Br. J. Pharmacol. (1994), **113**, 471–478

Inhibitory effect of strychnine on acetylcholine receptor activation in bovine adrenal medullary chromaffin cells

Gemma A.J. Kuijpers, Leoncio A. Vergara, [1]Soledad Calvo & [2]*Gal Yadid

Laboratory of Cell Biology and Genetics, National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD, U.S.A. and *Dept. of Life Sciences, Bar-Ilan University, Israel

1 Strychnine, which is known as a potent and selective antagonist of the inhibitory glycine receptor in the central nervous system, inhibits the nicotinic stimulation of catecholamine release from bovine cultured adrenal chromaffin cells in a concentration-dependent (1–100 μM) manner. At 10 μM nicotine, the IC_{50} value for strychnine is approximately 30 μM. Strychnine also inhibits the nicotine-induced membrane depolarization and increase in intracellular Ca^{2+} concentration.

2 The inhibitory action of strychnine is reversible and is selective for nicotinic stimulation, with no effect observed on secretion elicited by a high external K^+ concentration, histamine or angiotensin II.

3 Strychnine competes with nicotine in its effect, but does not modify the apparent positive cooperativity of the nicotine binding sites. In the absence of nicotine, strychnine has no effect on catecholamine release. Glycine does not affect catecholamine release nor the inhibitory action of strychnine on this release.

4 These results suggest that strychnine interacts with the agonist binding site of the nicotinic acetylcholine receptor in chromaffin cells, thus exerting a pharmacological effect independently of the glycine receptor.

Keywords: Strychnine; acetylcholine; glycine; nicotine; glycine receptor; chromaffin, secretion; nicotinic ACh receptor

Introduction

The convulsive alkaloid, strychnine, has been established as a specific antagonist of the postsynaptic receptor for the inhibitory neurotransmitter, glycine, in the central nervous system (Curtis *et al.*, 1971; Young & Snyder, 1973; Fostholm & Rotter, 1985). The strychnine recognition site appears to be located on the 48 kDa subunit of the glycine receptor (Graham *et al.*, 1983; Pfeiffer *et al.*, 1984), which has significant structural and amino acid sequence homology with the nicotinic acetylcholine receptor polypeptide family (Grenningloh *et al.*, 1987).

In addition to its interaction with the glycine receptor, strychnine has been shown to interfere with cholinergic transmission. Several decades ago it was demonstrated that strychnine has a depressant effect on the response of the superior cervical ganglion and the neuromuscular junction to nerve impulses and to acetylcholine (ACh) (Lanari & Luco, 1939; Alving, 1961; Landau, 1967).

More recently, it was found that strychnine inhibits nicotine-activated currents in a recombinant insect α_7 acetylcholine receptor subunit expressed in oocytes (Marshall *et al.*, 1990), as well as in the functionally expressed rat brain α_7 nicotinic ACh receptor (Seguela *et al.*, 1993). The function of native chick ciliary ganglion α_7 ACh receptor is also inhibited by strychnine (Zhang *et al.*, 1994). Furthermore, strychnine inhibits the ACh-induced electrophysiological response of chick cochlear hair cells mediated by a novel cholinoceptor (Fuchs & Murrow, 1992). Strychnine also inhibits the binding of α-bungarotoxin to native and homomeric chick α_7 neuronal ACh receptor (Anand *et al.*, 1993), and to the cholinoceptor of *Aplysia* ganglionic neurones and rat brain neurones (Ono & Salvaterra, 1981). The antagonistic effects of strychnine on cholinoceptor, i.e., nicotinic receptor activation may be explained by a structural similarity of the nicotine and strychnine molecules (Beers & Reich, 1970).

The chromaffin cell has been extensively studied as a cholinergic neurosecretory system, and activation of the nicotinic receptor in these cells leads to the Ca^{2+}-dependent exocytotic release of catecholamines, adenosine triphosphate (ATP), chromogranins and enkephalins (Livett, 1984). Recently, Yadid *et al.* (1989, 1993) showed that bovine adrenal medullary chromaffin cells possess a high affinity [^{3}H]-strychnine binding site. These authors also showed that glycine and milacemide, a glycine prodrug, can evoke catecholamine release from slices of adrenal medullary tissue and from freshly isolated chromaffin cells (Yadid *et al.*, 1991; 1992).

These data led us to examine the effects of strychnine and glycine in the bovine chromaffin cell in more detail, and search for an interaction of strychnine with the nicotinic receptor in these cells.

Methods

Cell preparation and cell culture

Bovine adrenal glands were obtained from the slaughterhouse and kept on ice for ca. 60 min. Chromaffin cells were isolated from the adrenal medulla by three successive collagenase perfusions and purification on a Percoll gradient essentially as described by Kuijpers *et al.* (1989). Cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 nutrient medium (Sigma Chem. Co., St. Louis, MO, U.S.A.), buffered with 15 mM HEPES and 21 mM $NaHCO_3$, containing 10% foetal bovine serum (Biofluids, Rockville, MD, U.S.A.) and antibiotics (penicillin, 100 units ml^{-1}, streptomycin, 100 μg ml^{-1}, Fungizone, 250 ng ml^{-1}). Cells were cultured at a density of 0.5×10^6 cells cm^{-2} in 24-well culture plates (Costar Corp., Cambridge, MA, U.S.A.), and they attached to the plates within a few hours after plating. Since DMEM contains 0.3 mM glycine, in some studies on the effects of glycine, cells were cultured in glycine-free Minimal Essential Medium Eagle's (EMEM) medium (Biofluids), containing 10% dialyzed foetal bovine serum.

[1] Present address: Department of Pharmacology, Faculty of Medicine, University of Alicante, Spain.
[2] Author for correspondence at present address: Bar Ilan University, Ramat Gan, Israel 55900.

For intracellular calcium and membrane potential studies, cells were cultured on round coverslips (diameter, 25 mm) or in tissue culture dishes (diameter, 35 mm), respectively, in the absence of Fungizone, for 3–5 days.

Secretion studies

After 3–7 days of culture, cells were taken from the incubator and washed four to six times with a physiological salt solution (standard release medium, SRM) containing (mM): NaCl 150, KCl 5.6, $MgCl_2$ 1, NaH_2PO_4/Na_2HPO_4 1.5, $CaCl_2$ 2.2, glucose 10, HEPES 10 and 0.25% BSA (pH 7.35) over a 30–45 min time period. After washing, cells were incubated in SRM with the appropriate additions. Incubations were carried out by replacing SRM solutions completely for each experimental period. Cells were stimulated with nicotine or other agonists, or with K^+ for 10 min. Stimulation with K^+ was carried out by replacing 47.4 mM NaCl in the medium with 47.4 mM KCl. After stimulation the medium containing secreted catecholamines was removed from the well and diluted in concentrated acetic acid (final concentration of acetic acid, 10%). The cells were then lysed in 10% acetic acid and all samples were stored at −70°C until analysis of catecholamine content by the trihydroxyindole (THI) method. In one experiment samples were stored in 0.4% perchloric acid (PCA), and catecholamine analysis was performed by high performance liquid chromatography (h.p.l.c.) as well as by the THI method, as described below.

