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# Blockade of nicotinic receptor-mediated release of dopamine from striatal synaptosomes by chlorisondamine and other nicotinic antagonists administered *in vitro*

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**1** Central nicotinic receptor function examined *in vitro*, by measuring nicotine-induced [$^3$H]-dopamine release from rat striatal synaptosomes.

**2** The agonists (−)-nicotine, acetylcholine, 1,1-dimethyl-4-phenylpiperazinium (DMPP) and cytisine ($10^{-7}$–$10^{-4}$ M) all increased [$^3$H]-dopamine release in a concentration-dependent manner. Cytisine did not produce a full agonist response, compared to the other agonists.

**3** The actions of nicotine, acetylcholine and cytisine were largely dependent on external $Ca^{2+}$. In contrast, DMPP ($10^{-5}$ and $10^{-4}$ M) evoked a marked release of [$^3$H]-dopamine even in the absence of $Ca^{2+}$. Nevertheless, in the presence of external $Ca^{2+}$, responses to DMPP were completely blocked by the nicotinic antagonists chlorisondamine and mecamylamine ($5 \times 10^{-5}$ M); in the absence of external $Ca^{2+}$, blockade was only partial.

**4** Chlorisondamine, mecamylamine and dihydro-β-erythroidine ($10^{-8}$–$10^{-4}$ M) produced a concentration-dependent block of responses to nicotine ($10^{-6}$ M). Approximate $IC_{50}$ values were 1.6, 0.3 and $0.2 \times 10^{-6}$, respectively. Chlorisondamine and mecamylamine blocked responses to nicotine ($10^{-7}$–$10^{-4}$ M) insurmountably, whereas dihydro-β-erythroidine behaved in a surmountable fashion.

**5** The occurrence of use-dependent block was tested by briefly pre-exposing the synaptosomes to nicotine during superfusion with antagonist, and determining the response to a subsequent nicotine application. Consistent with a possible channel blocking action, brief pre-exposure to agonist increased the antagonist potency of chlorisondamine (approximately 25 fold). No significant use-dependent block was detected with dihydro-β-erythroidine.

**Keywords:** Nicotine; acetylcholine; cytisine; DMPP; chlorisondamine; mecamylamine; dihydro-β-erythroidine; nicotinic receptors; calcium dependence; surmountable antagonism

## Introduction

Chlorisondamine (CHL) is a nicotinic receptor antagonist possessing a ganglionic profile of action (Grimson *et al.*, 1955; Plummer *et al.*, 1955; Schneider & Moore, 1955). The bisquaternary structure of CHL appears to impede passage across the blood brain barrier, but a single administration of the drug, given either directly into the cerebral ventricles or in a sufficiently high dose systemically, results in a pharmacologically selective and remarkably persistent (several weeks) blockade of 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; Corrigall *et al.*, 1992; El-Bizri & Clarke, 1994; Clarke *et al.*, 1994). In contrast, ganglionic blockade induced by CHL is transient (Grimson *et al.*, 1955; Plummer *et al.*, 1955; Schneider & Moore, 1955; Clarke *et al.*, 1994).

The mechanisms underlying the central nicotinic blockade associated with CHL administration have not been elucidated. Reports of central blockade by CHL have relied almost exclusively on behavioural measures that reflect central actions of nicotine. However, two recent *in vitro* studies reported that CHL abolished nicotine-induced inhibition of dopamine uptake in striatal tissue slices (Izenwasser *et al.*, 1991) and nicotine-induced rubidium efflux from mouse striatal synaptosomes (Marks *et al.*, 1993).

The additional observation that CHL does not inhibit high-affinity binding of [$^3$H]-acetylcholine or [$^3$H]-nicotine to rat brain membranes (Schwartz *et al.*, 1982; Clarke *et al.*, 1984) suggests that this antagonist does not act at the agonist recognition site(s) situated on the nicotinic receptor macromolecule. Indeed, at autonomic ganglia, CHL appears to act as a nicotinic receptor channel blocker, producing a block, the onset of which is both voltage- and use-dependent (Traber *et al.*, 1967; Alkadhi & McIsaac, 1974). A channel blocking mechanism has also been proposed for CHL at non-mammalian nicotinic receptors (Lingle, 1983a,b; Neely & Lingle, 1986).

