentage of glycine-induced current peak amplitudes in the absence of strychnohexamine (mean \pm SEM, n = 9-18).

Figure 1

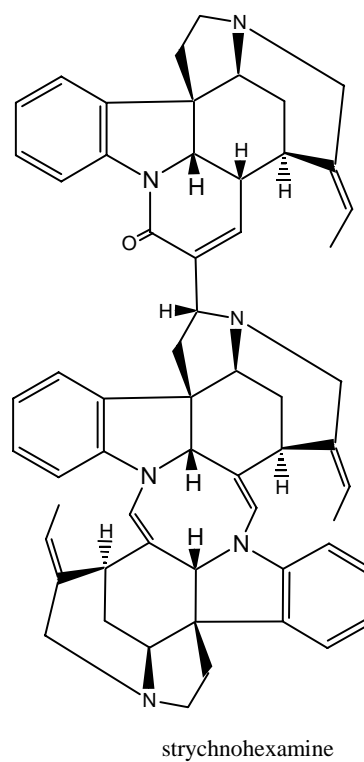
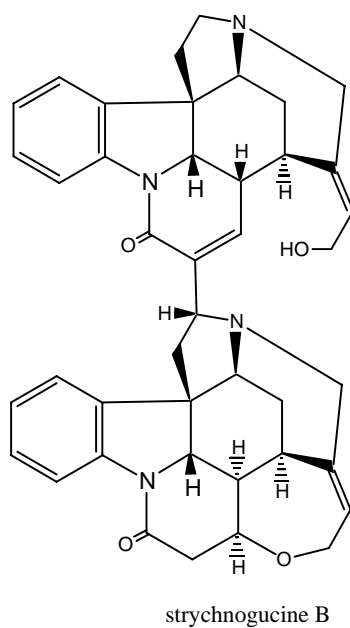
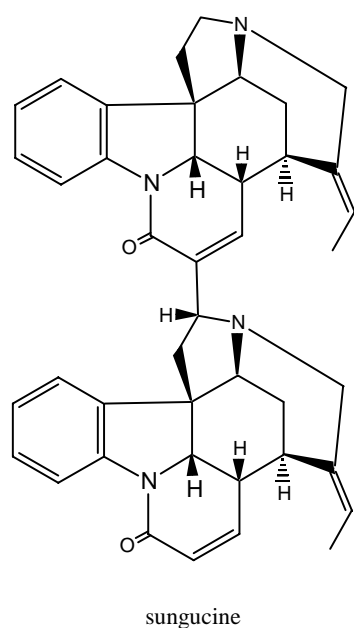
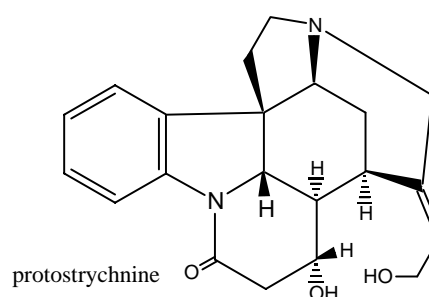
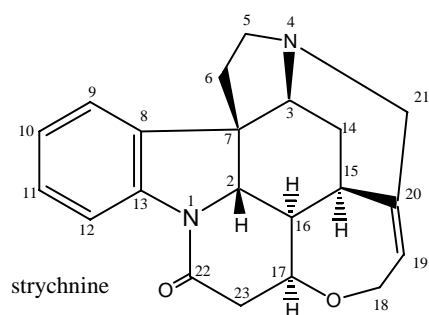


