The pharmacology of the nicotinic antagonist, chlorisondamine, investigated in rat brain and autonomic ganglion

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1 A single administration of the ganglion blocker, chlorisondamine (10 mg kg⁻¹, s.c.) is known to produce a quasi-irreversible blockade of the central actions of nicotine in the rat. The mechanism of this persistent action is not known. It is also unclear whether chlorisondamine can block neuronal responses to excitatory amino acids and whether chronic blockade of nicotinic responses also occurs in the periphery.

2 Acute administration of chlorisondamine (10 mg kg⁻¹, s.c.) to rats resulted in a blockade of central nicotinic effects (ataxia and prostration) when tested 1 to 14 days later, but caused no detectable cell death in tissue sections sampled throughout the rostrocaudal extent of the brain which were stained in order to reveal neuronal degeneration.

3 Long-term blockade of central nicotinic effects by chlorisondamine was not associated with significant alterations in the density (B_{max}) of high-affinity [³H]-nicotine binding to forebrain cryostat-cut sections.

4 In cultured dissociated mesencephalic cells of the foetal rat, chlorisondamine and mecamylamine inhibited [³H]-dopamine release evoked by N-methyl-D-aspartate (NMDA, 10⁻⁴ M), but only at high concentrations (IC₅₀ approx. 600 and 70 µM, respectively). A high concentration of chlorisondamine (10⁻³ M) had no effect on responses to quisqualate (10⁻⁵ M) and only slightly reduced responses to kainate (10⁻⁴ M). Mecamylamine (10⁻³ M) was ineffective against both agonists.

5 In adult rat hippocampal slices, chlorisondamine depressed NMDA receptor-mediated synaptically-evoked field potentials, but again only at high concentrations (10⁻⁴–10⁻³ M). Synaptic responses that were mediated by non-NMDA excitatory amino acid receptors were less affected.

6 In rat isolated superior cervical ganglion, electrically-evoked synaptic transmission was reduced 1 h after acute *in vivo* administration of chlorisondamine (0.1 mg kg⁻¹, s.c.). However, *in vivo* administration of a higher dose (10 mg kg⁻¹, s.c.) did not significantly affect ganglionic transmission when tested two weeks later, despite the continued presence of central nicotinic blockade.

7 These results indicate that the persistent CNS nicotinic blockade by chlorisondamine is not accompanied by changes in nicotinic [³H]-nicotine binding site density or by neuronal degeneration in the brain; that at doses sufficient to produce nicotinic receptor blockade, chlorisondamine acts in a pharmacologically selective manner; and that chronic central blockade is not accompanied by long-term peripheral ganglionic blockade.

Keywords: Chlorisondamine; nicotinic receptors; nicotine; mecamylamine; ganglion blockers; NMDA receptors; dopamine release

Introduction

Chlorisondamine (CHL) is a nicotinic antagonist of the ganglion blocking class and exerts negligible effects at nicotinic cholinergic receptors of the skeletal muscle endplate (Plummer *et al.*, 1955; Grimson *et al.*, 1955). Consistent with its bisquaternary structure, chlorisondamine does not appear to penetrate the CNS readily. However, behavioural studies in rats have shown that chlorisondamine, when administered centrally, exerts an extremely long-lasting blockade of the central actions of nicotine (Clarke & Kumar, 1983; Clarke, 1984; Reavill *et al.*, 1986; Fudala & Iwamoto, 1987; Kumar *et al.*, 1987; Mundy & Iwamoto, 1988; Clarke & Fibiger, 1990; Corrigan *et al.*, 1992). For example, a single administration of chlorisondamine (0.2–5.0 µg, i.c.v.) produced a dose-dependent blockade of the locomotor stimulant effect of nicotine (Clarke, 1984) which appears to reflect a site of action in the brain rather than the spinal cord (Clarke *et al.*, 1988). The central blockade resulting from chlorisondamine administration showed little sign of waning even at 5 weeks after a single administration. A similarly persistent blockade

occurred after systemic administration of a high dose (10 mg kg⁻¹, s.c.) (Clarke, 1984; El-Bizri & Clarke, 1994b). Although there is *in vitro* evidence that chlorisondamine blocks CNS nicotinic receptors acutely (Izenwasser *et al.*, 1991; Marks *et al.*, 1993; El-Bizri & Clarke, 1994a), the mechanism underlying its long-term blocking action is not understood.

The extreme persistence of the blocking effect raises the possibility that chlorisondamine destroys certain neurones, perhaps targeting those neurones which express nicotinic receptors. From a light microscopic examination of Nissl-stained sections from rat brain, Kumar *et al.* (1987) tentatively suggested that chlorisondamine is unlikely to act as a neurotoxin. In order to re-evaluate this possibility, we have now employed a more sensitive method which selectively reveals degenerating neurones. Since chlorisondamine administration produces insensitivity to nicotine, we have also tested whether persistent blockade by chlorisondamine is accompanied by a reduced density of brain nicotinic receptors and whether systemic administration of a high dose of chlorisondamine, sufficient to induce long-term central blockade, is accompanied by persistent ganglion block in the periphery.