Nicotine has been shown to induce the release of several neurotransmitters in the CNS via a direct action on nerve terminals (Balfour, 1982; Wonnacott *et al.*, 1989). This phenomenon has been repeatedly described in the nigrostriatal dopaminergic system, where micromolar concentrations of nicotine stimulate the release of dopamine in a receptor-mediated manner, both *in vitro* (Giorguieff *et al.*, 1976; 1977; Giorguieff Chesselet *et al.*, 1979; Westfall *et al.*, 1987; Rapier *et al.*, 1988; 1990; Rowell & Wonnacott, 1990; Izenwasser *et al.*, 1991; Grady *et al.*, 1992) and *in vivo* (Giorguieff *et al.*, 1976; Imperato *et al.*, 1986). Thus, the ability of nicotine to promote dopamine release from striatal synaptosomes provides a reliable *in vitro* assay for CNS nicotinic receptor function. We have used this assay to examine the mechanism by which acute *in vitro* administration of CHL produces blockade of CNS nicotinic responses.

## Methods

Male Sprague-Dawley rats (Charles River, St Constant, Quebec), weighing 200–250 g, were maintained on a 12/12 h light-dark cycle. Rats were house four per cage, and food and water were available *ad libitum*. Subjects were allowed to accommodate to the housing conditions for 4 days after arrival, and were drug-naive at the start of each experiment.

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### *Dopamine release from superfused synaptosomes*

Rats were decapitated, and both striata (total wet weight 160–200 mg) were immediately dissected in ice-cold 0.32 M sucrose/5 mM HEPES adjusted to pH 7.5 with NaOH. Striata were homogenized in 20 vol of the sucrose/HEPES solution (12 up and down strokes, at 850 RPM, in a 0.25 mm clearance glass/Teflon homogenizer). The homogenate was centrifuged at 1000 *g* for 10 min at 4°C. Supernatant was recentrifuged at 12000 *g* for 20 min at 4°C. The final pellet, consisting of the crude synaptosomal (P2) fraction, was resuspended in the superfusion buffer (5 ml $g^{-1}$ of wet tissue weight). The superfusion buffer (SB) was composed of the following (mM): NaCl 128, KCl 2.4, $CaCl_2$ 3.2, $KH_2PO_4$ 1.2, $MgSO_4$ 0.6, HEPES 25, D-glucose 10, L-ascorbic acid 1 and pargyline 0.1 adjusted with NaOH to pH 7.5. The synaptosomal preparation was incubated with [$^3$H]-dopamine ($0.12 \times 10^{-6}$ M) for 10 min at 37°C. The synaptosomes were then centrifuged at 1000 *g* for 5 min at RT and the pellet was gently resuspended in an equal volume of SB.

### *Superfusion*

The apparatus comprised 16 identical channels. Each channel consisted of a length of Tygon or silicone tubing (0.8 mm i.d.) leading to and from a retention chamber comprising a polypropylene filter unit (Millipore Corp., Bedford, MA, U.S.A.), fitted with a 13 mm diameter A/E glass fibre filter (1 μm pore size, Gelman Sciences Inc., Ann Arbor, MI, U.S.A.). The superfusate was continuously pumped downward through the chamber, at a rate of 0.4 ml $min^{-1}$, via a peristaltic pump (Masterflex, Cole-Parmer, Chicago, IL, U.S.A.) positioned downstream to the chamber. Upstream to the chamber two 1 ml syringes (Becton Dickinson, NJ, U.S.A.) were connected to the superfusion tubing via 25 gauge needles. The first syringe was used in order to introduce the drug (or SB for control channels), and the second to introduce buffer containing 20 mM KCl. In order to reduce the possibility of contaminants, all syringes used in these experiments were washed with bleach, followed by thorough rinsing with distilled water.

Each experiment comprised several assays. At the start of each assay, channels were thoroughly rinsed by superfusion with distilled water followed with SB. Next, 100 μl of the synaptosomal suspension was injected into the tubing immediately upstream to the superfusion chamber; synaptosomes were retained within the chamber on the filter. Following a superfusion period of 25 min, 28 samples per channel were collected in consecutive 1 min intervals into polypropylene minivials (Sarstedt, Montreal, Canada) containing 2 ml of scintillation fluid (Hi-Safe III, Fisher Scientific, Montreal, Canada). Following an initial collection period of 10 min, a 1 min (0.4 ml) pulse of drug or SB (control channels) was injected. In some experiments, this was followed 10 min later by a 1 min (0.4 ml) pulse of buffer containing high KCl (20 mM) given to all channels. Superfusate samples were collected for a further 8 min. Finally, the filters holding the synaptosomes were removed in order to measure residual radioactivity. Samples were measured in a liquid scintillation counter (Wallac 1410, LKB, Sweden).