All experiments were done at room temperature.

Catecholamine analysis

Catecholamines were determined using a modification of the original THI method (von Euler & Floding, 1955; Kelner *et al.*, 1985). Standards were solutions of adrenaline in the appropriate diluent. In one experiment, catecholamines and DOPAC were also measured by h.p.l.c. For the THI method, catecholamines were converted to fluorescent trihydroxyindole products by potassium ferricyanide and measured in a spectrofluorometer with excitation set at 412 nm and emission at 523 nm.

For h.p.l.c., samples were filtered through 45 μm pore filters, diluted and injected into an h.p.l.c. apparatus coupled to an electrochemical detector. Separation of catecholamines was achieved by reverse phase liquid chromatography through a 5 μm column (4.6 mm i.d. × 250 mm) (Altex Ion Pair Ultrasphere C-18) with a column temperature of 30°C. Mobile phase consisted of 2.1 l water, 3.2 g Na-HEPES, 0.2 g EDTA, 16 ml triethylamine, 12 ml 85% *o*-phosphoric acid (all h.p.l.c. grade chemicals from Fisher Scientific, Fairlawn, NJ. U.S.A.) and 60 ml acetonitrile (Burdick & Jockson, Muskegon, MI, U.S.A.). Electrochemical detection was carried out with an EiCOM CB-100 analytical cell and a Waters 460 detector. The oxidation potential was set between 0.64 and 0.67 V. The mobile phase was pumped at 0.8 ml min^{-1}.

Intracellular calcium measurements

Chromaffin cells plated on coverslips were loaded with 5 μM indo-1 acetoxymethyl ester (Indo-1/AM) in a Krebs solution (KRB) for 120 min at room temperature. A stock solution of Indo-1/AM (1 mM) was previously prepared in anhydrous dimethylsulphoxide (DMSO) containing 20% Pluronic F127. The composition of KRB was (mM): NaCl 140, KCl 5, $CaCl_2$ 2.6, $MgCl_2$ 1, $NaHCO_3$ 5, glucose 5 and Na-HEPES 5 (pH 7.4). Coverslips were mounted on the stage of an inverted Diaphot-TMD microscope (Nikon, Inc.), and excited with a super high pressure mercury light source (Nikon, Inc.) and 340 nm filter. Emitted light was measured simultaneously at 410 nm and 485 nm by two separate photomultiplier tubes using the Nikon dual photometry system. Data were collected using the FastInCA programme developed at the Center for Image Analysis (College of Medicine, University of Cincinnati, Cincinnati, OH, U.S.A.) in collaboration with Nikon, Inc.

Membrane potential recording

The membrane potential was measured by the nystatin-perforated patch clamp technique (Horn & Marty, 1988). The composition of the pipette solution was (mM): KCl 70, K-aspartate 70, $MgCl_2$ 1, NaCl 10, K-HEPES 10 and nystatin 0.25 mg ml^{-1}, pH 7.2. The bath was continuously perfused with KRB at a rate of 5 ml min^{-1}. Nicotine and/or strychnine were added to the perfusion solution at the appropriate times. A signal was recorded using the EPC-7 patch clamp amplifier in current clamp mode, and the data were digitized on line using a TL-1 DMA interface and the Axotape programme (Axon Instruments, Foster City, CA, U.S.A.).

Drugs used

Nicotine (free base), histamine dihydrochloride, glycine (free base), and angiotensin II (human) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Oxotremorine-M (oxotremorine methiodide) was obtained from Research Biochemicals Inc., Natick, MA, U.S.A.

Data and statistical analysis

Results on catecholamine secretion obtained by the THI method were expressed as percentage of total cellular catecholamine content of the start of the experiment, or as percentage of control or maximal response. Within an experiment, incubations were always done in triplicate and the results were averaged. The data shown are averages with s.e.mean of n individual experiments. Results from one experiment obtained by h.p.l.c. were expressed as picogram of catecholamine per supernatant sample volume, and given as averages with s.e.mean of three determinations. Statistical analysis was carried out by Student's two-tailed t test for paired measurements, or a two-way analysis of variance (ANOVA).

Results

Bovine cultured chromaffin cells released 11–32% of their total catecholamine content upon stimulation with a nicotine concentration causing maximal release (10 μM). Basal, unstimulated, catecholamine release varied between 0.1 and 1.8% of the total cell content. Strychnine (1–100 μM) induced a dose-dependent inhibition of nicotine-stimulated catecholamine release. At a nicotine concentration of 10 μM, the inhibition by strychnine ranged from 18% at 10 μM strychnine to 88% at 100 μM strychnine, with an IC_{50} value for strychnine of approximately 30 μM (Figure 1). Strychnine concentrations up to 100 μM did not affect a non-receptor-mediated secretory response, caused by membrane depolarization due to elevation of the extracellular K^+ concentration from 5.6 mM to 53 mM (Figure 1). Strychnine did not affect the basal release of catecholamines.

The effect of strychnine on nicotine-stimulated catecholamine release was very rapid and the extent of the inhibition induced by 35 μM strychnine was not significantly different for strychnine preincubation times in the range of 0–120 s (Figure 2). The effect was also reversible, since repeated washing of the cells with SRM after a 2 min incubation with 35 μM strychnine fully restored the subsequent secretory response of the cells to nicotine (Figure 2). Analysis of the various catecholamines adrenaline, noradrenaline, dopamine, 5-HT (5-hydroxytryptamine) and the dopamine metabolite DOPAC (3,4-dihydroxyphenylacetic acid) by h.p.l.c. showed that strychnine inhibits the nicotine-stimulated release of all these amines, albeit to different extents, with inhibition being

most profound in the case of adrenaline (82%) and least in the case of noradrenaline (28%) (Figure 3).

The dose-response curves relating nicotine concentration to secretory response in the presence and absence of strychnine are shown in Figure 4. This figure clearly shows that the effect of strychnine on catecholamine release is competitive, and that the strychnine-induced inhibition can be overcome by increasing the nicotine concentration. The nicotine concentration at which half maximal secretion occurs is shifted by strychnine (30 μM) from ca. 5 μM to ca. 15 μM. Analysis of the dose-response curves revealed that the Hill coefficient for nicotine-stimulated catecholamine release is 2.55 ± 0.50 (average ± s.e.mean, $n = 3$), and is not changed significantly by strychnine (10–30 μM) (Table 1). Strychnine also inhibited competitively the secretion induced by the cholinoceptor agonist, oxotremorine-M (Oxo-M) (Figure 5). At 100 μM Oxo-M, the IC_{50} value for strychnine was approximately

