Strychnine: A Potent Competitive Antagonist of \( \alpha \)-Bungarotoxin-Sensitive Nicotinic Acetylcholine Receptors In Rat Hippocampal Neurons

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ABSTRACT

In our study, evidence is provided that strychnine, a competitive antagonist at glycine-gated \( \text{Cl}^- \) channels, is also a potent competitive antagonist at native \( \alpha \)-\( \gamma \)-containing, \( \alpha \)-bungarotoxin-sensitive nicotinic acetylcholine receptor (nAChRs). To address the effects of strychnine on two types of nicotinic responses, the whole-cell mode of the patch-clamp technique was applied to rat hippocampal neurons in culture. Type IA and type II nicotinic currents evoked by acetylcholine (ACh) were inhibited by strychnine in a concentration-dependent manner with \( IC_{50} \)s of 1.2 and 38 \( \mu \)M, respectively. Strychnine (2 \( \mu \)M) decreased the peak amplitude of the \( \alpha \)-bungarotoxin-sensitive type IA current in a voltage-independent manner and prolonged the decay phase of this current. The concentration-response curve for ACh in evoking type IA current showed a parallel shift to the right in the presence of strychnine (2 \( \mu \)M); the \( EC_{50} \) for ACh was increased from 0.4 to 0.8 mM. These findings suggest that strychnine acts as a competitive antagonist of ACh at the \( \alpha \)-\( \gamma \) nAChRs that subserve type IA current. In contrast, the inhibition by strychnine of type II current was strongly voltage-dependent, and the decay phase of this current was markedly accelerated by the toxin, suggesting an open-channel blockade by strychnine of the \( \alpha \)-\( \beta \) nAChRs subserve type II currents. Preexposure of the neurons to strychnine enhanced its ability to decrease the peak amplitude of type II currents, indicating that the toxin may also act on \( \alpha \)-\( \beta \) nAChR channels that are not open. It is concluded that strychnine is a potent competitive antagonist of ACh at neuronal \( \alpha \)-\( \gamma \) nAChRs and a noncompetitive antagonist at the \( \alpha \)-\( \beta \) nAChR.

Although strychnine (fig. 1) is a well-known competitive antagonist of glycine at glycine-gated \( \text{Cl}^- \) channels, its binding to glycine-resistant sites has been recognized for many years. For instance, numerous reports have provided evidence that strychnine can inhibit cholinergic transmission at the neuromuscular junction and in sympathetic ganglia (Lanari and Luco, 1939; Alving, 1961). A presynaptic nicotinic mechanism appears to account for the ability of the toxin (1–100 \( \mu \)M; Koelle and McKinstry, 1976) to block the release of ACh from sympathetic ganglia (Collier and Katz, 1970) and to block the release of catecholamines from adrenal medullary chromaffin cells (10–30 \( \mu \)M; Kuijpers et al., 1994). However, it is unlikely that strychnine poisoning could be accounted for by these effects, because they are observed at concentrations that are considerably greater than those at which the alkaloid exerts its excitatory and depressant effects in the CNS (Franz, 1975). Nevertheless, findings that strychnine produces spiking in the cortex (Amato et al., 1969; García Ramos et al., 1977), where glycine receptors are rare (Frostholm and Rotter, 1985), strongly support the notion that some of the toxic effects of this alkaloid can be accounted for by its action on receptors other than the glycine receptors in the CNS.

In contrast to the previously noted low potency of strychnine at nonneuronal nAChRs, the toxin has been shown to have potent actions on certain subtypes of neuronal nAChRs. On the outer hair cells of the vestibular system, strychnine is even more potent than curare or \( \alpha \)-BGT in antagonizing the ABBREVIATIONS: ACh, acetylcholine; nAChR, nicotinic acetylcholine receptor; \( \alpha \)-BGT, \( \alpha \)-bungarotoxin; CNS, central nervous system; GABA, \( \gamma \)-amino butyric acid; MEM, minimum essential medium; HEPES, N-[2-hydroxyethyl]piperazine-N-[2-ethane sulfonic acid]; DHβE, dihydro-\( \beta \)-erythroidine; ATP-βS, adenosine 5'-triphosphate-regenerating solution; EGTA, ethyleneglycol-bis-(\( \beta \)-aminoethyl ether)-N,N,N',N'-tetraacetic acid; \( t_d \), decay-time constant; \( IC_{50} \), agonist concentration that evokes 50% of the maximal response; \( K_d \), antagonist concentration that reduces by 50% the maximal response; \( n_H \), Hill coefficient; ANOVA, analysis of variance; \( K_a \), apparent affinity of an antagonist for the receptor; \( K_a \), apparent affinity of an agonist for the receptor.
nicotinic actions of ACh (Bartolami et al., 1993; Erostegui et al., 1994; Guth et al., 1994). The anticholinergic effects of strychnine in the auditory system (Guth et al., 1994) have been observed at concentrations as low as 0.01 μM (Doi and Ohmori, 1993), and strychnine binds to the neuronal nAChRs of the outer hair cells with a $K_i$ of approximately 35 nM (Lawoko et al., 1995). Of interest, in addition to the outer hair cell nAChRs, which are likely to be made up of the α9 subunit (Elgoyhen et al., 1994), homomorphic α7 nAChRs, homomorphic α8 nAChRs and homomorphic α9 nAChRs ectopically expressed in Xenopus oocytes are highly sensitive to inhibition by toxicologically relevant concentrations of strychnine (Anand et al., 1993; Elgoyhen et al., 1994; Gerzanich et al., 1994; Peng et al., 1994).