In each assay, data were collected simultaneously from all 16 channels. Two rats provided striatal tissue for each assay. Care was taken to include control (SB only) channels in all assays, and to counterbalance treatment conditions across channels and across individual assays. In experiments using $Ca^{2+}$-free medium, an equivalent molar concentration of $Mg^{2+}$ and EGTA (2.25 mM) was substituted. In experiments with antagonists, these were present throughout each assay, including the 25 min washout period prior to the sample collection.

Throughout the paper we shall refer to the tritium released as dopamine release, since it has been established that in similar synaptosomal preparations preloaded with [$^3$H]-dopamine, tritium released by nicotinic agonists or by depolarization largely corresponds to dopamine itself (Rapier *et al.*, 1988).

### *Data analysis*

Basal release was defined as the mean radioactivity released (d.p.m./min) over the six 1 min samples collected immediately prior to drug or SB administration. Basal [$^3$H]-dopamine release corresponded to $3 \times 10^{-15}$ mol $mg^{-1}$ of original wet tissue. For each channel, the release occurring in each 1 min collection period was calculated as a percentage of basal release; evoked release was taken as the peak value that occurred in the first three periods after a drug challenge. $EC_{50}$ and $IC_{50}$ values were determined by linear interpolation of points either side of 50% of maximal effect. Drug effects were examined by analysis of variance, using commercial software (Systat, Evanston, IL, U.S.A.). Multiple comparisons with a control group were made with Dunnett's test (Dunnett, 1955); other multiple comparisons were made by Student's *t* test with Bonferroni's correction (Glantz, 1992).

### *Drugs*

Chemicals and supplies were as follows: [8-$^3$H]-dopamine (specific activity 20–40 Ci $mmol^{-1}$; New England Nuclear, Boston, MA, U.S.A.). (−)-nicotine hydrogen tartrate, cytisine, 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), diisopropylfluorophosphate (DFP) (Sigma Chemical Corp., St. Louis, MO, U.S.A.), acetylcholine hydrochloride, pargyline hydrochloride, atropine sulphate (Research Biochemicals Inc., Natick, MA, U.S.A.), mecamylamine hydrochloride (Mec) and dihydro-β-erythroidine (DHBE) (gifts of Merck and Co, Rahway, NJ, U.S.A.), chlorisondamine chloride (CHL) (gift of Ciba-Geigy, Summit, NJ, U.S.A.). Other chemicals and reagents were purchased from commerical sources. For *in vitro* administration, drugs were dissolved in superfusion buffer (SB). Stock solutions of drugs were prepared twice a week, and stored at −40°C. Dilutions were freshly made prior to use for each assay.

## Results

### *Concentration-dependent effects of nicotine on striatal [$^3$H]-dopamine release*

(−)-Nicotine ($3.2 \times 10^{-8} - 10^{-4}$ M) increased [$^3$H]-dopamine release in a concentration-dependent manner (Figure 1), with an estimated $EC_{50}$ of $1.6 \times 10^{-7}$ M. Significant effects occurred at $10^{-7}$ M and above (Dunnett's test: $P < 0.02$), and a maximal effect was achieved at $10^{-6}$ M.

### *Effects of nicotinic agonists and high $K^+$ buffer on striatal [$^3$H]-dopamine release*

In the first set of assays, four nicotinic agonists (nicotine, ACh, cytisine and DMPP) were tested at $10^{-7}$–$10^{-4}$ M. The superfusion medium contained DFP ($10^{-4}$ M) and atropine sulphate ($10^{-6}$ M) in order to inhibit hydrolysis of ACh and to prevent activation of muscarinic cholinoceptors, respectively. All four agonists increased [$^3$H]-dopamine release in a concentration-dependent manner (Figure 2). Cytisine did not behave as a full agonist (Figure 2b). Neither ACh nor DMPP appeared to produce maximal effects within the range of concentrations tested (Figure 2c and 2d). With the exception of DMPP (Figure 2d), removal of external $Ca^{2+}$ from SB abolished agonist-induced [$^3$H]-dopamine release. Thus, in the absence of external $Ca^{2+}$, DMPP significantly evoked release ($P < 0.001$), but the other agonists did not ($P > 0.6$ for each agonist). Omission of external $Ca^{2+}$ reduced basal [$^3$H]-dopamine release by 63%.