Figure 2

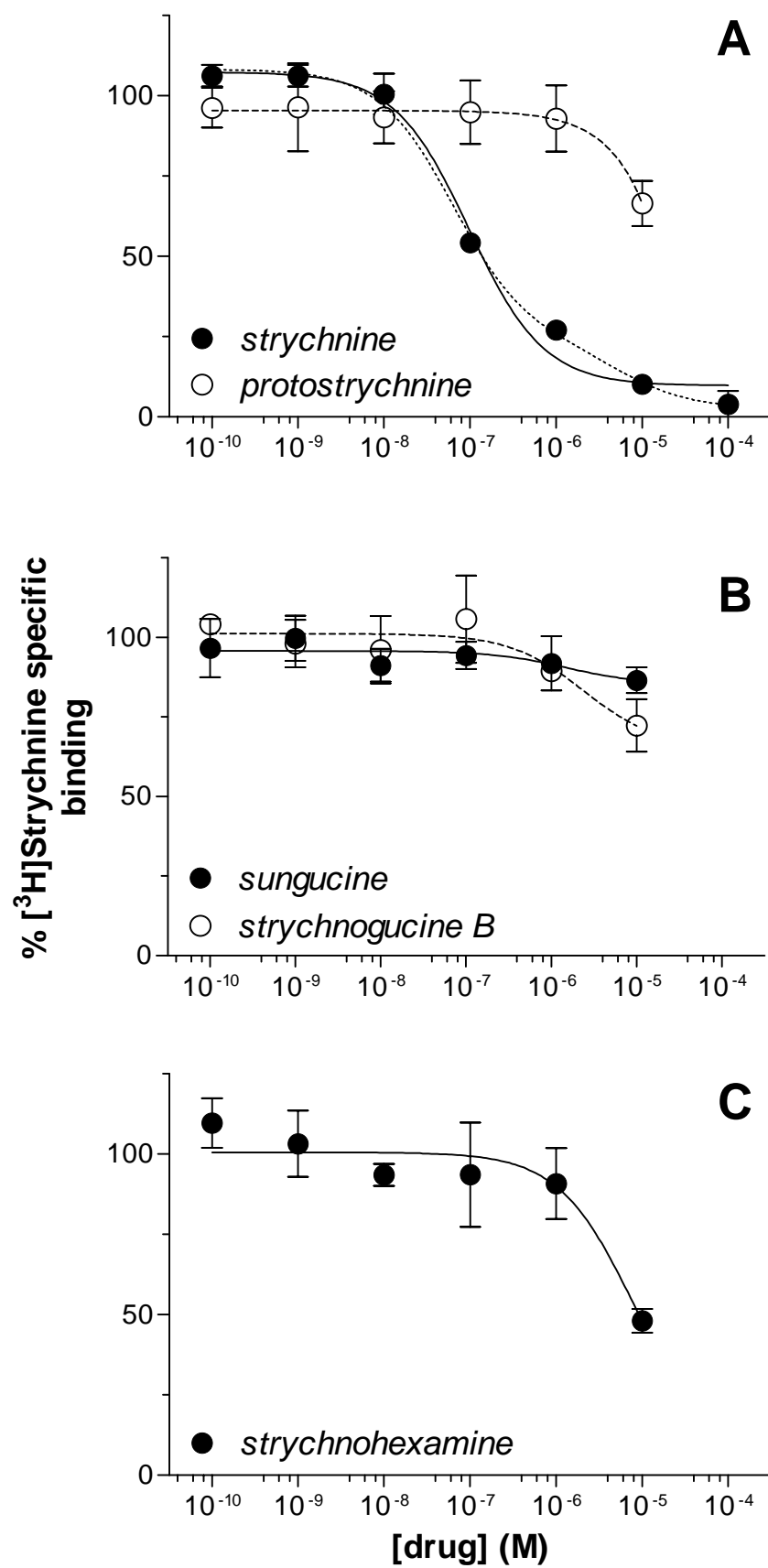


Figure 3

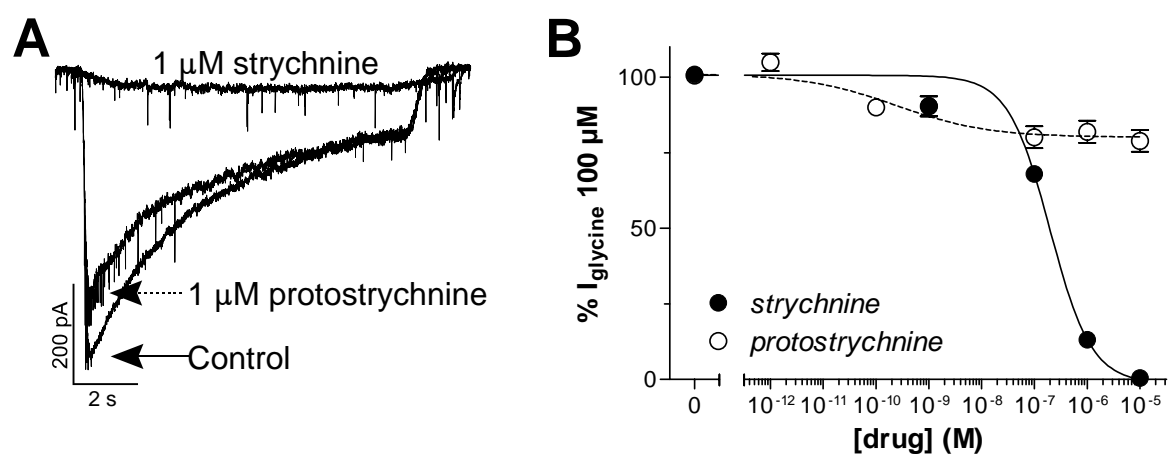