Studies directed at determining the ability of centrally acting compounds to interact with native neuronal nAChRs have been helpful in improving the understanding of the functions of these receptors in the CNS. For instance, studies of the interactions of epibatidine, α-conotoxin-ImI and amantadine with neuronal nAChRs have suggested that different subtypes of these receptors are likely to be involved in analgesia, convulsions and Parkinson’s disease. In fact, 1) epibatidine, a toxin isolated from the skin of the frog Epipedobates tricolor and known to cause analgesia when injected i.p. in mice (Badio and Daly, 1994), is a potent agonist at nAChRs containing nAChRs in hippocampal neurons (Alkondon and Albuquerque, 1993); 2) α-conotoxin-ImI, a toxin isolated from the venom of Conus imperialis and shown to cause convulsions when injected intracerebroventricularly in mice and rats (Johnston et al., 1995), is a competitive antagonist selective for native α7-bearing nAChRs (Pereira et al., 1996) and 3) at therapeutically relevant concentrations, amantadine, a drug used to treat Parkinson’s disease, acts as a noncompetitive antagonist of ACh at α7-containing nAChRs in hippocampal neurons (Matsubayashi et al., 1997).

Considering that, 1) homomorphic α7 nAChRs heterologously expressed in Xenopus oocytes are sensitive to blockade by strychnine (Peng et al., 1994), 2) convulsions are among the well-documented toxic effects of strychnine, 3) hippocampal foci can be developed as a consequence of exposure to strychnine (Baker, 1965), and 4) α7-bearing nAChRs, which are expressed in the majority of the hippocampal neurons (Alkondon and Albuquerque, 1993), appear to be involved in convulsions, we decided to investigate the interactions of the toxin with the different subtypes of nAChRs (including the α7-bearing receptors) present on hippocampal neurons. As reported previously, hippocampal neurons can respond to nicotinic agonists with at least one of three pharmacologically and kinetically distinct types of nicotinic currents, namely types IA, II and III that arise from the activation of nAChRs composed of α7, α4β2, and α3β4 subunits, respectively (Alkondon and Albuquerque, 1993; Alkondon et al., 1994; Ishihara et al., 1995; Barbosa et al., 1996).

Our study demonstrates that strychnine inhibits both α7 nAChR-mediated type IA currents and α4β2 nAChR-mediated type II currents recorded from rat hippocampal neurons. It is likely that some of the symptoms of strychnine-induced intoxication are associated with its ability to block α7 nAChRs in the CNS, because we provide evidence that the concentrations at which strychnine inhibits the activation of native α7 nAChRs present on hippocampal neurons are similar to those at which it interacts with glycine receptors in the CNS.

Materials and Methods

Tissue culture. Cultured hippocampal neurons were prepared by a procedure similar to that described previously (Alkondon and Albuquerque, 1993). The hippocampi of 17- to 18-day-old rat fetuses (Sprague-Dawley strain) were dissected out, minced and incubated with 0.25% trypsin (Gibco BRL, Grand Island, NY) for 30 min at 36°C. Using a sterile Pasteur pipette, hippocampal neurons were dissociated and plated at a density of approximately 700,000 cells per 35-mm culture dish precoated with collagen (Vitrogen 100, Celtix Laboratories, Palo Alto, CA). The cells were cultured in an incubator at 36°C in a water-saturated, 10%-CO₂/90%-air atmosphere. The medium surrounding the cells, which consisted of MEM (Gibco BRL) enriched with fetal bovine serum (10%; Gibco BRL), horse serum (10%, Gibco BRL), glutamine (2 mM, Sigma Chemical Co., St. Louis, MO) and DNase (40 μg/ml, Sigma), was replaced twice a week with fresh medium consisting of MEM supplemented with horse serum (10%) and glutamine (2 mM). On the 7th day after plating the cells, uridine (final concentration = 6.7 μg/ml) and 5-fluoro-2-deoxyuridine (final concentration = 13.3 μg/ml) were added for 24 hr to the culture medium to inhibit the proliferation of nonneuronal cells. Neurons cultured for 14 to 30 days were used throughout this study.

Whole-cell current recording. Nicotinic currents were recorded from hippocampal neurons according to the standard whole-cell patch-clamp technique using an LM-EPC-7 patch-clamp system (List Electronic, Darmstadt, FRG) (Hamill et al., 1981). Patch pipettes were pulled from borosilicate capillary glass and had resistances when filled with internal solution. The series resistance of the patches was 10 to 25 MΩ and was not compensated. Currents were filtered at 3 kHz and either sampled directly by a microcomputer using the program pCLAMP (Axon Instruments, Foster City, CA) or stored for off-line analysis on videocassette tapes after passage through a pulse code modulation device (Neuro-Corder DR-384; Neuro-Data Instruments Corp., New York, NY). All experiments were performed at room temperature (20–22°C).