High $K^+$ (20 mM) buffer increased [$^3$H]-dopamine release to a peak value of 378 ± 19.5% ($n = 8$) of baseline. Removal of external $Ca^{2+}$ from SB resulted in $K^+$-evoked release of 173 ± 14.6% ($n = 8$), corresponding to a 74% decrease. In this experiment, omission of external $Ca^{2+}$ reduced basal [$^3$H]-dopamine release by 57%.

### *Nicotinic blockade by* in vitro *chlorisondamine, mecamylamine and dihydro-β-erythroidine*

The effects of CHL, Mec and DHBE on responses to nicotine ($10^{-6}$ M) and to hypertonic $K^+$ (20 mM) were examined. Each antagonist was tested in a different set of assays. All three antagonists ($10^{-8}$–$10^{-4}$ M) produced a concentration-dependent blockade of [$^3$H]-dopamine release evoked by $10^{-6}$ M nicotine (Figure 3). Approximate $IC_{50}$ values were: CHL ($1.6 \times 10^{-6}$ M), Mec ($0.3 \times 10^{-6}$ M) and DHBE ($0.2 \times 10^{-6}$ M). CHL pretreatment also significantly increased basal release ($P < 0.025$), but differences from the control group were small (<20%), and did not reach significance at any concentration of CHL ($P > 0.05$). Neither Mec nor DHBE significantly altered basal release ($P > 0.5$ for each).

### *Surmountable vs insurmountable blockade by* in vitro *chlorisondamine, mecamylamine and dihydro-β-erythroidine*

CHL, Mec and DHBE at concentrations = $IC_{50}$ (see above) were tested against graded concentrations of nicotine ($10^{-7}$–$10^{-4}$ M). The partial blockade that was produced by CHL was not overcome by increasing concentrations of nicotine ($10^{-7}$–$10^{-4}$ M) (Figure 4a). Similar results were obtained with Mec (Figure 4b). In contrast, blockade by DHBE was completely reversed by increasing concentrations of nicotine (Figure 4c). None of the antagonists altered dopamine release induced by high $K^+$ buffer; [$^3$H]-dopamine release (mean ± s.e.mean) after administration of high $K^+$ buffer in control channels compared to those perfused with CHL, Mec and DHBE were, respectively: 195 ± 6%, 195 ± 7%, 206 ± 5% and 180 ± 6% ($n = 30$).

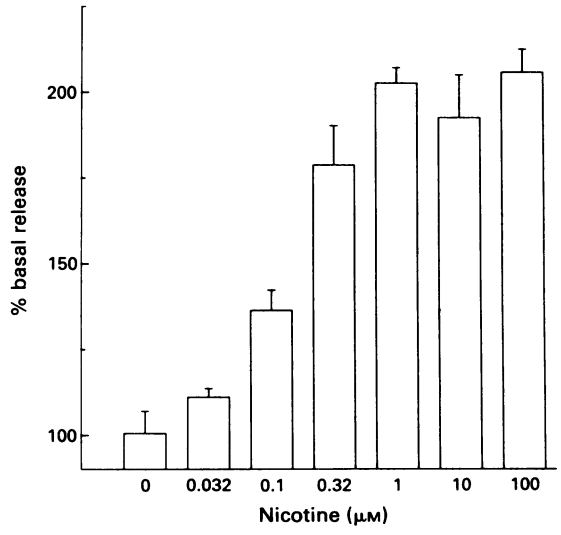



**Figure 1** Effects of (−)-nicotine ($3.2 \times 10^{-8}$–$10^{-4}$ M) on [$^3$H]-dopamine release from striatal synaptosomes. Synaptosomes were superfused with SB for 35 min prior to administration of a 1 min pulse of (−)-nicotine or SB (control). The vertical axis represents the mean peak (±s.e.mean) release, calculated as a percentage of basal release ($n = 6$–9).