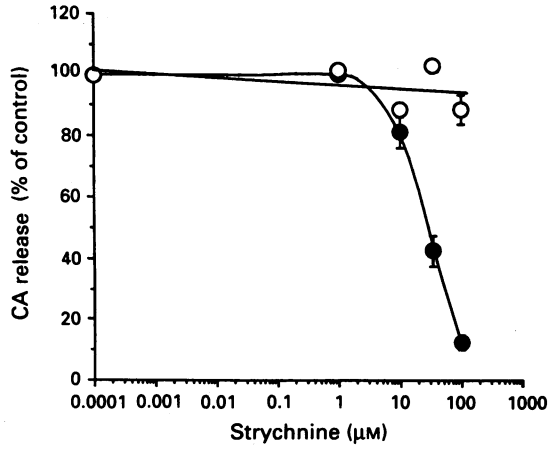

Figure 1 Effect of strychnine (1–100 μM) on catecholamine (CA) release stimulated by nicotine (10 μM) (●) and K^+ (53 mM) (○) from chromaffin cells. Data are expressed as percentage of control release in the absence of strychnine, and are averages ± s.e.mean of 2–9 (nicotine) and 1–3 (K^+) experiments. All data represent net release, i.e., basal release was subtracted from total release to give the response to agonist alone. Basal CA release was 0.78 ± 0.14% (average ± s.e.mean, $n = 13$) of total cellular CA content at the start of the incubation. Control release was, for nicotine (10 μM): 17.7 ± 2.2% of total cellular CA, and for K^+ (53 mM): 6.4 ± 0.5% of total CA ($n = 12$ and $n = 4$, respectively). Curves are significantly different from each other ($P < 0.001$, ANOVA). For all experiments, the total cellular catecholamine content varied between 30 and 100 nmol/10^6 cells.

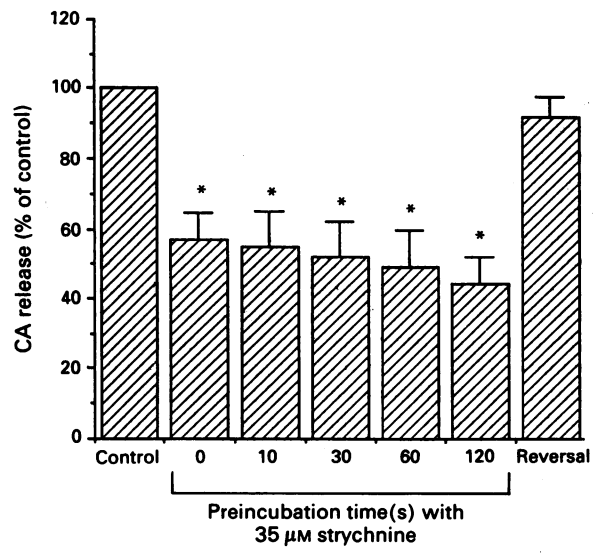

Figure 2 Effect of preincubation time(s) with strychnine on nicotine-stimulated catecholamine (CA) release. Cells were preincubated with strychnine (35 μM) for the indicated period of time, and then stimulated with nicotine (10 μM). The response was expressed as a percentage of control release induced by nicotine alone. Data given are average ± s.e.mean of four experiments. The reversal indicates CA release of cells in response to nicotine (10 μM), after they had been preincubated with strychnine (35 μM) for 120 s and then washed 5–8 times with SRM (5 min total). Basal secretion was 0.66 ± 0.08% of total cellular CA, and control release stimulated by nicotine was 14 ± 4% of total CA ($n = 4$). *Significant difference from control ($P < 0.001$, Student's t test). Effects of different preincubation times (0–120 s) were not significantly different from each other.

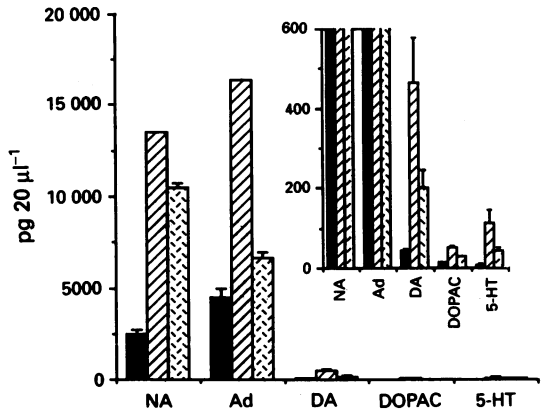

Figure 3 Effect of strychnine on catecholamine and catecholamine metabolite secretion, NA = noradrenaline; Ad = adrenaline; DA = dopamine; DOPAC = 3,4-dihydroxyphenylacetic acid; 5-HT = 5-hydroxytryptamine. Cells were incubated with SRM (solid columns), nicotine (2 μM) (hatched columns) or nicotine in the presence of strychnine (30 μM) (stippled columns). Samples of the cell media from three different wells, collected after 10 min of incubation, were assayed by h.p.l.c. and electrochemical detection. The data are given as pg 20 μl^{-1} cell medium (mean ± s.e.mean; $n = 3$). Insert shows same graph, but at smaller abscissa scale. The total cellular catecholamine contents of the differently treated cells were: (control, SRM) 62.6 ± 2.7, (nicotine) 55.8 ± 0.9, and (nicotine + strychnine) 63.7 ± 0.5 nmol/10^6 cells ($n = 3$).

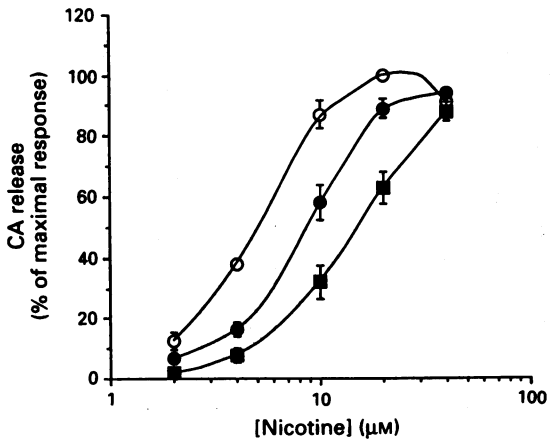

Figure 4 Effect of strychnine on dose-response curves for nicotine-stimulated catecholamine (CA) release from chromaffin cells. Cells were incubated with nicotine alone (○) or with nicotine in the presence of 10 μM strychnine (●) or 30 μM strychnine (■). Data given are averages ± s.e.mean of three experiments. Results are expressed as percentage of the maximal response with 20 μM nicotine (17.6 ± 3.2% of total cellular CA). Basal secretion was 0.84 ± 0.27% of total cellular CA ($n = 3$). The control response is significantly different from the response in the presence of 10 μM strychnine ($P < 0.005$, ANOVA), and the latter is significantly different from the response in the presence of 30 μM strychnine ($P < 0.01$, ANOVA).

Table 1 Effect of strychnine on Hill coefficient for nicotine-stimulated catecholamine release from bovine chromaffin cells

Nicotine (control)	*Nicotine + strychnine* (10 μM)	*Nicotine + strychnine* (30 μM)
2.55 ± 0.50	2.31 ± 0.33	2.48 ± 0.44

Hill coefficients are calculated for catecholamine release response elicited by nicotine (2–20 μM) (control) or nicotine (2–20 μM) + strychnine (10 and 30 μM).