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Methods

Male adult Sprague-Dawley rats (Charles River, Quebec) were maintained on food and water *ad libitum*. They were housed in groups of two or three subjects per cage, randomly allocated with respect to drug treatment, in a room illuminated from 07 h 00 min to 19 h 00 min. Behavioural testing was carried out between 09 h 00 min and 16 h 00 min. All rats were initially drug-naïve.

Evaluation of possible neuronal degeneration following chlorisondamine treatment

Fifteen rats (220 to 330 g) were randomly allocated to five groups ($n = 3$). Four groups received chlorisondamine (10 mg kg^{-1} , s.c.) and were tested behaviourally for central nicotinic blockade 1, 3, 7, or 14 days later. The fifth group received saline s.c. and was tested after 3 days. Following behavioural testing, each animal was killed for histological assessment of neuronal degeneration.

Production of excitotoxic lesions At each survival time, one or two additional rats were infused intracerebrally with the excitotoxin, N-methyl-D-aspartate (NMDA) in order to provide positive controls for nicotinic behavioural effects and for neuronal degeneration. Briefly, animals were anaesthetized with sodium pentobarbitone (55 mg kg^{-1} , i.p.) and mounted in a stereotaxic device (David Kopf, Tujunga, CA), with the tooth bar positioned 3.3 mm below the interaural line. NMDA (0.1 M in 0.1 M sodium phosphate buffer, pH 7.4) was infused into the dentate gyrus ($0.25 \mu\text{l}$ over 5 min) and into ventral thalamus ($0.5 \mu\text{l}$ over 5 min), via 30 gauge stainless steel cannulae attached by polyethylene tubing to a syringe pump (Sage Instruments, Cambridge, MA, U.S.A.). The cannula was withdrawn after a further 3 min, and the wound sutured. Seizure activity was observed a few hours after surgery and was abolished by administration of 8 mg kg^{-1} , i.p. of diazepam. Animals were killed one day later.

Behavioural testing Behavioural tests served to confirm central nicotinic blockade by CHL. Rats were scored individually for prostration and ataxia induced by nicotine (0.8 mg kg^{-1} , s.c.); both effects are due to central actions of nicotine (Clarke, 1984). A plexiglass cage was employed, measuring 35 cm by 30 cm by 16 cm high, with a wire grill lid and a thin layer of sawdust underfoot. Rats were tested individually. At 2, 4, 6, 8, and 10 min after injection, the presence or absence of prostration and ataxia were separately noted within a 15 s observation period.

Detection of neuronal degeneration Animals were deeply anaesthetized with sodium pentobarbitone (65 mg kg^{-1} , i.p.) and were perfused intracardially with ice-cold saline (1 min) followed by fixative (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, 20 min). Brains were removed, post-fixed in the same fixative for 3–8 days at 4°C , divided into three blocks, and cut on a freezing microtome. Serial $40 \mu\text{m}$ sections were taken at 0.5 mm intervals, through the entire rostrocaudal extent of the brain. Brain sections were stored for 3–4 days at 4°C in multiwell plates modified with polypropylene mesh bottoms to allow incubation in fixative. The silver staining procedure of Gallyas *et al.* (1980) was used, as modified by Nadler & Evenson (1983). Processed sections were mounted on microscope slides, coverslipped with Permount mounting medium (Fisher Scientific Co., Fair Lawn, New Jersey), and examined by light microscopy at $100\times$ – $400\times$ magnification. Neuronal degeneration was detected by the presence of argyrophilic material.

[^3H]-nicotine binding following chlorisondamine treatment

Twenty-two rats (300 to 375 g) were randomly allocated and received CHL (10 mg kg^{-1}) or saline ($n = 11$ per group). Fourteen days later, four rats in each group were tested for nicotine-induced ataxia and prostration (see above). The remaining seven rats in each group did not receive nicotine and were killed at this time for radioligand binding analysis.

Brain nicotinic receptors were quantified by [^3H]-nicotine binding, as previously described (Clarke *et al.*, 1984). Slide-mounted coronal brain sections were used, since the ultimate intention was to map CHL-induced changes by autoradiography. However, initial results were essentially negative (see Results), and autoradiography was not performed. Cryostat sections were taken at two levels of the brain (10.6 and 4.2 mm anterior to interaural zero (Paxinos & Watson, 1986), corresponding to mid-striatal and posterior thalamic levels, respectively) and stored at -40°C until use. Thawed sections were labelled with [^3H]-(-)-nicotine in buffer, rinsed, dried, and scraped into vials for liquid scintillation counting. Non-specific binding was defined by the addition of 10^{-5} M (-)-nicotine ditartrate to the incubation.