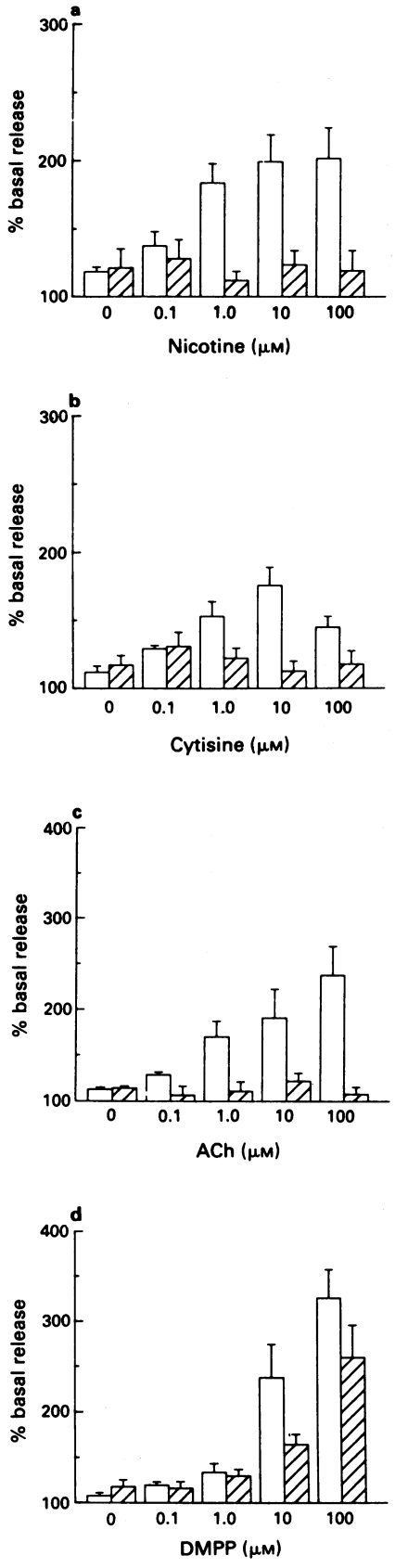
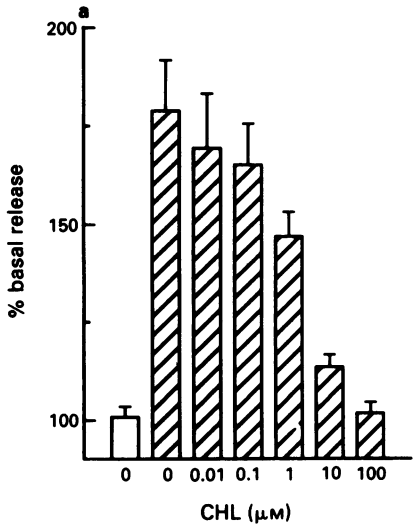
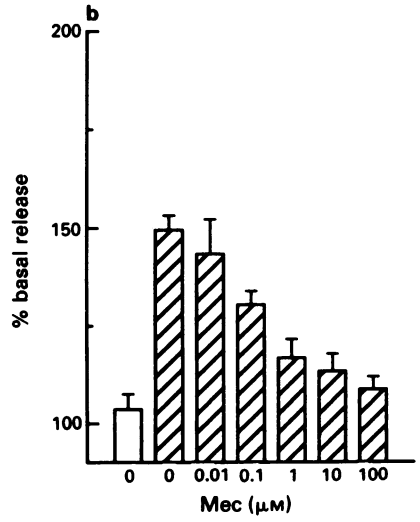
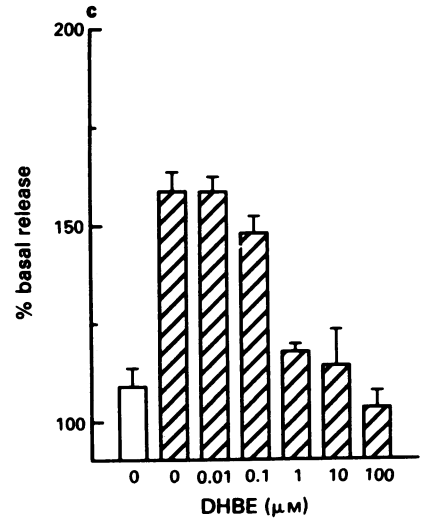
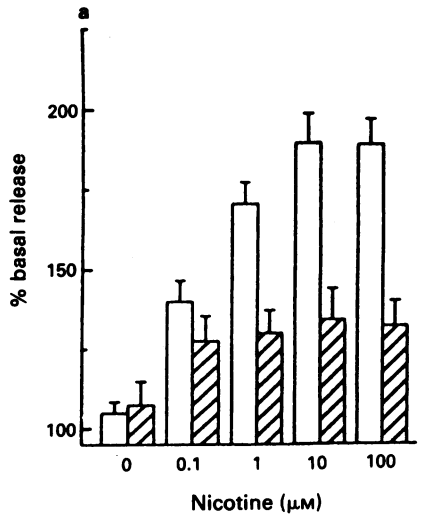