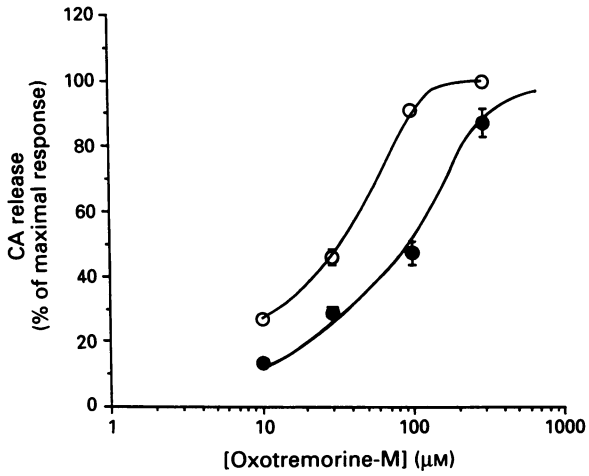

Figure 5 Effect of strychnine on dose-response curves for oxotremorine-M (Oxo-M)-stimulated catecholamine (CA) release. Cells were incubated with Oxo-M alone (○) or with Oxo-M in the presence of strychnine (35 μM) (●). Data given are averages ± s.e.mean of 2–4 experiments. Results are expressed as percentage of maximal secretion with 300 μM Oxo-M (15.3 ± 3.0%). Basal secretion was 1.05 ± 0.25% of total CA ($n = 4$). Control response is significantly different from response in presence of 35 μM Oxo-M ($P < 0.01$, ANOVA).

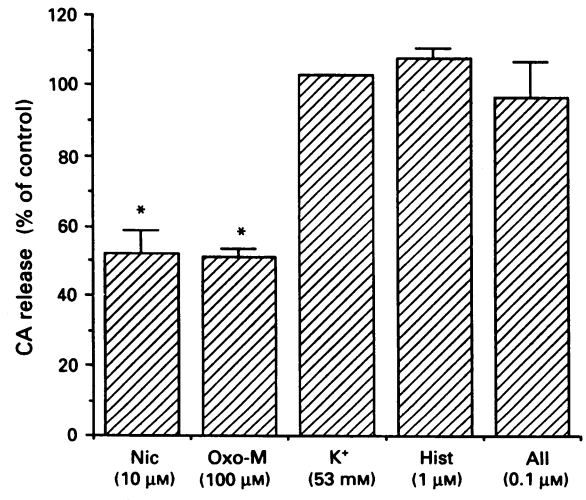

Figure 6 Effect of strychnine on catecholamine (CA) release induced by various agonists. Agonists were used at the concentrations indicated. Cells were washed, and then incubated with agonist in the absence or presence of strychnine (35 μM). Data are given as percentage of control release induced by the agonist in the absence of strychnine and are averages ± s.e.mean of n experiments. Control release was: nicotine (Nic) (10 μM): 15.2 ± 2.3% ($n = 8$); oxotremorine-M (Oxo-M) (100 μM): 12.3 ± 3.2% ($n = 3$); K^+ (53 mM): 4.99 ($n = 1$); angiotensin II (AII) (0.1 μM): 1.22 ± 0.06% ($n = 4$); histamine (Hist) (1 μM): 2.26 ± 0.40% ($n = 3$). *Significant difference from control ($P < 0.001$, Student's t test).

35 μM. Half maximal secretion occurs at ca. 35 μM Oxo-M in the absence of strychnine, and at ca. 90 μM in the presence of 35 μM strychnine.

In order to investigate the selectivity of the inhibitory effect of strychnine, we measured the catecholamine release of chromaffin cells in response to various agonists, in the absence and presence of strychnine (Figure 6). As described above, strychnine (1–100 μM) had no effect on secretory stimulation induced by depolarization of the cell membrane. Moreover, strychnine did not inhibit the catecholamine release induced by histamine (1 μM) or angiotensin II (0.1 μM) (Figure 6).

Glycine (0.1–1 mM) did not affect the inhibition of cholinoceptor-stimulated release by strychnine (35 μM). Moreover, glycine (1–1000 μM) did not have a significant effect on basal catecholamine release but it did slightly potentiate the nicotine-stimulated catecholamine release. In the latter study, cells were cultured in glycine-free EMEM medium (data not shown).

In order to localize the effect of strychnine in the cascade of events involved in receptor-activated catecholamine secretion, we studied the nicotine-induced changes in intracellular calcium concentration ($[Ca^{2+}]_i$) and membrane potential. Under resting conditions, $[Ca^{2+}]_i$ was 110 ± 9 nM (average ± s.e.mean, $n = 11$) and increased to 237 ± 36 nM ($n = 11$) upon stimulation of the cells by 10 μM nicotine (Figure 7a,b). After removal of the agonist from the cell medium, $[Ca^{2+}]_i$ gradually returned to resting values. When a second pulse of nicotine was applied in the presence of 40 μM strychnine, the response was significantly reduced to 61 ± 5% ($n = 7$) of the first response in the absence of strychnine (t test, $P < 0.05$) (Figure 7a). This effect of strychnine was reversible, as shown by the recovery of the response following a third exposure of the cells to nicotine alone, to 82 ± 2% ($n = 7$) of the first response. In control experiments, in which the cells were repeatedly stimulated with 10 μM nicotine in the absence of strychnine, the response of the cells to the second application of nicotine was similar to the first response (97 ± 1%; $n = 4$) (Figure 7b). Strychnine did not affect the basal level of $[Ca^{2+}]_i$ (Figure 7a).

Nicotine (10 μM) also induced a rapid depolarization of the cell membrane followed by a slower recovery phase (Figure 8a). The response of the membrane potential to nicotine was totally reversible. Up to four repetitive stimulations of a cell, separated by 3 min intervals, elicited similar responses, as shown in Figure 8b and no desensitization was evident under our experimental conditions. When strychnine (40 μM) was added simultaneously with nicotine during the second stimulation, the depolarizing response was significantly reduced (Figure 8a). This effect of strychnine was fully reversible, as a total recovery of the response was attained following a third stimulation of the cell with nicotine alone, shortly after washout of the strychnine from the medium (Figure 8a). Strychnine (40 μM) did not affect the resting membrane potential. The inhibitory effect of strychnine was very rapid, since the extent of the inhibition was the same after preincubation of the cell with strychnine for zero and 2 min (data not shown).

Discussion

In this study, we have obtained evidence that strychnine can interact with the peripheral nicotinic acetylcholine receptor expressed in adrenal medullary chromaffin cells. Strychnine blocks nicotine- and oxotremorine-M-stimulated catecholamine secretion from chromaffin cells, but does not affect non-cholinoceptor stimulation by agonists such as histamine and angiotensin II, or by elevation of the extracellular K^+ concentration. These results suggest that the inhibitory action of strychnine is specific for acetylcholine receptor activation. The reversibility of the strychnine effect suggests that the inhibitory action is mediated through a cellular receptor.