Radiochemical purity was tested by thin layer chromatography, since impure samples of [^3H]-nicotine can produce a second (non-specific) component of binding (Romm *et al.*, 1990). In addition, saturation experiments were performed in brains from control rats ($n = 2$), and CHL-treated animals ($n = 2$).

These experiments were performed on anterior brain sections (IA 10.6) and employed ten [^3H]-nicotine concentrations (0.1 – 32 nM). Since weighted-nonlinear curve fitting analysis of binding isotherms (LIGAND, Elsevier Biosoft; Munson & Rodbard, 1980) revealed only a single population of sites in each of these subjects, two-point Scatchard analysis (Dunn *et al.*, 1988) was performed on the remaining anterior brain sections, at 1 and 16 nM [^3H]-nicotine. Posterior brain sections (IA 4.2) were processed in this way several weeks later.

Responses to excitatory amino acids in cultured cells

Mesencephalic cell cultures were prepared as previously described (Mount *et al.*, 1989). Briefly, anteroventral mesencephalic tissue was dissected from the brains of Sprague-Dawley rat embryos (Charles River, Quebec, Canada) on day 15 of gestation. The tissue was mechanically dissociated and diluted to approximately 10^6 cells ml^{-1} in Dulbecco's modified Eagle's medium (DMEM; Gibco Canada, Burlington, Ont., Canada) containing nystatin (24 u ml^{-1}), KCl (20 mM), and 10% v/v heat-inactivated foetal calf serum (Gibco, Canada). Cells were grown on 24-well Multiwell plates coated with poly-D-lysine ($20 \mu\text{g ml}^{-1}$). Cultures were maintained at 37°C in humid air containing 10% CO_2 .

Release experiments followed a protocol previously described in detail (Mount *et al.*, 1990). Briefly, cells were rinsed with Krebs-Ringer-HEPES buffer (KRH), pH 7.4, with the following composition (mM): NaCl 125, KCl 4.8, HEPES 25, NaOH 5, MgSO_4 1.2, KH_2PO_4 1.2, D-glucose 5.6, CaCl_2 2.2, pargyline 0.1 and ascorbate 1.0. Cells were then loaded with [^3H]-dopamine (50 nM) for 20–30 min at 37°C , in the presence of $50 \mu\text{M}$ desipramine (Mount *et al.*, 1989). After [^3H]-dopamine loading, each well was rinsed four times with KRH at 23°C , until spontaneous release was stable (4–6% total [^3H]-dopamine content per 5 min).

When the effect of a single drug was tested on [^3H]-dopamine release, KRH buffer ($500 \mu\text{l}$ per well) was collected after a 5 min ('basal release') period, followed immediately by a 5 min ('stimulation') incubation in the presence of drug. When two drugs were tested, cells were exposed to the first drug (antagonist) during the first 5 min ('basal release') period, and then exposed to both drugs (antagonist and agonist) for the second 5 min ('stimulation') period. Radioactivity remaining in the cells was counted after extraction in

acidified ethanol; previous experiments have shown that 90% of both released and cellular radioactivity elutes with standards (Mount *et al.*, 1989). For data presentation, basal [^3H]-dopamine release was subtracted from drug-stimulated release, and the resulting value was expressed as a percentage of the total [^3H] content present in the cells during the relevant measurement period.

The effects of mecamlamine 10^{-4} M and CHL 10^{-3} M in the absence of agonist were examined by comparison with control tissue wells treated with KRH (Ringer-HEPES buffer) during the 'stimulation' period. Subsequently, the effects of the same concentrations of antagonist were studied on responses to quisqualate (Quis, 10^{-5} M), kainate (KA, 10^{-4} M), and NMDA (10^{-4} M). These concentrations of agonist have been found to produce half-maximal to maximal effects (Mount *et al.*, 1990). Finally, responses to NMDA (10^{-4} M) were examined in the presence of a range of concentrations of mecamlamine (10^{-5} – 10^{-3} M) or CHL (10^{-6} – 10^{-3} M). The number of wells per condition was 9–21.