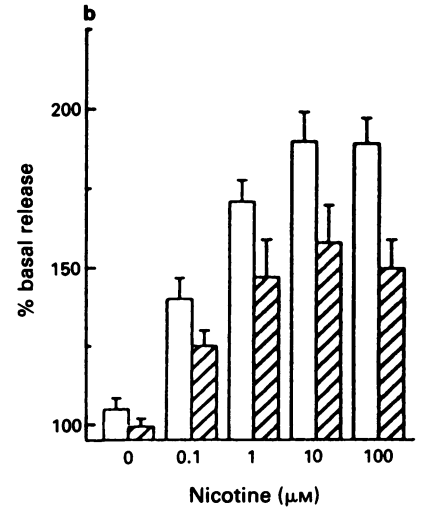

**Figure 2** Effects of nicotinic agonists ($10^{-7}$–$10^{-4}$ M) on [$^3$H]-dopamine release from striatal synaptosomes, in presence of external $Ca^{2+}$ (open columns) and absence of external $Ca^{2+}$ (hatched columns). Synaptosomes were superfused with SB for 35 min prior to administration of a 1 min pulse of the agonist or SB (controls). (a) (−)-Nicotine; (b) cytisine; (c) acetylcholine (ACh); (d) 1,1-dimethyl-4-phenylpiperazzinium (DMPP). The vertical axis represents the mean (±s.e.mean) peak release, calculated as a percentage of basal release ($n = 6$).



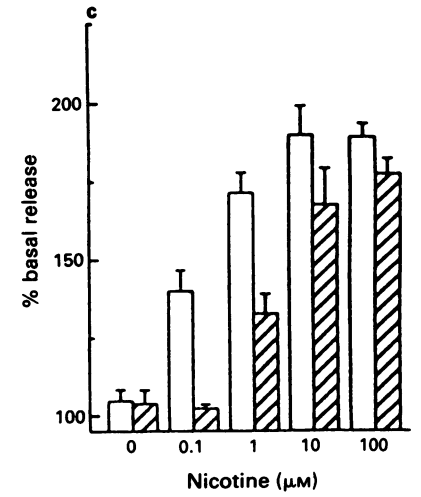




**Figure 3** Effects of antagonists ($10^{-8}$–$10^{-4}$ M) on [$^3$H]-dopamine release, from striatal synaptosomes, induced by a 1 min pulse of (−)-nicotine ($10^{-6}$ M). Antagonists were present in the SB throughout the superfusion period prior to the challenge with (−)-nicotine (hatched columns). Control channels (open columns) were superfused and challenged with SB only. (a) CHL; (b) mecamylamine (Mec); (c) dihydro-β-erythroidine (DHBE). The vertical axis represents the mean (±s.e.mean) peak release, calculated as a percentage of basal release ($n = 12$).

**Figure 4** Tests of surmountable vs insurmountable antagonism. Synaptosomes were superfused with a 1 min pulse of (−)-nicotine ($10^{-7}$–$10^{-4}$ M) or SB, following a 35 min period of superfusion with antagonist (hatched columns) or SB (open columns). Antagonists were present at approximate $IC_{50}$ concentrations determined against (−)-nicotine $10^{-6}$ M: (a) chlorisondamine (CHL, $1.2 \times 10^{-6}$ M); (b) mecamylamine (Mec, $0.3 \times 10^{-6}$ M); (c) dihydro-β-erythroidine (DHBE, $0.22 \times 10^{-6}$ M). The vertical axis represents the mean (±s.e. mean) peak release, calculated as a percentage of basal release ($n = 6$).