The external bath solution (340 mM) consisted of (in mM): NaCl, 165; KCl, 5; CaCl₂, 2; glucose, 10 and HEPES, 5; the pH of the solution was adjusted to 7.3. With NaOH. The neurons were perfused continuously at a rate of 1.5 to 2.0 ml/min with external solution containing atropine (1 μM) and tetrodotoxin (200 nM). In some experiments, specified in “Results,” DHP/E (0.1 μM) was added to the standard external solution described above. The patch pipettes were filled with ATP-RS, which has been shown to reduce the extent of the rundown of type IA current (Alkondon et al., 1994). The ATP-RS consisted of (in mM): CsCl, 60; CsF, 60; Cs-EGTA, 10; HEPES, 10; MgCl₂, 2; Tris adenosine 5'-triphosphate, 5 and Tris phosphocreatine, 20. After adding creatine phosphokinase (50 U/ml) to this solution, a small amount of CsOH (8 mM) was added to adjust the pH of the solution to 7.3. The final osmolality of the solution was 340 mOsm.

Drug applications. The drugs were delivered to the neurons according to the method described previously (Albuquerque et al.,
A U-shaped tube ("U-tube") with a pore of 250 to 400 μm in diameter at the apex was positioned about 50 μm directly above the neuronal soma. The input to the U-tube was connected to a manual switch valve, which was used to select the desired drug solution. The outlet of the U-tube was connected via an electric valve to a polyethylene tube for removal of drug solution from the neuronal surroundings. To prevent any leakage of the drug solution through the pore of the U-tube, the outlet was under continuous vacuum so that the drug solution and a small amount of the bath solution from the dish were removed when the valve was not activated. Upon activation of the valve by a 0.5 to 2-sec electric pulse, the drug solution flowed rapidly out of the apical pore and displaced completely the solution surrounding the neuronal soma and dendrites. Using this U-tube system, pulses of ACh alone or in an admixture with strychnine (0.1–300 μM) were applied to the neurons. Alternatively, in some experiments, strychnine was applied through the external bath perfusion system, in which case the same concentration of strychnine was added to the agonist-containing solution in the U-tube.

**Data analysis.** The peak amplitude, the rise time (10–90%) and the exponential decay-time constant (τ) of the whole-cell currents were determined using the pCLAMP program. EC50, IC50 and the nH values were determined with the Sigmaplot program according to the following equation for activation:

\[ I = I_{\text{max}} \times \frac{[\text{ACh}]^a}{[\text{ACh}]^a + EC_{50}^{\text{a}}} \]

and for inhibition:

\[ I = I_{\text{max}} - I_{\text{max}} \times \frac{[\text{strychnine}]^a}{[\text{strychnine}]^a + IC_{50}^{\text{a}}} \]

where I, I_{max}, [ACh] and [strychnine] were the peak whole-cell current, the maximum obtainable peak current, the concentration of ACh, and the concentration of strychnine, respectively. Values were expressed as the mean ± S.E.M.

**Drugs used.** Acetylcholine chloride, tetrodotoxin, atropine sulfate and strychnine hydrochloride were obtained from Sigma. Dihydro-β-erythroidine hydrobromide was a gift from Merck, Sharp & Dohme Research Laboratories (Rahway, NJ). ATP (Tris salt), phosphocreatine (di-Tris salt) and creatine phosphokinase (type I) were obtained from Sigma Chemical Co.

**Statistical analysis.** Two-way ANOVA was used to determine the significance of the results.

**Results**

Nicotinic whole-cell currents were elicited by pulse application (0.5–2 sec) of ACh via the U-tube to hippocampal neurons in culture. In this study, 80 of the 121 neurons tested responded to ACh with type IA current, characterized by rapid onset after the start of the ACh pulse and rapid decay in the continued presence of ACh (fig. 2, top row; Alkondon and Albuquerque, 1993). Type II current, characterized by its slow decay phase (fig. 2, bottom row; Alkondon and Albuquerque, 1993), was recorded from 40 neurons. Type IB current, which is comprised of the rapidly decaying type IA current and the slowly decaying type II current (Alkondon and Albuquerque, 1993; Alkondon et al., 1994), was recorded from only one neuron. In our study, the percent probability of finding hippocampal neurons that responded to ACh with type II currents was somewhat higher than that reported previously (Alkondon and Albuquerque, 1993, 1995; Alkondon et al., 1994).

**Effects of strychnine on ACh-induced currents recorded from hippocampal neurons in culture.** Application of an admixture of ACh (1 mM) plus strychnine (10 μM) to neurons that responded to ACh (1 mM) with type IA currents resulted in activation of currents whose amplitudes were approximately 60% smaller than those of currents evoked by ACh alone (fig. 2). The effect of strychnine on type IA currents was completely reversible within 1 min after washing of the neurons with external solution. In contrast, the peak amplitude and the kinetics of ACh (1 mM)-evoked type II currents were not affected when strychnine (10 μM) was applied to the neurons exclusively in admixture with ACh (1 mM) (fig. 2). Thus, strychnine was apparently more selective in inhibiting the a7 nAChR activity subserving type IA currents than in inhibiting the a4b2 nAChR activity subserving type II currents.

**The potency and the mechanism of action of strychnine on type IA nicotinic currents recorded from hippocampal neurons.** Because a single hippocampal neuron can express both α7 and α4b2 nAChRs (Alkondon and Albuquerque, 1993), contamination of type IA currents by slowly decaying currents corresponding to the activation of α4b2 nAChRs was avoided by the continuous perfusion of the neurons with DHβE (0.1 μM)-containing external solution. At 0.1 μM, DHβE selectively inhibits the activation of whole-cell currents subserved by α4b2 nAChRs (Alkondon and Albuquerque, 1993). Except in experiments where the voltage dependence of the effect of strychnine on nicotinic currents was addressed, the neurons were held at −60 mV.