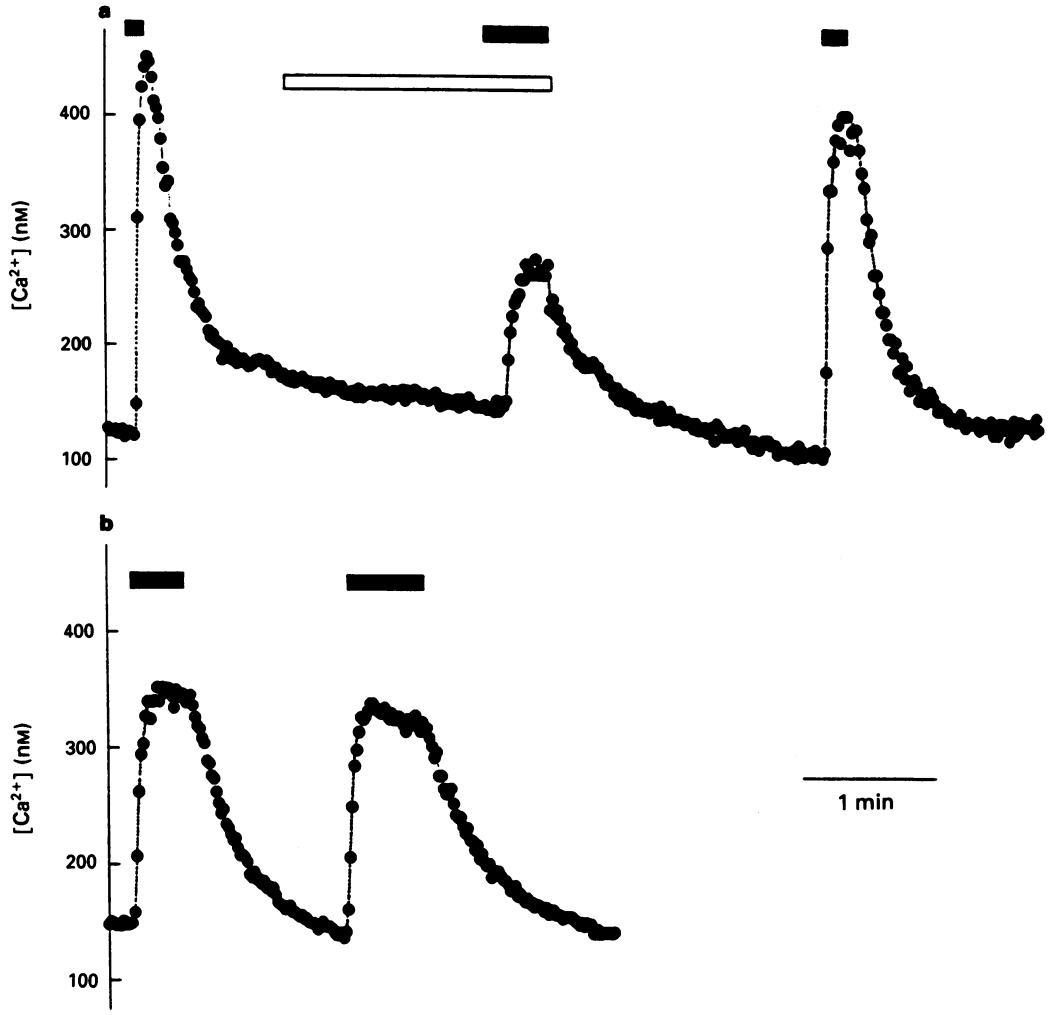

Figure 7 Effect of strychnine on intracellular Ca^{2+} transients in response to nicotine stimulation. Cells were (a) incubated with nicotine (10 μM), followed by nicotine + strychnine (40 μM), and then nicotine again, or (b) incubated with nicotine, followed by nicotine alone again. Preincubation time with strychnine was 80 s. Results shown are from single experiments, and are representative for 7 (a) and 4 (b) experiments. Solid bars indicate application of nicotine, open bars indicate application of strychnine.

The competitiveness of the effect with cholinoceptor agonists indicates that strychnine may interact with the ACh binding site itself, or with a site distal but very close to the receptor, thereby reducing its apparent affinity for cholinoceptor agonists. The rapid and reversible inhibition by strychnine of the increase in membrane potential and $[Ca^{2+}]_i$ induced by nicotine, is in agreement with the results obtained in the secretion studies, and supports the conclusion that strychnine interferes with an early step in the nicotinic acetylcholine receptor mediated cell stimulation.

The results of our study are in accordance with the older findings that strychnine and ACh can act in an antagonistic manner. Bonnet (1938) showed that in crayfish peripheral ganglia, ACh antagonizes the paralytic effects of strychnine. Similarly, Lanari & Luco (1939), Alving (1961) and Landau (1967) demonstrated that strychnine has a curare-like, depressant action on the sympathetic superior cervical ganglion and the neuromuscular junction stimulated by ACh or nerve stimulation.

The inhibition of the Oxo-M-induced catecholamine release by strychnine can be interpreted in light of recent findings by Shirvan *et al.* (1991), that in the chromaffin cell the nicotinic ACh receptor is activated by the otherwise muscarinic agonist Oxo-M. Shirvan *et al.* (1991) argued that nicotine and Oxo-M occupy different sites on this unique cholinoceptor. Our results indicate that these two sites cannot be distinguished in our study, since nicotine- and Oxo-M-stimulated CA release appear to be about equally sensitive to inhibition by strychnine.

The Hill coefficient for the nicotine-induced secretory response is 2.55. This suggests a positive cooperativity between the nicotine (ACh) binding sites at the nicotinic ACh receptor on chromaffin cells. Hill coefficients of 1.5–2.7 have been found in skeletal muscle fibres (Maelicke, 1984), and have been taken to indicate a positive cooperativity between two or more agonist binding sites at the ACh receptor. The lack of an effect of strychnine on the Hill coefficient thus suggests that strychnine, although interfering with the secretory response elicited by nicotine via an effect on its binding site, does not affect the apparent cooperative interaction between the ACh binding sites.

Our h.p.l.c. measurements suggest that strychnine induces a differential inhibition of the cholinoceptor mediated release of various catecholamines from chromaffin cells. In particular, the release of noradrenaline is reduced to a much lesser extent than the release of adrenaline. The latter finding may be due to a difference in sensitivity between the ACh receptors on adrenaline-and noradrenaline-containing cells.