Figure 4

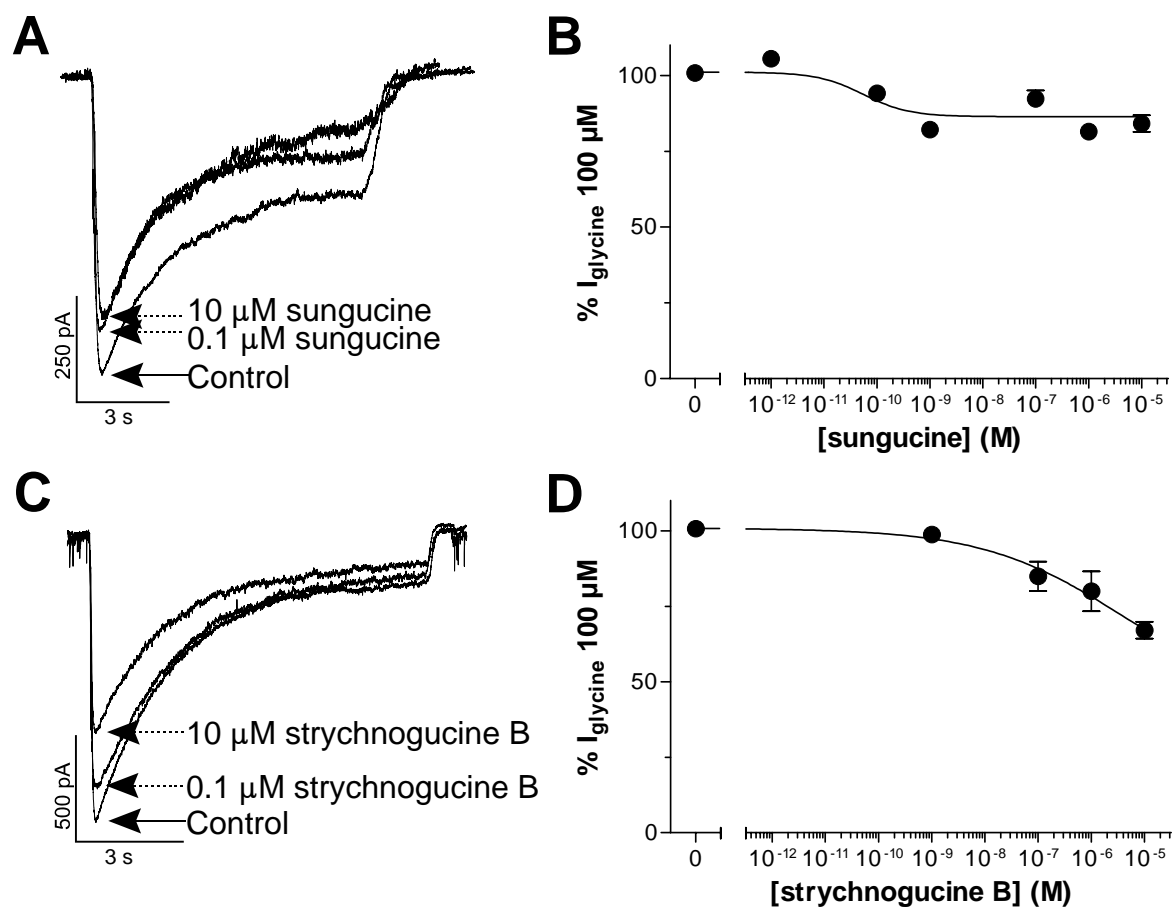


Figure 5