To determine the apparent potency of strychnine in inhibiting the nAChR activity subserving type IA currents, neurons that responded to 1-sec pulses of ACh (1 mM) with type IA current were exposed 1 min later to a pulse of an admixture of ACh (1 mM) plus one of various concentrations of strychnine. The reversibility of the effect of strychnine on type IA currents was tested subsequently by the application of ACh (1 mM) to the neurons. These experiments were performed on neurons exposed to concentrations of strychnine ranging from 0.1 to 30 μM. The peak amplitude of type IA current was decreased by strychnine in a concentration-dependent manner; the IC50 for the toxin was approximately 1.9 μM (fig. 3A; table 1).

To verify whether the toxin interacts with a7 nAChR channels that are not opened, after the recording of ACh (1 mM)-evoked type IA current, the neurons were continuously perfused with strychnine (0.1–30 μM)-containing external solution, and 10 min after the perfusion had begun, they were exposed to an admixture of ACh (1 mM) plus the proper concentration of strychnine. Under this experimental condition, strychnine decreased the peak amplitude of type IA currents in a concentration-dependent manner, and the IC50 for the toxin was about 1.2 μM (fig. 3A; table 1). The IC50 for strychnine were significantly different according to the ANOVA (F = 8.4; P < .01). The apparent potency of strychnine in inhibiting the nAChR activity subserving type IA currents increased 1.6-fold when the neurons were preexposed to the toxin, a finding that may indicate an action on the nAChR channels that are not opened.

Based on the IC50 determined above, 2 μM strychnine was selected for studies to probe the mechanism of inhibition of type IA currents by the toxin. The peak amplitude of type IA currents evoked by 0.5-sec pulses of ACh increased as the concentration of ACh was increased from 0.05 to 10 mM (fig. 3B). When each test concentration of ACh was applied to the
neurons as an admixture with strychnine (2 μM), the peak amplitude of type IA currents was smaller than the peak amplitude of the currents evoked by the corresponding concentration of ACh in the absence of the toxin, and the magnitude of the blockade by strychnine of type IA currents decreased with increasing concentrations of the agonist. The results obtained from experiments in 25 neurons were combined by normalizing the peak amplitude of type IA currents to the amplitude of the currents elicited by 10 mM ACh in the absence of strychnine. The ACh concentration-response relationship in the absence of strychnine yielded an EC₅₀ of 0.4 mM for ACh in evoking type IA currents (fig. 3B; table 2). In the presence of strychnine (2 μM), the ACh concentration-response curve was shifted to the right in a parallel manner (fig. 3B), yielding an EC₅₀ of about 0.8 mM for ACh in eliciting type IA currents (table 2). Strychnine did not alter the maximal response to ACh, but decreased the apparent potency of ACh in evoking type IA currents.

In a second set of experiments the neurons were perfused for 10 min with strychnine (2 μM)-containing external solution after the control responses were recorded. Under this experimental condition, the peak amplitude of type IA currents evoked by subsequent application of an admixture of ACh plus strychnine was smaller than that of the currents evoked by the corresponding concentration of ACh in the absence of the toxin. Further, the magnitude of the inhibitory effect of strychnine decreased with increasing concentrations of ACh, such that the concentration-response relationship for ACh in evoking type IA currents was shifted to the right in the presence of strychnine. Based on the results obtained from this set of experiments in which the neurons were allowed to equilibrate with strychnine before their exposure to the admixture of ACh plus the toxin, there was no change in the maximal response of the receptors to ACh, but the apparent EC₅₀ for ACh in evoking type IA currents increased from 0.4 to 0.8 mM (see table 1). The findings from this two series of experiments indicated that ACh and strychnine compete for the agonist binding site on the α₇ nAChR.

Analysis of the voltage dependence of the effects of strychnine on the peak amplitude of type IA currents and investigation of the effects of the toxin on the kinetics of these currents provided further evidence that strychnine acts as a competitive antagonist at the α₇ nAChR on hippocampal neurons. Type IA currents evoked by 0.5-sec pulses of ACh (0.3 mM) were recorded from seven neurons as the holding potential was changed from −120 mV to +60 mV in 20 mV steps. Then, 10 min after perfusing the neurons with strychnine (1 μM)-containing solution, an admixture of ACh plus strychnine...
strychnine (1 μM) was applied through the U-tube to the same neurons (fig. 4A). The data were combined by normalizing all the responses relative to the peak amplitude of the ACh (0.3 mM)-evoked currents at −120 mV (fig. 4B). The ratio of the amplitude of type IA currents evoked by ACh in the presence of strychnine to the amplitude of the currents evoked by ACh alone did not change significantly at any holding potential (fig. 4C). Therefore, the reduction by strychnine (1 μM) of the peak amplitude of type IA current was voltage independent.

The effects of increasing concentrations of strychnine (0.3–3 μM) on the decay and the rising phase of type IA currents were studied after 10-min perfusion of the neurons with solution containing one of the test concentrations of the toxin. For illustrative purposes, the nicotinic currents recorded in the presence of strychnine were normalized to the corresponding control currents (fig. 5). The rise time of ACh (0.3 mM)-evoked currents ranged from 15 to 27 msec and was prolonged in a concentration-dependent manner by strychnine (table 3).