The lack of an effect of glycine on catecholamine release contradicts previous findings from other laboratories (Yadid *et al.*, 1991; 1992), that glycine can induce the release of catecholamines in the absence of other agonists. This discrepancy could be due to the different ways in which the cells were prepared. In this study we used isolated chromaffin

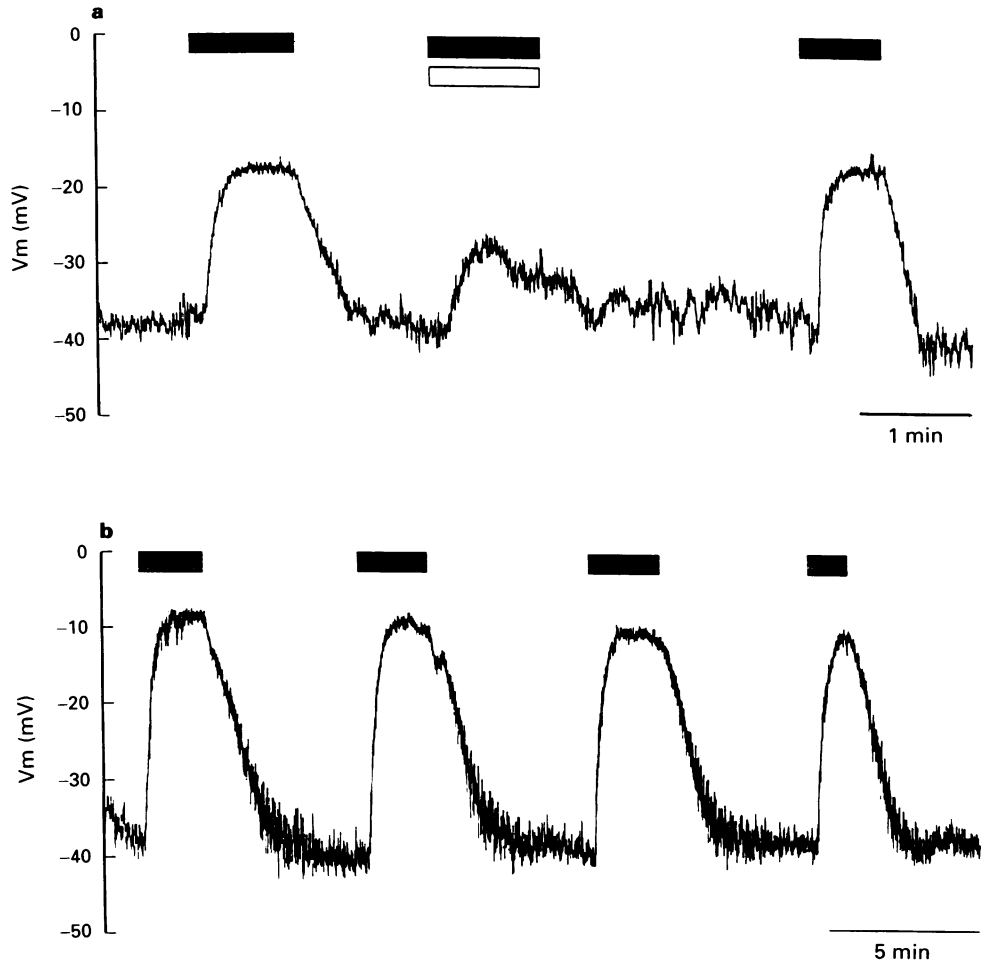

Figure 8 Effect of strychninė on membrane potential elevation induced by nicotine. Cells were (a) activated by nicotine (10 μM), followed by nicotine (10 μM) + strychnine (40 μM), and then nicotine (10 μM) again, or (b) repeatedly activated by nicotine (10 μM). Results shown are from single experiments, and are representative for three experiments. Solid bars indicate application of nicotine, open bars indicate application of strychnine.

cells, which were cultured for 3–7 days, whereas in the experiments of Yadid *et al.* (1991, 1992) freshly isolated chromaffin cells or slices of adrenal medulla were used. The exposure of the cells to serum or other factors during the cell culture might affect the expression of a functional strychnine-sensitive glycine receptor in our cells. At any rate, the absence of an effect of glycine on the inhibitory effect of strychnine reported in this study, suggests that strychnine exerts its effect through a direct blockade of the nicotinic acetylcholine receptor.

The inhibitory effect we describe occurs at relatively high, micromolar concentrations of strychnine. Blockade of cholinergic transmission at the sympathetic ganglion and neuromuscular junction has also been reported to take place in the concentration range of 10–80 μM (Lanari & Luco, 1939), 2–130 μM (Landau, 1967) or 20–100 μM (Alving, 1961). At these concentrations the drug is likely to have its lethal effect through paralysis of the neuromuscular junctions (Wall *et al.*, 1955). However, the concentration needed to inhibit a cholinoceptor-mediated response appears to depend on what type of ACh receptor is studied. In a recent study, it was found that 0.1 μM strychnine blocks the nicotine-activated current in a recombinant insect α-like nervous system ACh receptor expressed in oocytes (Marshall *et al.*, 1990). Also, an IC_{50} value of 0.35 μM was reported for strychnine inhibition of the current response to nicotine of a functionally expressed rat brain homooligomeric α_7 nicotinic receptor (Seguela *et al.*, 1993). In chick cultured ciliary ganglion neurones, containing native α_1 ACh receptors, Zhang *et al.* (1994) found that 1–10 μM strychnine inhibits the rapid and slow component of the nicotinic response. Moreover, in a novel cholinoceptor from chick isolated cochlear hair cells (Fuchs & Murrow, 1992), strychnine blocks the ACh response for 80–100% at concentrations of 0.3–3 μM.

With respect to binding to the nicotinic ACh receptor, K_i values for strychnine, reflecting its ability to compete with α-bungarotoxin (αBuTX) binding, of 5.4 and 6.9 μM have been reported for native brain α_7 receptor and α_7 homomers expressed in oocytes, respectively (Anand *et al.*, 1993). Similarly, K_i values of 6.8 μM and 2.2 μM have been reported for the cholinoceptor in *Aplysia* ganglia and rat brain membranes (Ono & Salvaterra, 1981). However, strychnine appears to be a more potent competitive inhibitor of αBuTX binding in central nervous tissue than in peripheral tissue (Schmidt & Raferty, 1974; Ono & Salvaterra, 1981). Taken together, these results are in accordance with our findings that strychnine, at micromolar concentrations, can competitively antagonize nicotinic receptor responses.

The K_d values reported in studies on the binding of strychnine to the glycine receptor are in the nanomolar range (Young & Snyder, 1973; 1974; Marvizon *et al.*, 1986; Yadid *et al.*, 1989). However, the concentration of strychnine needed to inhibit a functional glycine receptor-mediated response appears to be in the range of 1–10 μM (Mercuri *et al.*, 1990; Ito & Cherubini, 1991; Droge & Tao, 1993), or even 10–100 μM (Wu *et al.*, 1992). This discrepancy may partly be explained by the fact that the K_d values found in binding studies can be several orders of magnitude smaller than

suggested by dose-response studies under physiological conditions (Maelicke & Prinz, 1983; Paul *et al.*, 1993). Thus, the effects of strychnine in cholinoceptor systems can occur at similar concentrations as in glycine receptor systems.

Apart from its interaction with the glycine receptor, strychnine has been shown, although less effectively, to interfere with GABA-mediated neuronal inhibition (Curtis *et al.*, 1968; 1971; Davidoff *et al.*, 1969; Safronov *et al.*, 1989). In addition, interactions between bicuculline, a specific $GABA_A$ receptor antagonist, and specific [^{3}H]-strychnine binding have been observed (Goldinger & Muller, 1980; Marvizon *et al.*, 1986).