Synaptic responses in hippocampal slice

Hippocampal slices (425 μm thick) were prepared from adult male Sprague-Dawley rats and maintained at 30°C in a recording chamber. The upper surface of the slice was exposed to a flow of moist 95% O_2 and 5% CO_2 , and the lower surface rested on a nylon mesh exposed to oxygenated artificial cerebrospinal fluid (ACSF) of the following composition (mM): NaCl 124, KCl 5.0, NaHCO_3 25.6, CaCl_2 2.0, NaH_2PO_4 1.2, MgSO_4 1.0 or 0.0 and D-glucose 10. The ACSF was perfused through the chamber at 1 ml min^{-1} . Field potentials were recorded in the pyramidal (CA1) cell body layer by a micropipette (10–20 M Ω) filled with 4 mM NaCl. Stimulating pulses (0.1 ms) were delivered by a bipolar electrode in the stratum radiatum to activate the cells orthodromically. Unless stated otherwise, the intensity of stimulus was adjusted to produce a population spike with an amplitude corresponding to 70% to 90% of maximum. Chlorisondamine and DL-2-amino-5-pentanoic acid (APV) were dissolved in ACSF and perfused through the recording chamber. A microcomputer-based system was used for data acquisition and analysis, as previously described (Theoret *et al.*, 1984).

In a typical experiment, magnesium-free medium was used. During a 5 min baseline period, electrical stimulation was applied every 10 s, consisting of single pulses of 0.1 ms duration. Evoked responses to the same stimulus were recorded 20–28 min and again 45–53 min after the start of perfusion with the drug dissolved in ACSF. In some experiments, a second, higher concentration of drug was tested in the same fashion, with no intervening wash-out period. Three distinct population spikes were identified and quantified, by expressing the peak voltage of each as a percentage of its individual pre-drug baseline.

Twenty-three rats were used, weighing 150 to 200 g. The temporal stability of the preparation was tested in three control experiments in which a slice was perfused with Mg-free medium for between 4 and 6 h.

Transmission in superior cervical ganglion

Twenty-three rats, weighing 195 to 270 g, were used. To examine the chronic effects of CHL, 20 rats were randomly allocated to two groups and received CHL (10 mg kg^{-1}) or saline ($n = 10$ per group). Twelve days later, four rats in each group were tested for nicotine-induced ataxia and prostration (as described previously). The remaining six rats in each group did not receive nicotine and were killed at this time for electrophysiological studies. The acute effect of CHL on ganglionic transmission was examined in an additional three rats that received a lower dose of CHL (0.1 mg kg^{-1} , s.c.) only 1 h before they were killed.

Superior cervical ganglia were dissected under ether anaes-

thesia and transferred to a bath containing 5 ml Krebs solution (composition (mM): NaCl 120, KCl 4.6, CaCl_2 2.4, MgSO_4 1.2, NaHCO_3 25, KH_2PO_4 1.2 and D-glucose 9.9) bubbled with 95% O_2 /5% CO_2 at pH 7.4. The preganglionic and internal carotid (postganglionic) nerves were aspirated into glass suction electrodes fitted with Ag/AgCl electrodes for stimulation and recording, respectively (Dunant, 1967; Quirk & Lamarca, 1982). Supramaximal square wave pulses of 1.0 ms duration were applied at 0.2 Hz to the stimulating electrode from a stimulator via an isolation unit (Grass Instrument Co., Quincy, MA). The evoked potentials recorded from the internal carotid nerve were displayed on an oscilloscope (Tektronix, Portland, OR, U.S.A.) and permanent records were made on polaroid film. Data were analysed as peak postganglionic population action potentials.

Drugs

(–)-Nicotine di-(+)-tartrate (Sigma) was dissolved in 0.9% w/v NaCl solution (saline) and neutralized to $\text{pH } 7.2 \pm 0.1$ with NaOH. Chlorisondamine Cl (gift of CIBA-Geigy, Summit, New Jersey) was dissolved in saline. All systemic injections were subcutaneous (s.c.), given in the flank in a volume of 1 ml kg^{-1} . Doses of nicotine and CHL refer to the base; doses of other drugs are of the salt. [^3H]-dopamine and [^3H](–)-nicotine (specific activity 20 and 68.8 Ci mmol^{-1} , respectively) were purchased from New England Nuclear (Boston, MA, U.S.A.). N-methyl-D-aspartic acid (NMDA), kainic acid (KA), quisqualic acid (Quis) and DL-2-amino-5-pentanoic acid (APV) were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.). Diazepam was obtained from Sabex Pharmaceuticals (Boucherville, Canada). Other chemicals were reagent grade and were obtained commercially.

Analysis of data

Data were analysed by ANOVA or by *t* tests. For comparisons with a single control group, Dunnett's test was used (Dunnett, 1955). Probability values are for 2-tailed tests.

Results

Evaluation of possible neuronal degeneration following chlorisondamine treatment

Administration of nicotine resulted in prostration and ataxia in all 10 rats that had not received chlorisondamine. All ten subjects became prostrate in the first 2 min after injection. From 2 to 4 min post injection, three subjects were still prostrated, and all subjects were ataxic. Ataxia persisted throughout all subsequent 2 min periods, except for one rat which recovered in the final period (8–10 min). In contrast, none of the 15 rats that had received chlorisondamine manifested these behavioural effects of nicotine; three of these rats had received chlorisondamine 24 h before.