### *Use-dependent vs non use-dependent blockade by* in vitro *chlorisondamine, mecamylamine and dihydro-β-erythroidine*

The effects of pre-exposure to nicotine on the potency of CHL, Mec and DHBE were tested. Each antagonist was tested in a different set of assays. Channels were perfused with SB or with SB containing antagonist ($10^{-8}$–$10^{-5}$ M), throughout the experiment. Half of the perfusion channels received an initial 0.4 ml pulse of nicotine ($10^{-6}$ M) whilst the remainder received an equivalent pulse of SB. Thirty-five minutes later, channels were randomly allocated to receive a 0.4 ml challenge with nicotine ($10^{-6}$ M) or SB.

In channels that did not receive antagonist, pre-exposure to a brief pulse of nicotine reduced the effect of a subsequent nicotine dose. The reduction was similar in each set of assays: 32.3% for CHL, 45.4% for Mec, and 35.7% for DHBE. In order to control for this reduction in the response to nicotine, values of peak release were normalized within each set of assays, and were thus expressed in relation to SB alone or nicotine (0% and 100%, respectively).

Pre-exposure of the synaptosomes to nicotine altered the effect of CHL ($P<0.025$), resulting in a leftward shift of the CHL dose-response curve (Figure 5a). The estimated $IC_{50}$ value of CHL was reduced from $1.8 \times 10^{-6}$ M to $6.8 \times 10^{-8}$ M, a 26-fold difference.

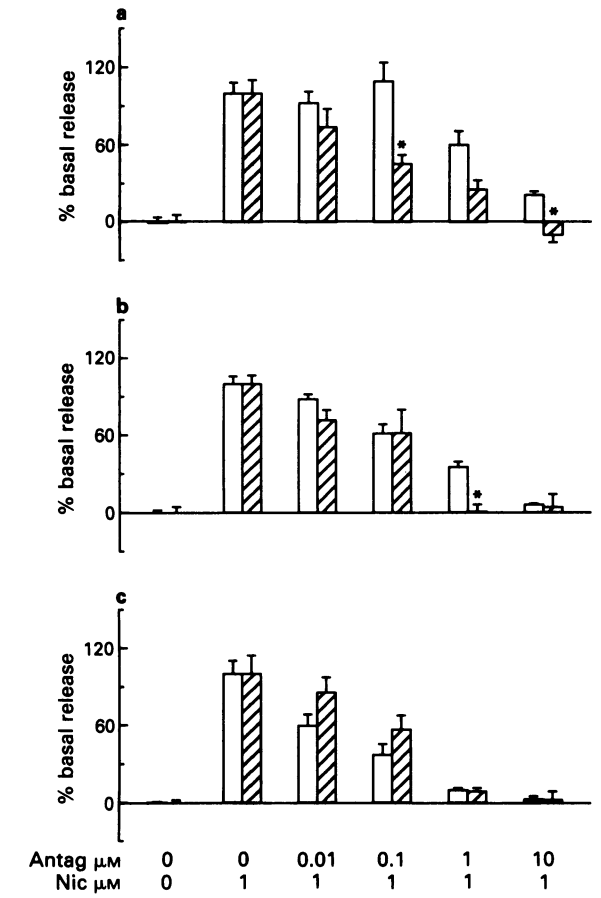



**Figure 5** Tests for use-dependent blockage. Synaptosomes were superfused throughout each assay with SB or SB containing antagonist ($10^{-8}$–$10^{-5}$ M): (a) chlorisondamine (CHL); (b) mecamylamine (Mec); (c) dihydro-β-erythroidine (DHBE). Half of the superfusion channels received an initial 1 min pulse of (−)-nicotine ($10^{-6}$ M) (hatched columns), the remaining channels receiving a pulse of SB (open columns). Thirty min later, channels were randomly allocated to receive a 1 min pulse of nicotine ($10^{-6}$ M) or SB. The vertical axis represents the mean (± s.e.mean) peak release evoked by the challenge dose, calculated as a percentage of basal release (a, $n = 12$; b, $n = 5$; c, $n = 5$). *Different from channels not prestimulated with nicotine at same concentration of antagonist ($P<0.01$).