It has been suggested that strychnine can affect membrane ion permeability changes (Phillis & York, 1967; De Groat, 1970; Curtis *et al.*, 1971; Freeman, 1972). Indeed, recent studies have shown that at high micromolar concentrations (10–300 μM), strychnine can block a number of ion channels, e.g., K^+ and Na^+ channels in nervous tissues (Shapiro, 1977a,b; Cahalan & Almers, 1979; Yamamoto, 1986), and Ca^{2+} channels in brain and heart muscle (Ramos, 1974; O'Neill & Bolger, 1990). The fact that the inhibitory effect of strychnine on nicotine-stimulated catecholamine release, described in this paper, is competitive with nicotine, and the fact that K^+-stimulated release is unaffected, however, argues against a blockade of non-receptor coupled ion channels as the mechanism for the inhibition.

In conclusion, the results of our study demonstrate that strychnine specifically inhibits nicotine-stimulated catecholamine secretion from chromaffin cells, most likely by competitively antagonizing the activation of the chromaffin cell nicotinic ACh receptor by its agonists. The chemical similarities between the nicotine and strychnine molecules (Beers & Reich, 1970), as well as the homology between the well-characterized strychnine-binding subunit on the inhibitory glycine receptor and nicotinic acetylcholine receptor polypeptides (Greeningloh *et al.*, 1987), are in accordance with the hypothesis that strychnine can bind to the nicotinic ACh receptor, either at or close to the agonist binding site. Since nicotinic ACh receptors are thought to be members of a gene family of ligand-gated ionotropic receptors including neuronal and muscle nicotinic receptors, $GABA_A$ receptors, and glycine receptors (Grenningloh *et al.*, 1987; Schofield *et al.*, 1987), and since strychnine can interfere with the physiological events mediated through any of these receptor proteins, we suggest that strychnine interacts with an allosteric site common to the ion channel forming transmitter receptors.

We thank Dr H.B. Pollard for helpful discussion during the course of this work, and for critically reading the manuscript.

References

ALVING, B.O. (1961). The action of strychnine at cholinergic junctions. *Arch. Int. Pharmacodyn. Ther.*, **131**, 123–150.

ANAND, R., PENG, X. & LINDSTROM, J. (1993). Homomeric and native α_7 acetylcholine receptors exhibit remarkably similar but non-identical pharmacological properties, suggesting that the native receptor is a heteromeric protein complex. *FEBS Lett.*, **327**, 241–246.

BEERS, W.H. & REICH, E. (1970). Structure and activity of acetylcholine. *Nature*, **228**, 917–922.

BONNET, V. (1938). Action of strychnine and acetylcholine on neuronic rhythmicity in crustaceans. *Compt. Rend. Soc. Biol.*, **127**, 804–806.

CAHALAN, M.D. & ALMERS, W. (1979). Block of sodium conductance and gating current in squid giant axons poisoned with quaternary strychnine. *Biophys. J.*, **27**, 57–74.

CURTIS, D.R., DUGGAN, A.W. & JOHNSTON, G.A.R. (1971). The specificity of strychnine as a glycine antagonist in the mammalian spinal cord. *Exp. Brain Res.*, **12**, 547–565.

CURTIS, D.R., HOSLI, L. & JOHNSTON, G.A.R. (1968). A pharmacological study of the depression of spinal neurones by glycine and related amino acids. *Exp. Brain Res.*, **6**, 1–18.

DAVIDOFF, R.A., APRISON, M.H. & WERMAN, R. (1969). The effects of strychnine on the inhibitory effects of glycine and γ-aminobutyric acid. *Int. J. Neuropharmacol.*, **8**, 191–194.

DE GROAT, W.C. (1970). The effects of glycine, GABA and strychnine on sacral parasympathetic preganglionic neurones. *Brain Res.*, **18**, 542–544.

DROGE, M.H. & TAO, Y. (1993). Glycine effects on in vitro motor pattern generation in mouse spinal cord. *Neurosci. Lett.*, **158**, 139–142.

VON EULER, U.S. & FLODING, I. (1955). A fluorometric method for differential estimation of adrenaline and noradrenaline. *Acta Physiol. Scand.*, **33**, (Suppl. 118), 45–56.

FOSTHOLM, A. & ROTTER, A. (1985). Glycine receptor distribution in mouse CNS: autoradiographic localization of [^{3}H]strychnine binding sites. *Brain Res. Bull.*, **15**, 473–486.

FREEMAN, A.R. (1972). Axon membrane effects of inhibitory synaptic blocking agents. *Fed. Proc.*, **31**, 333.

FUCHS, P.A. & MURROW, B.W. (1992). A novel cholinergic receptor mediates inhibition of chick cochlear hair cells. *Proc. R. Soc. B.*, **248**, 35–40.

GOLDINGER, A. & MULLER, W.E. (1980). Stereospecific interaction of bicuculline with specific [^{3}H]-strychnine binding to rat spinal cord synaptosomal membranes. *Neurosci. Lett.*, **16**, 91–95.

GRAHAM, D., PFEIFFER, F. & BETZ, H. (1983). Photoaffinity labelling of the glycine receptor of the rat spinal cord. *Eur. J. Biochem.*, **131**, 519–525.

GRENNINGLOH, G., RIENITZ, A., SCHMITT, B., METHFESSEL, C., ZENSEN, M., BEYREUTHER, K., GUNDELFINGER, E.D. & BETZ, H. (1987). The strychnine-binding subunit of the glycine receptor shows homology with nicotinic acetylcholine receptor. *Nature*, **328**, 215–220.

HORN, R. & MARTY, A. (1988). Muscarinic activation of ionic currents measured by a new whole-cell recording method. *J. Gen. Physiol.*, **92**, 145–159.

ITO, S. & CHERUBINI, E. (1991). Strychnine-sensitive glycine responses of neonatal rat hippocampal neurones. *J. Physiol.*, **440**, 67–83.

KELNER, K.L., LEVINE, R.A., MORITA, K. & POLLARD, H.B. (1985). A comparison of trihydroxyindole and HPLC/electrochemical methods for catecholamine measurement in adrenal chromaffin cells. *Neurochem. Int.*, **7**, 373–378.

KUIJPERS, G.A.J., ROSARIO, L. & ORNBERG, R.L. (1989). Role of intracellular pH in secretion from adrenal medullar chromaffin cells. *J. Biol. Chem.*, **264**, 698–705.

LANARI, A. & LUCO, J.V. (1939). The depressant action of strychnine on the superior cervical sympathetic ganglion and on skeletal muscle. *Am. J. Physiol.*, **126**, 277–282.

LANDAU, E.M. (1967). The effect of strychnine on the neuromuscular junction of the rat. *Life Sci.*, **6**, 2515–2517.