Processed brain sections from control (saline-treated) and CHL-treated rats were indistinguishable. Consistent with previous reports (Gallyas *et al.*, 1980; Nadler & Evenson, 1983), sections possessed a general golden-brown appearance (Figure 1), with three types of silver grain distribution: (1) some sections from all levels of the brain were overlaid with a sparse and even distribution of silver grains, which was anatomically nonspecific and inconsistently observed across adjacent sections, and hence was probably the result of random silver precipitation; (2) within some magnocellular neurones in the vestibular nuclei of the brain stem, and (3) a characteristic pattern of grains was seen running along blood vessels. None of these silver grain distributions suggested neuronal degeneration.

Clear signs of neuronal degeneration were, however, observed in rats lesioned with NMDA (Figure 1a,b,c and d). Degenerating cell bodies and processes containing grey or

Figure 1 Coronal sections (40 μm thick) of rat brain stained by the method of Gallyas *et al.* (1980) in order to reveal degenerating neurones. Panel (a) ($\times 100$ magnification) shows the hippocampus and dentate gyrus of a rat which had received a unilateral (left side) infusion of the excitotoxin NMDA. The pyramidal and dentate granule cell layers are stained black on the lesioned side. The dorsomedial hippocampal area in panel (a) is shown at higher magnification ($\times 200$) in panel (b) where black-stained pyramidal and granule cell bodies and some processes are visible. Panels (c–h) are at $\times 400$ magnification; the calibration bar in (c) indicates 100 μm . Panel (c) shows several stained neurones in the ventral thalamus of a rat that had sustained an NMDA lesion in this area; degenerating cell bodies and processes are visible. Panels (e) and (g) show corresponding images from rats which had received systemic saline or chlorisondamine, respectively, three days before they were killed; only golden-brown staining of cells and neurophil is seen. Panels (d), (f) and (h) show neurones in the hippocampal pyramidal cell layer from NMDA-lesioned, saline- and chlorisondamine-treated rats, respectively; again, no degeneration is observed except in the lesioned animal. A colour photograph is available from the authors upon request.

black granular deposits were readily identified in both dentate gyrus and ventral thalamus. Degenerating axons were also clearly visible (Figure 1c and d).

Tritiated nicotine binding following chlorisondamine treatment

Behavioural tests confirmed blockade of the central nicotinic actions that were observed in control animals. Thus, none of the four CHL-treated rats displayed ataxia or prostration, whereas all four of the control rats did.

Non-linear regression analysis of specific binding revealed a single population of sites in each of the four rats tested. The two saline-treated control animals yielded apparent K_D and B_{max} values of 5.9 and 4.7 nM and 15.4 and 14.0 fmol/section, respectively. The corresponding values in CHL-treated rats were 4.7 and 3.7 nM, and 14.3 and 12.3 fmol/section. Scatchard plots illustrating these data are shown in Figure 2. For all four rats, Hill coefficients were near unity (range 0.99–1.06).

Subsequent analyses included data from all subjects. B_{max} values extrapolated from 2-point Scatchard analyses did not differ significantly between saline- and CHL-treated groups, either at the anterior brain level (mean $\pm$ s.e.mean, respectively: 16.7 ± 0.8 and 14.9 ± 1.1 ; $t = 1.33$, d.f. 12, $P > 0.1$), or at the posterior level (respectively: 13.9 ± 1.0 and 15.1 ± 1.2 ; $t = 0.77$, d.f. 12, $P > 0.1$). Values of the apparent K_D differed significantly between saline- and CHL-treated groups at the anterior brain level (respectively: 6.13 ± 0.36 and 4.71 ± 0.36 ; $P < 0.02$). However, at the posterior level, no significant difference was found (respectively: 3.71 ± 0.31 and 4.10 ± 0.43 ; $P > 0.1$).

Responses to excitatory amino acids in cultured cells

When given alone, high concentrations of CHL (10^{-3} M) and mecamylamine (10^{-4} M) had no effect on [3 H]-dopamine release ($P > 0.4$). The mean ($\pm$ s.e.mean) release (in excess of basal) for KRH-treated, mecamylamine-treated, and CHL-treated samples was $-0.07\% \pm 0.12\%$, $0.19\% \pm 0.17\%$, and $0.03\% \pm 0.13\%$, of total cellular [3 H]-dopamine stores, respectively.