Of 14 neurons tested, 11 neurons responded to ACh with type IA currents that showed single-exponential decays with time constants (τ) of 15.2 to 52.1 msec under the control condition. Although strychnine prolonged the decay phase of these currents, they still showed a single exponential decay in the presence of the toxin. The decay phase of the currents recorded from the other three neurons were better fitted to double-exponential decay functions under the control condition; the fast decay component had τf of 14.6 to 22.7 msec and the slow decay components had τs of 219 to 294 msec. These three currents also showed double-exponential decays in the presence of strychnine (0.3 μM), and the τ values were increased by the toxin in a concentration-dependent manner. The effect of various concentrations of strychnine on the fast decay component of all ACh-evoked type IA currents is shown in table 3.

The effect of membrane potential on the strychnine-induced prolongation of the decay phase of type IA currents was studied in neurons that were perfused for 10 min with strychnine (1 μM)-Containing solution before their exposure to ACh plus strychnine. The responses of the neurons were recorded at membrane potentials that ranged from −120 to −20 mV in steps of 20 mV. Four neurons responded to ACh with currents that had a single-exponential decay with τ values of 21 to 40 msec. Three other neurons responded to ACh with type IA currents that had a double-exponential decay: the fast decay component had τf of 14 to 31 msec and the slow decay component had τs of 91 to 958 msec. In the analysis, only the τs was considered when averaging the results. In the presence of strychnine (1 μM), the decay phase of the currents recorded from these seven neurons was better fitted by a single-exponential function, and was prolonged in a voltage-independent fashion (fig. 5).

**The potency and the mechanism of action of strychnine as an inhibitor of the α4β2 nAChR activity subserving type II currents in hippocampal neurons.** Be-

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**Fig. 3.** Concentration-dependent inhibition of type IA current by strychnine and the mechanism underlying the action of the toxin. A, Concentration-response relationship for the inhibitory effect of strychnine on type IA currents. The peak amplitude of ACh (1 mM)-evoked currents was taken as 100% and was used to normalize the peak amplitude of the currents evoked in the presence of strychnine. The curves were fitted to the Hill equation. Symbols and bars represent the mean ± S.E.M. of results obtained from four to seven neurons. Holding potential, −60 mV. The IC₅₀ and the nH values for strychnine obtained are shown in table 1. B, Effect of strychnine on the concentration-response relationship for ACh in evoking type IA currents. In each experiment, the peak amplitude of currents evoked by ACh (10 mM) was taken as 100% and was used to normalize the peak amplitude of currents evoked by other concentrations of ACh in the presence or in the absence of strychnine. Each symbol and bar represent the mean ± S.E.M. (n = 5–25). The EC₅₀ and nH for ACh in evoking type IA currents are shown in table 2.

**TABLE 1**

<table>
<thead>
<tr>
<th>Current</th>
<th>Strychnine Exposure</th>
<th>IC₅₀ (μM)</th>
<th>Hill Coefficient (nH)</th>
<th>Kᵢ Calculated (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type IA</td>
<td>U-tube</td>
<td>1.9 ± 0.4 ⁴</td>
<td>1.0 ± 0.1</td>
<td>1.11</td>
</tr>
<tr>
<td></td>
<td>U-tube + bath</td>
<td>1.2 ± 0.2</td>
<td>1.0 ± 0.1</td>
<td>0.68</td>
</tr>
<tr>
<td>Type II</td>
<td>U-tube</td>
<td>118 ± 15</td>
<td>1.5 ± 2.3</td>
<td>n.d. ³</td>
</tr>
<tr>
<td></td>
<td>U-tube + bath</td>
<td>38 ± 4</td>
<td>1.5 ± 0.1</td>
<td>n.d. ³</td>
</tr>
</tbody>
</table>

⁴ Mean ± S.E.M.  
³ n.d., Not determined.

**TABLE 2**

<table>
<thead>
<tr>
<th>Condition</th>
<th>EC₅₀ (mM)</th>
<th>Hill Coefficient (nH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACh</td>
<td>0.41 ± 0.03 ⁵</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>ACh + strychnine (2 μM) in U-tube</td>
<td>0.80 ± 0.05</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>ACh + strychnine (2 μM) in the U-tube and the bath</td>
<td>0.78 ± 0.10</td>
<td>1.2 ± 0.1</td>
</tr>
</tbody>
</table>

⁵ Mean ± S.E.M.
cause type II currents elicited by ACh (1 mM) were insensitive to strychnine (10 μM) (see fig. 2), the effects of the toxin on these currents were investigated at concentrations 10-fold higher than those used to test the inhibition of the type IA current.

The amplitudes of the currents evoked by the application of an admixture of ACh (0.3 mM) plus increasing concentrations of strychnine (1–300 μM) to eight neurons that responded to ACh (0.3 mM) with type II currents were smaller than those of the currents evoked by ACh alone; this effect of strychnine, which was reversible within 1 min after washing of the neurons with external solution, was also concentration-dependent (fig. 6). The concentration-response relationship revealed that the IC50 for strychnine in blocking type II currents was about 118 μM (fig. 6; table 1). In a second set of experiments, neurons that responded to ACh with type II currents were continuously perfused with strychnine-containing external solution for 10 min before their exposure to an admixture of ACh plus the corresponding concentration of the toxin. Under this experimental condition, the apparent potency of strychnine in reducing the peak amplitude of type II currents was substantially increased, indicating that the toxin is able to interact with α4β2 nAChR channels that are not open; the IC50 for strychnine was approximately 38 μM (fig. 6; table 1), i.e., 0.3 times the IC50 obtained when strychnine was applied to the neurons exclusively via the U-tube. It is unlikely that strychnine acts as a competitive antagonist at the α4β2 nAChRs, because its effect on type II currents had the same magnitude regardless of whether ACh was used at the concentration of 0.3 or 1 mM.