LIVETT, B.G. (1984). Adrenal medulary chromaffin cells in vitro. *Physiol. Rev.*, **64**, 1103–1161.

MAELICKE, A. (1984). Biochemical aspects of cholinergic excitation. *Angew. Chem. Int. Ed. Engl.*, **23**, 195–221.

MAELICKE, A. & PRINZ, H. (1983). The acetylcholine receptor-ion channel complex: Linkage between binding and response. In *Modern Cell Biology*, Vol. 1 (Satir, B.H., series ed.), pp. 171–197. New York: Alan R. Liss, Inc.

MARSHALL, J., BUCKINGHAM, S.D., SHIGAI, R., LUNT, G.G., GOOSEY, M.W., DARLISON, M.G., SATTELLE, D. & BARNARD, E. (1990). Sequence and functional expression of a single α-subunit of an insect nicotinic acetylcholine receptor. *EMBO J.*, **9**, 4391–4398.

MARVIZON, J.C.G., VAZQUEZ, J., CALVO, M.G., MAYOR, F., RUIZ-GOMEZ, A., VALDIVIESO, F. & BENAVIDES, J. (1986). The glycine receptor: pharmacological studies and mathematical modelling of the allosteric interaction between the glycine- and strychnine-binding sites. *Mol. Pharmacol.*, **30**, 590–597.

MERCURI, N.B., CALABRESI, P. & BERNARDI, G. (1990). Effects of glycine on neurons in the rat substantia nigra zona compacta: In vitro electrophysiological study. *Synapse*, **5**, 190–200.

O'NEILL, S.K. & BOLGER, G.T. (1990). The effects of strychnine on the regulation of voltage-dependent calcium channels by dihydropyridines in brain and heart. *Pharmacol. Biochem. Behav.*, **35**, 833–840.

ONO, J.K. & SALVATERRA, P.M. (1981). Snake α-toxin effects on cholinergic and non cholinergic responses of Aplysia Californica neurons. *J. Neurosci.*, **1**, 259–270.

PAUL, I., BASILE, A.S., ROJAS, E., YOUDIM, M.B.H., DE COSTA, B., SKOLNICK, P., POLLARD, H.B. & KUIJPERS, G.A.J. (1993). Sigma receptors modulate nicotinic receptor function in adrenal chromaffin cells. *FASEB J.*, **7**, 1171–1178.

PFEIFFER, F., SIMLER, R., GRENNINGLOH, G. & BETZ, H. (1984). Monoclonal antibodies and peptide mapping reveal structural similarities between the subunits of the glycine receptor of the spinal cord. *Proc. Natl. Acad. Sci. U.S.A.*, **81**, 7224–7227.

PHILLIS, J.W. & YORK, D.H. (1967). Strychnine block of neural and drug-induced inhibition in the cerebral cortex. *Nature*, **216**, 922–923.

RAMOS, J.G. (1974). On the mechanism of strychnine spikes. *Acta Physiol. Lat. Am.*, **24**, 124–133.

SAFRONOV, B.V., BAEV, K.V., BATUEVA, I.V., RUSIN, K.I. & SUDEREVSKAYA, E.I. (1989). Peculiarities of receptor-channel complexes for inhibitory mediators in the membranes of lamprey spinal cord neurons. *Neurosci. Lett.*, **102**, 82–86.

SCHMIDT, J. & RAFERTY, M.A. (1974). The cation sensitivity of the acetylcholine receptor from Torpedo californica. *J. Neurochem.*, **23**, 617–623.

SCHOFIELD, P.R., DARLISON, M.G., FUJITA, N, BURT, D.R., STEPHENSON, F.A., RODRIGUEZ, H., RHEE, L.M., RAMACHANDRAN, J., REALE, V., GLENCORSE, T.A., SEEBURG, P.H. & BARNARD, E.A. (1987). Sequence and functional expression of the $GABA_A$ receptor shows a ligand-gated receptor super-family. *Nature*, **328**, 221–227.

SEGUELA, P., WADICHE, J, DINELEY-MILLER, K., DANI, J.A. & PATRICK, J.W. (1993). Molecular cloning, functional properties, and distribution of rat brain α_7: a nicotinic cation channel highly permeable to calcium. *J. Neurosci.*, **13**, 596–604.

SHAPIRO, B.I. (1977a). Effects of strychnine on the potassium conductance of the frog node of ranvier. *J. Gen. Physiol.*, **69**, 897–914.

SHAPIRO, B.I. (1977b). Effects of strychnine on the sodium conductance of the frog node of ranvier. *J. Gen. Physiol.*, **69**, 915–926.

SHIRVAN, M.H., POLLARD, H.B. & HELDMAN, E. (1991). Mixed nicotinic and muscarinic features of cholinergic receptor coupled secretion in bovine chromaffin cells. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 4860–4864.

WALL, P.D., MCCULLOCH, W.S., LETTVIN, J.Y. & PITTS, W.H. (1955). Effects of strychnine with special reference to spinal afferent fibres. *Epilepsy*, **4**, 29–39.

WU, W., ZISKIND-CONHAIM, L. & SWEET, M. (1992). Early development of glycine- and GABA-mediated synapses in rat spinal cord. *J. Neurosci.*, **12**, 3935–3945.

YADID, G., MAOR, G., YOUDIM, M.B.H., SILBERMAN, M. & ZINDER, O. (1993). Autoradiographic localization of strychnine-sensitive glycine receptor in bovine adrenal medulla. *Neurochem. Res.*, **18**, 1051–1055.

YADID, G., YOUDIM, M.B.H. & ZINDER, O. (1989). High affinity strychnine binding to adrenal medulla chromaffin cell membranes. *Eur. J. Pharmacol.*, **175**, 365–366.

YADID, G., ZINDER, O. & YOUDIM, M.B.H. (1991). Effect of the prodrug milacemide (2-N-pentylaminoacetamide) on catecholamine secretion from isolated adrenal chromaffin cells. *Br. J. Pharmacol.*, **104**, 760–764.

YADID, G., ZINDER, O. & YOUDIM, M.B.H. (1992). Preferred release of epinephrine by glycine from adrenal chromaffin cells. *Eur. J. Pharmacol.*, **221**, 389–391.

YAMAMOTO, D. (1986). Dynamics of strychnine block of single sodium channels in bovine chromaffin cells. *J. Physiol.*, **370**, 95–407.

YOUNG, A.B. & SNYDER, S.H. (1973). Strychnine binding associated with receptor of central nervous system. *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 2832–2836.

YOUNG, A.B. & SNYDER, S.H. (1974). Strychnine binding in rat spinal cord membranes associated with the glycine synaptic receptor: cooperativity of glycine interactions. *Mol. Pharmacol.*, **10**, 790–809.

ZHANG, Z., VIJAYARAGHAVAN, S. & BERG, D.K. (1994). Neuronal acetylcholine receptors that bind α-bungarotoxin with high affinity function as ligand-gated ion channels. *Neuron*, **12**, 167–177.

(Received March 9, 1994
Revised June 2, 1994
Accepted June 20, 1994)