Release of [3 H]-dopamine in response to Quis 10^{-5} M was not significantly affected by these high concentrations of mecamylamine and CHL ($P > 0.2$). Quis-evoked release was $4.25\% \pm 0.27\%$ (no antagonist), $3.90\% \pm 1.21\%$ (CHL) and $3.53\% \pm 0.82\%$ (mecamylamine). KA-evoked release was $13.3 \pm 0.6\%$ (no antagonist), $11.0 \pm 0.7\%$ (CHL) and $11.7 \pm 0.6\%$ (mecamylamine). The small effect of CHL on the KA response was statistically significant ($P < 0.05$). Both mecamylamine and CHL reduced NMDA- (10^{-4} M)-induced [3 H]-dopamine release in a concentration-related manner (mecamylamine: $P < 0.001$; CHL: $P < 0.001$; Figure 3). Mecamylamine produced a significant attenuation at 10^{-4} M and complete blockade at 10^{-3} M. CHL produced a significant, albeit incomplete, attenuation of the NMDA response only at 10^{-3} M ($P < 0.01$). IC_{50} values of approximately 70 μ M for mecamylamine and 600 μ M for CHL were obtained (mean of three experiments).

Synaptic responses in hippocampal slice

In magnesium-free medium, baseline evoked responses were multiphasic (Figure 4). Addition of magnesium (1.0 mM) or APV (100 μ M) to the superfusion medium abolished secondary spikes (Figure 4). These findings confirm previous observations suggesting that the primary and secondary spikes are mediated by non-NMDA and NMDA excitatory amino acid receptors, respectively (Coan & Collinridge, 1987).

Control experiments indicated that the slice preparation was stable throughout the observation period. The mean values of the primary spike at the three measurement times

Figure 2 Scatchard plots of [3 H]-nicotine binding to cryostat-cut forebrain sections. Concentrations of radioligand were 0.1–32 nM. Two animals (a) had received an injection of saline 2 weeks before they were killed; two animals (b) had received a systemic dose of chlorisondamine (10 mg kg^{-1} , s.c.), sufficient to produce persistent central nicotinic blockade.

Figure 3 Effects of chlorisondamine and mecamylamine on the release of [3 H]-dopamine evoked by N-methyl-D-aspartate (NMDA: 10^{-4} M) in rat cultured mesencephalic cells. The vertical axis shows mean ($\pm$ s.e.mean, $n = 9$ –21) antagonist-stimulated release, expressed as a percentage of residual cellular 3 H content (see text). Compared to controls (no antagonist: open columns), both chlorisondamine (solid columns) and mecamylamine (hatched columns) reduced or blocked responses to NMDA, but only at high concentrations.

were, respectively, 103, 123 and 93% of baseline. Corresponding values for secondary spikes were 120, 126 and 90%. Tertiary spikes appeared less stable (85, 117 and 66%, respectively).

Chlorisondamine had no effect on the magnitude of primary (non-NMDA) responses (Figure 5). In contrast,

Figure 4 Representative CA1 hippocampal population spikes in response to electrical stimulation of the stratum radiatum. In normal artificial cerebrospinal fluid (ACSF), only one population spike was seen (a); after a period of superfusion in Mg^{2+} -free medium, two later spikes appeared (b) which could then be abolished by a high concentration of chlorisondamine (10^{-3} M; c); recovery occurred after withdrawal of drug (d). In a second experiment, the later negative components occurring in Mg^{2+} -free medium (e) were abolished by superfusion with D,L-2-amino-5-pentanoic acid (10^{-4} M; f). Recovery occurred upon wash-out (g). Subsequently, a lower concentration of chlorisondamine (10^{-4} M) had little or no effect (h). Calibration scale: 5 mV (vertical), 10 ms (horizontal).

Figure 5 Effects of chlorisondamine on CA1 population spikes in hippocampal slices. The vertical axis shows the mean ($\pm$ s.e.mean, $n = 3-7$) magnitude of electrically-evoked primary (open columns), secondary (solid columns) and tertiary (hatched columns) population spikes as a percentage of pre-drug baseline. Chlorisondamine reduced secondary and tertiary spikes in a concentration-dependent manner, but had little or no effect on the magnitude of primary spikes.

Table 1 Effect of chlorisondamine on nicotinic receptor-mediated transmission in rat superior cervical ganglion

Treatment	Survival	n	Action potential amplitude (mV)
Saline	1 h	3	0.57 ± 0.13
Chlorisondamine	1 h	5	$0.19 \pm 0.08^*$
Saline	12 days	9	0.47 ± 0.09
Chlorisondamine	12 days	10	0.57 ± 0.07

Chlorisondamine was given s.c. (0.1 mg kg^{-1} with 1 h survival; 10 mg kg^{-1} with 12 days survival). Values represent mean $\pm$ s.e.mean. Significance of difference from control ganglia: $*P < 0.05$.

secondary spikes were reduced ($P < 0.001$); they were unaffected by lower concentrations (10^{-6} and 10^{-5} M), but were somewhat decreased at 10^{-4} M and abolished at 10^{-3} M (Figure 5). Tertiary spikes were similarly affected (Figure 5). The blockade produced by the highest concentration was followed during a wash-out period of 45 to 60 min duration; recovery from blockade was observed in 4 out of 5 experiments, and partial recovery occurred in the remaining experiment.