The mechanisms by which strychnine inhibited the activation of type II currents were further investigated by examining the kinetics of the currents in the presence and in the absence of the toxin and by investigating the voltage dependence of the effect of the toxin on the peak amplitude of these currents.

In agreement with earlier studies (Alkondon and Albuquerque, 1993, 1995; Alkondon et al., 1994), ACh (0.3 mM)-

![Fig. 5. Concentration-dependent prolongation of decay phase of type IA current by strychnine. Top traces, Sample recordings of type IA currents evoked by application of ACh (0.3 mM) in the absence and in the presence of strychnine (0.3–3 μM) to neurons held at −60 mV. Each pair of traces was obtained from one neuron. To illustrate the effects of strychnine on the kinetics of type IA currents, the peak amplitudes of the currents evoked by ACh in the presence of strychnine were scaled to match those of the currents evoked by ACh alone. Bottom graph, The relationship between the membrane potentials and the decay-time constants of the currents evoked by ACh alone.](image)

### Table 3

| Strychnine-induced changes in the kinetics of type IA currents |
|---|---|---|
| [Strychnine] (μM) | Rise Time (% of Control) | Decay-Time Constant (% of Control) |
| 0.3 | 107 ± 11 (6)* | 128 ± 5 (6) |
| 1 | 132 ± 18 (4) | 188 ± 12 (4) |
| 3 | 168 ± 26 (4) | 388 ± 138 (4) |

*Mean ± S.E.M. (number of neurons). Under control conditions, the rise time of ACh (0.3 mM)-evoked type IA currents ranged from 15 to 27 msec (n = 14). The decay phase of currents recorded from 11 neurons was best fitted by a single-exponential function and the decay-time constant of these currents ranged from 15.2 to 52.1 msec. The decay phase of the currents recorded from three neurons was fitted by a double-exponential function, and the τ2 of these currents, which varied from 14.6 to 22.7 msec, was used in averaging the results.
evoked type II currents displayed a very strong inward rectification (fig. 7). Four neurons that responded to ACh with type II currents were continuously perfused for 10 min with strychnine (30 μM)-containing external solution, and then exposed to the admixture of ACh plus strychnine (fig. 7A). The peak amplitude of all currents was normalized to the peak amplitude of the ACh-evoked current at -120 mV and used to plot the current-voltage relationship. The reduction by strychnine of the peak amplitude of type II currents became more intense as the membrane potential was made more negative (fig. 7B). The ratio of the peak current amplitude in the presence of strychnine to the corresponding amplitude in the absence of the toxin also showed the toxin’s voltage-dependent effect on the peak amplitude of type II currents (fig. 7C).

When strychnine (10, 30 or 100 μM) was applied to the neurons exclusively as an admixture with ACh (0.3 mM), in addition to the reduction of the peak current amplitude, there was an acceleration of the decay phase of the ACh-evoked type II currents (fig. 8A). The decay phases of the currents recorded from 3 of 10 neurons sampled were better fitted to a single-exponential function either in the absence or in the presence of strychnine, and the decay phase of the currents recorded from the remaining seven neurons was fitted by a double exponential function either in the presence or in the absence of the toxin. The decay-time constant of type II currents evoked by ACh was shortened by strychnine in a concentration-dependent manner (table 4; fig. 8A). In addition, perfusion of the neurons with strychnine does not appear to affect the interaction of the toxin with the open state.
ACh (0.3 mM)-evoked type II currents ranged from 22.5 to 49.5 msec (presence of the highest tested concentration of strychnine serving type II currents. Also, as illustrated on table 4, in the presence of the highest tested concentration of strychnine plus toxin (fig. 8B). These findings suggest that strychnine can interact with the open state of the nAChR channel suberving type IA currents.

Discussion

Our study revealed that strychnine, a toxin well known for its convulsant effects and for its ability to block glycine-activated Cl⁻ channels, acts as a competitive nicotinic antagonist at the α7 nAChRs, which give rise to type IA currents in hippocampal neurons, and inhibits via noncompetitive mechanisms the α4β2 nAChR activity. On the basis of the IC₅₀ values obtained in our study, strychnine appears to be 30 times more potent in blocking type IA currents (IC₅₀ = 1.2 μM) than in blocking type II currents (IC₅₀ = 38 μM).

Mechanism of action of strychnine on the α7 nAChRs suberving type IA currents. Studies of the concentration-response relationship for ACh in evoking type IA currents in the absence and in the presence of 2 μM strychnine (a concentration of strychnine that is close to its IC₅₀ in blocking type IA current) demonstrated that the toxin decreases the apparent potency of ACh; strychnine increased the EC₅₀ for ACh from 0.4 to 0.8 mM. In addition, neither the maximal responsiveness of the α7 nAChRs subserving type IA currents nor the Hill coefficient for ACh was affected by the toxin. These results suggested that strychnine competes with ACh for the agonist binding site(s) on the α7 nAChRs. These findings that 1) the reduction of the peak amplitude of type IA currents by strychnine was voltage independent and 2) the decay phase and the rise time of type IA currents were prolonged by the toxin favor the notion that the toxin is a competitive antagonist of ACh at the α7 nAChRs.