Transmission in superior cervical ganglion

In behavioural tests, nicotine induced prostration and ataxia in the four control (saline-treated) rats, but not in four rats treated 14 days before with CHL. Ptosis, which occurred within minutes of injection of CHL, was absent in all animals 24 h later.

Acute administration of CHL (0.1 mg kg^{-1}) 1 h prior to the animal's death significantly reduced the magnitude of postganglionic potentials ($P < 0.05$; Table 1). In contrast, administration of the much higher dose of CHL (10 mg kg^{-1}) two weeks beforehand had no significant effect on postganglionic potentials ($P > 0.3$ compared to saline-treated controls).

Discussion

A single administration of chlorisondamine, in a sufficient dose, produces an extremely long-term blockade of all the centrally-mediated behavioural effects of nicotine that have been examined to date. Such effects include locomotor stimulation and locomotor depression (Clarke, 1984), depression of operant behaviour and the nicotine cue (Kumar *et al.*, 1987), conditioned taste aversion (Reavill *et al.*, 1986), conditioned place aversion (Fudala & Iwamoto, 1987), nicotine-induced impairment of autoshaping response (Mundy & Iwamoto, 1988), and nicotine intravenous self-administration (Corrigall *et al.*, 1992). The present observations add nicotine-induced ataxia and prostration to this list, and additionally show that central nicotinic blockade was complete within 24 h of chlorisondamine administration.

Does chlorisondamine cause neuronal degeneration?

Little or no neuronal degeneration was detected in chlorisondamine-treated rats, despite evidence of central nicotinic blockade in the same animals tested just before death. The absence of observable pathology does not appear to reflect an insensitive method, since clear signs of axonal and somatodendritic degeneration were seen in the brains of subjects lesioned with NMDA.

Could chlorisondamine have destroyed a population of neurones which, though small, may be crucial for the mediation of the behavioural effects of nicotine? The extensive nature of our survey, in which one thousand brain sections

were processed and examined, makes this possibility unlikely. Furthermore, strong evidence exists that the locomotor stimulant effect of nicotine is mediated through a direct action on mesolimbic dopaminergic neurones (Clarke, 1990); since this behavioural effect is blocked persistently by chlorisondamine (Clarke, 1984), it is noteworthy that no signs of degeneration were observed in dopamine-containing systems in the present study.

Does chlorisondamine alter nicotinic receptor number?

Persistent central nicotinic blockade resulting from chlorisondamine administration was not associated with a decreased density of nicotinic receptors, as assessed by high-affinity [3 H]-nicotine binding at two rostrocaudal levels of the brain. There was an indication that binding affinity was slightly altered by chlorisondamine, but this finding must be viewed with caution since it was only obtained at one of the two brain levels examined. High affinity [3 H]-nicotine binding sites represent a prevalent subtype of nicotinic receptors in rat brain (Lindstrom *et al.*, 1987) which are believed to be a major target for subconvulsive doses of nicotine (Clarke, 1987; London *et al.*, 1988). It would thus appear unlikely that chlorisondamine produces its persistent blockade by regulating the number of central nicotinic receptors. However, this possibility cannot be ruled out completely, since other populations of central nicotinic receptors exist that are not labelled with high affinity by [3 H]-nicotine (Clarke *et al.*, 1985; Couturier *et al.*, 1990; Schulz *et al.*, 1991).

Two weeks of chronic nicotine treatment *in vivo* results in an upregulation of high affinity agonist binding sites in rodent brain (Wonnacott, 1990). This paradoxical effect has been attributed to the development of a form of functional antagonism mediated by receptor desensitization (Marks *et al.*, 1983; Schwartz & Kellar, 1983). In contrast, in the present study, no such upregulation was observed after an equivalent period of demonstrable and complete nicotinic blockade, a blockade furthermore that is insurmountable (El-Bizri & Clarke, 1994b). Our observations therefore suggest that nicotine-induced functional blockade may not be the mechanism by which nicotine upregulates its own receptors.

Is chronic central blockade by chlorisondamine unrelated to nicotinic receptors?

Although there is evidence that chlorisondamine can block central nicotinic receptors acutely (Izenwasser *et al.*, 1991; El-Bizri & Clarke, 1994a), the possibility must be considered that chlorisondamine may be exerting its long-term block independently of nicotinic receptors. This appears unlikely for three reasons. Firstly, chlorisondamine persistently blocks diverse behavioural effects of nicotine that are probably not all mediated by the same neuronal systems with the CNS. Secondly, the long-term blocking action of chlorisondamine does not, as far as is known, extend to behavioural effects of non-nicotinic agents (Clarke, 1984; Reavill *et al.*, 1986; Kumar *et al.*, 1987). Thirdly, *in vivo* administration of CHL results in a block of nicotinic responses tested *in vitro*, yet fails to alter responses to hypertonic K^+ or to (+)-amphetamine (El-Bizri & Clarke, 1994b).