Some insights regarding the kinetics of interaction of strychnine with the α7-containing nAChRs that give rise to type IA currents are suggested from the present results. An increase in the apparent potency of strychnine (change in IC₅₀ from 1.9 to 1.2 μM) to inhibit ACh-evoked type IA currents was observed when the method of application of strychnine was changed to include a period of preincubation of the neurons with the toxin (see fig. 3A). Considering that the activation and inactivation rates of the α7 nAChRs subserving type IA currents are very rapid, at the saturating concentration of the agonist used in our experiments it is likely that the equilibrium of the receptors with strychnine is not fully achieved when the toxin is applied to the neurons exclusively during the agonist pulse, and that would account for the increase in the apparent potency of strychnine when the receptors are allowed to preequilibrate with the toxin before their exposure to the admixture of strychnine plus ACh. In fact, it has been shown that at a given agonist concentration, the potency of a competitive antagonist depends on the rates of antagonist-receptor association and dissociation and the rates of receptor activation and inactivation (Benveniste et al., 1990; 1991). That strychnine prolongs the rising and decay phases of type IA currents suggests a rapid dissociation of the antagonist from the receptor and a resistance of the receptor-antagonist bound complex to desensitization. Thus, when ACh displaces strychnine from the agonist-binding site(s) on the α7 nAChR, the receptor can be activated by the agonist. In contrast to strychnine, methlylycaconitine has slower kinetics of dissociation as evidenced by the lack of effect on the rising or the decay phase of the currents.

of the α4β2 nAChR channel, given that the magnitude of the effect of strychnine on the decay phase of type II currents was not altered by allowing the neurons to equilibrate with strychnine before their exposure to the admixture of ACh plus toxin (fig. 8B). These findings suggest that strychnine can interact with the open state of the nAChR channel suberving type II currents. Also, as illustrated on table 4, in the presence of the highest tested concentration of strychnine (i.e., 100 μM), the rise time of type II currents was accelerated, a finding that could be accounted for by the substantial acceleration of the decay phase of the currents.

Fig. 8. Concentration-dependent effect of strychnine on the decay phase of type II currents. The peak amplitudes of type II currents evoked by ACh (0.3 mM) in the presence of increasing concentrations of strychnine were scaled to match the peak amplitudes of the currents evoked by ACh alone. Each pair of traces was obtained from one neuron. A, Strychnine was applied to the neurons exclusively as an admixture with ACh. B, The admixture of strychnine plus ACh was applied to the neurons after their 10-min perfusion with strychnine-containing external solution. Holding potential, −60 mV. The quantification of the effect of strychnine applied to the neurons via the bath and the U-tube on the decay phase of type II currents is shown in table 4.

TABLE 4
Strychnine-induced changes in the kinetics of type II currents

<table>
<thead>
<tr>
<th>[Strychnine] (μM)</th>
<th>Rise Time (ms)</th>
<th>Decay-time Constant (τ)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(% of Control)</td>
<td>(% of Control)</td>
</tr>
<tr>
<td>10</td>
<td>79.7 ± 20.6</td>
<td>90.8 ± 18.8</td>
</tr>
<tr>
<td>30</td>
<td>89.5 ± 13.5</td>
<td>τ₁ 75.7 ± 22.9</td>
</tr>
<tr>
<td></td>
<td>84.6 ± 14.3</td>
<td>τ₂ 48.4 ± 12.1</td>
</tr>
<tr>
<td>100</td>
<td>46.1 ± 11.3</td>
<td>τ₃ 47.3 ± 11.8</td>
</tr>
</tbody>
</table>

a Mean ± S.E.M. (number of neurons). Under control conditions, the rise time of ACh (0.3 mM)-evoked type II currents ranged from 22.5 to 49.5 msec (n = 10).

b The decay phase of the ACh-evoked currents recorded from the first group of neurons was fitted by a single-exponential function and the decay-time constant of these currents ranged from 469 to 630 msec. The decay phase of the ACh-induced currents recorded from neurons of the other two experimental groups was fitted by a double-exponential function; the fast and the slow decay-time constants (τ₁ and τ₂) of these currents varied from 100 to 195 msec and from 997 to 1850 msec, respectively.
of the α7-nAChRs-mediated type IA currents (Alkondon et al., 1992).

To compare the apparent potency of strychnine as a competitive nicotinic antagonist at the α7 nAChRs in hippocampal neurons to the reported apparent potency of the toxin as a competitive antagonist at other receptors, the $K_a$ of the toxin for the α7 nAChRs of hippocampal neurons was determined according to the equation of Cheng and Prusoff (1973):

$$K_a = \frac{IC_{50}}{1 + [L]/K_d}$$

Using this equation, where [L] and $K_d$ are the concentration of the agonist and its affinity for the selected receptor, $IC_{50}$ and $K_a$ are the concentration of strychnine producing 50% inhibition of response and its apparent affinity, respectively, we estimated that the $K_a$ for strychnine as a competitive antagonist of ACh at the α7 nAChRs was 0.68 μM (table 1). This $K_a$ value for strychnine in blocking type IA currents is close to the $K_a$ for the toxin-induced inhibition of rat α7 nAChR homomers ectopically expressed in oocytes (0.14 μM, calculated from data in Séguela et al., 1993), as well as human α7 and α8 homomers ectopically expressed in oocytes (0.46 and 0.72 μM, calculated from data in Gerzanich et al., 1994).

**Mechanism of action of strychnine on the α4β2 nAChRs subserveing type II currents.** The acceleration of the decay phase of type II currents and the voltage-dependent reduction of the peak amplitude of these currents by strychnine strongly suggest that the toxin acts as an open-channel blocker of the α4β2 nAChR. This change in the decay phase of type II currents had the same magnitude regardless of whether the neurons were allowed to equilibrate with strychnine or were rapidly exposed to the admixture of this toxin with ACh. However, the apparent potency of strychnine in reducing the peak amplitude of type II currents was 3-fold higher when the neurons were perfused with strychnine-containing external solution before their exposure to the admixture of ACh plus strychnine, thus indicating that, in vivo, strychnine can also interact with α4β2 nAChRs that are not open.

**Structure-activity relationships of strychnine.** The pharmacophore for molecules that interact with the glycine receptor appears to bear two negative regions contributed by a coplanar amide nitrogen and phenyl group and by a positive region at the opposite end of the molecule borne by a nitrogen with its adjacent carbon. The antagonist property of strychnine is carried in the carbonyl oxygen of the molecule (Aprison et al., 1995; Galvez-Ruano et al., 1995). In contrast, it has been proposed that strychnine’s role as a nicotinic antagonist is met by the cationic nitrogen and the ether oxygen (fig. 1), both of which form a hydrogen bond with the nicotinic receptor (Beers and Reich, 1970; Sheridan et al., 1986). It is unclear whether the same pharmacophore would operate for the rat α7-containing nAChR. Nevertheless, this class of semi-rigid compounds may provide further insight to the structure of the neuronal nAChRs bearing the α7 subunit.

Regarding receptor specificity of strychnine, the glycine, GABA and nicotinic receptors are homologous members of a receptor superfamily (Betz, 1990). Strychnine binds to the glycine receptor α subunit at two domains that are homologous to the agonist binding regions of the nAChR (Vandenbergh et al., 1992). In the first domain, Y161 is invariant in the receptor superfamily. The ligand-binding domain just external to the M1 transmembrane segment is suggested to have amino acids in the antiparallel β-sheeted sheet arrangement ($K^W Y^Y N^T$) with K200 and Y202 being important for strychnine binding, and Y202 and T204 important for glycine binding (Rajendra et al., 1995). Adjacent to the ACh-binding domain of rat and chick nAChR α subunits, the rat nAChR α2, α4 and α7 subunits have the sequences $K^X Y^Y C^C, K^Y Y C^C$ or $R^Y Y C^C$ (Wada et al., 1989; Séguela et al., 1993), respectively. Other nAChR α subunits have different, but possibly related, nearby sequences: mouse, chick and Torpedo α1 have $K^W Y^Y$ (Schoepfer et al., 1990), chick α7 has $R^Y E^F$ and chick α8 has $K^N L Y^V$ (Schoepfer et al., 1990). Based on these sequences, one would expect that rat brain nAChRs would all be sensitive to strychnine, and that muscle-type receptors and chick brain α7 and α8 nAChRs would be less sensitive to strychnine. Indeed, according to our results, the $K_a$ for strychnine as an inhibitor of the α7 nAChRs in rat hippocampal neurons was found to be approximately 0.68 μM, whereas, according to previous studies the $K_a$ for strychnine as an inhibitor of the activation of the chick α7 nAChRs has been reported to be approximately 10-fold higher, i.e., about 6 μM (Anand et al., 1993).

**The relevance of the neuronal nAChR sensitivity to inhibition by strychnine.** The toxic effects of strychnine have been associated with its ability to block glycine receptors. However, based on our study, the $K_a$ for strychnine as a competitive antagonist of ACh at the α7 nAChRs is in the submicromolar range, as is the affinity of the toxin for the glycine receptors. Direct radioligand analysis has shown that strychnine binds to the glycine receptor with $K_a$ of 32 nM (Saitoh et al., 1994), and according to a pA2 analysis, strychnine interacts with glycine receptors with a $K_d$ of 270 nM (Itu and Cherubini, 1991). In many studies, strychnine concentrations of 1 to 10 μM (Mercuri et al., 1990; Ito and Cherubini, 1991) or 10 to 50 μM (Wu et al., 1992) have been used to inhibit glycine-agonist-mediated responses. Thus, at these concentrations generally used, the toxin may block not only the function of the glycine receptors but also that of neuronal nAChRs in the CNS. Considering that the degree of inhibition of receptors in vivo will be determined by the level of each endogenous agonist versus its affinity for its receptor, a challenge is raised to the common belief that the toxic effects of strychnine are mediated by its inhibitory action on glycine receptors. The finding that the activity of the α7-bearing nAChRs in the rat hippocampus was particularly sensitive to inhibition by strychnine supports the concept that these receptors are likely to be involved in controlling the overall neuronal excitability (Sargent, 1993; Lindstrom, 1995; Role and Berg, 1996; Albuquerque et al., 1997).

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