Are mecamylamine and chlorisondamine selective nicotinic antagonists?

Mecamylamine has been very widely used as an antagonist of the central effects of nicotine. Surprisingly, its pharmacological selectivity has not been well established. Indeed, mecamylamine has been shown to reduce responses mediated by glutamate receptors in several species and tissues (MacLeod *et al.*, 1984; Olney *et al.*, 1987; O'Dell & Christensen, 1988; Snell & Johnson, 1989). In most cases, high concentrations of mecamylamine have been used.

In the present experiments, mecamylamine antagonized NMDA receptor-mediated responses, but concentrations greater than $10\text{ }\mu\text{M}$ were required. Consistent with electrophysiological findings (Bijak *et al.*, 1991), mecamylamine did not affect responses to quisqualate. The central concentrations of mecamylamine that are achieved following systemic administration are not known. However, a dose of 1 mg kg^{-1} , which is usually sufficient to block central actions of systemically administered nicotine (Clarke, 1987), would attain a concentration of around $10\text{ }\mu\text{M}$ if evenly distributed. This estimate is consistent with *in vitro* tests showing that such a concentration produces a near-total blockade of nicotinic responses (El-Bizri & Clarke, 1994a). Thus, mecamylamine, at the doses usually encountered in behavioural studies (i.e. not exceeding 1 mg kg^{-1}), can probably be regarded as a moderately selective nicotinic antagonist.

We also found that chlorisondamine exerted only minimal effects on responses mediated by kainate or quisqualate receptors, but reduced NMDA receptor-mediated responses. Complete blockade of NMDA receptor-mediated actions required millimolar concentrations in both preparations. In the hippocampal slice, partial attenuation was evident at a concentration of 10^{-4} M chlorisondamine, but in the cultured cells, no clear effect was observed at 10^{-4} M or below. The basis for this apparent difference in potency is not known, but possibly reflects a difference in the receptor subtypes present, the effective concentrations of the agonist at the receptors, or the temporal characteristics of synaptically released *versus* exogenously applied agonist.

As with mecamylamine, the concentrations of chlorisondamine required to block the central actions of systemically applied nicotine are not known. A dose of $5\text{ }\mu\text{g}$ (14 nmol) given intracerebroventricularly is sufficient to produce a central blockade (Clarke, 1984), and assuming that this dose were entirely retained in the CNS and uniformly distributed, the resulting concentration would certainly be no more than $3\text{ }\mu\text{M}$. This estimate is consistent with *in vitro* tests in which near-total block occurred at $1\text{ }\mu\text{M}$ chlorisondamine in the presence of concurrent nicotinic stimulation (El-Bizri & Clarke, 1994a). Under these conditions, mecamylamine and chlorisondamine were approximately equipotent, whereas mecamylamine was some ten fold more potent than chlorisondamine in antagonizing NMDA responses. These observations suggest that chlorisondamine may well be a more selective nicotinic antagonist than the more widely-used mecamylamine.

Selective central block by chlorisondamine

Clinically, chlorisondamine is considered to have a somewhat longer duration of action than other common ganglion blockers (Grimson *et al.*, 1955). Nevertheless, we have shown that the ganglion blocking action is shorter-lived than the central blockade exerted by this drug. Indeed, the absence of ptosis noted on the day after drug administration suggests that ganglion blockade may have dissipated within a matter of hours.

Although the mechanism by which chlorisondamine produces its long-term central blockade is not fully understood (see El-Bizri & Clarke, 1994b), several differences may be noted between central and peripheral responses examined to date. Firstly, the prevalent subtypes of nicotinic receptor located on peripheral neurones appear to differ from those that are prevalent in the CNS (Deneris *et al.*, 1991). Secondly, in the periphery, block of nicotinic cholinergic transmission has been measured, whereas in brain tissue, only block of responses to endogenous agonists has been examined. Thirdly, the possibility exists that chlorisondamine is trapped within the CNS by the blood-brain barrier; this issue is investigated in a companion paper (El-Bizri & Clarke, 1994b).

It appears that systemic administration of a high dose of chlorisondamine provides a means of achieving long-term

antagonism of the central actions of nicotine in the absence of lasting ganglionic blockade, at least in rats. There is some reason to hope that a CNS-selective nicotinic receptor blocker would help people give up tobacco smoking and maintain abstinence (Clarke, 1991). Chlorisondamine may provide a starting point in the search for such a drug.

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