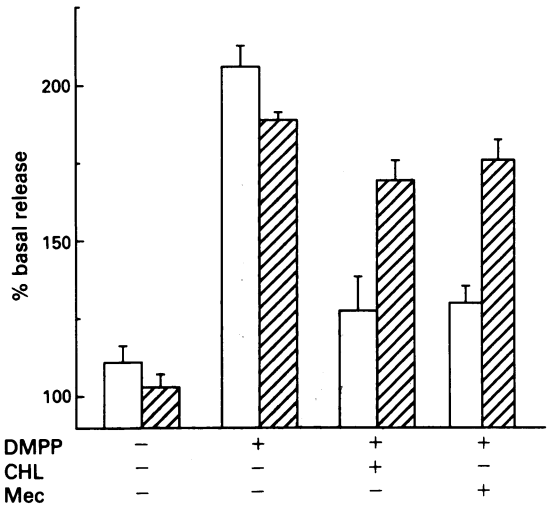



**Figure 6** Effects of nicotinic antagonists chlorisondamine (CHL, $5 \times 10^{-5}$ M) and mecamylamine (Mec, $5 \times 10^{-5}$ M) on responses to 1,1-dimethyl-4-phenylpiperazinium (DMPP, $10^{-5}$ M), in presence of $Ca^{2+}$ (open columns) and in absence of $Ca^{2+}$ (hatched columns). Antagonists were superfused for 35 min prior to the administration of a 1 min pulse of DMPP or SB. The vertical axis represents the mean (± s.e.mean) peak release, calculated as a percentage of basal release ($n = 4$).

Nicotine pre-exposure did not significantly alter the effects of Mec as assessed by ANOVA ($P>0.2$), but a significant effect was observed at one concentration of antagonist (Figure 5b). No such effect was evident with DHBE ($P>0.2$; Figure 5c). Estimated $IC_{50}$ values for Mec, with and without pre-exposure to nicotine, were $1.6 \times 10^{-7}$ and $2.1 \times 10^{-7}$ M, respectively. Corresponding values for DHBE were $1.4 \times 10^{-7}$ and $2.6 \times 10^{-8}$ M.

Basal release prior to test pulse was significantly altered by CHL ($P<0.025$), but not by nicotine pre-exposure ($P>0.2$). However, as in Experiment 3, the effect of CHL was small; a marginally significant change (23% increase over control) occurred at only one concentration ($10^{-5}$ M; $P<0.05$). Neither Mec nor DHBE significantly altered basal release ($P>0.1$ for each).

### *Effects of mecamylamine and chlorisondamine on DMPP-induced release in $Ca^{2+}$-free medium*

Channels were randomly allocated to three groups which were perfused, with either PB alone. PB containing Mec ($5 \times 10^{-5}$ M), or CHL ($5 \times 10^{-5}$ M). Within each group, half the channels were perfused in the presence of external $Ca^{2+}$, and half in its absence. After 35 min, a 0.4 ml pulse of DMPP ($10^{-5}$ M) or PB (control channels) was given.

DMPP ($10^{-5}$ M) induced [$^3$H]-dopamine release even in the absence of external $Ca^{2+}$ (Figure 6; see also Figure 2d). In the presence of $Ca^{2+}$, both Mec ($5 \times 10^{-5}$ M) and CHL ($5 \times 10^{-5}$ M) greatly attenuated the DMPP response (Figure 6). In contrast, these antagonists only slightly reduced the effect of DMPP in the absence of external $Ca^{2+}$ (Figure 6). This difference was confirmed by ANOVA, which revealed a significant interaction between antagonist treatment (control vs Mec vs CHL) and external $Ca^{2+}$ ($P<0.001$).

## Discussion

Nicotine-induced dopamine release has been well documented in striatal synaptosome-enriched preparations (Rapier *et al.*, 1988; 1990; Rowell & Wonnacott, 1990; Grady *et al.*, 1992) and slices (Giorguieff *et al.*, 1976; 1977; Giorguieff-

Chesselet *et al.*, 1979; Westfall *et al.*, 1987; Izenwasser *et al.*, 1991). Two mechanisms of action have been identified. At very high concentrations of nicotine ($10^{-3}$ M or higher) dopamine release is evoked in a $Ca^{2+}$-independent manner (Marien *et al.*, 1983; Westfall *et al.*, 1987; Grady *et al.*, 1992), by a mechanism that is not blocked by classical nicotinic antagonists such as (+)-tubocurarine and Mec (Westfall *et al.*, 1987; Grady *et al.*, 1992); it has been suggested that a tyramine-like depleting action may be involved (Westfall *et al.*, 1987). In contrast, dopamine release evoked by lower concentrations of nicotine ($10^{-4}$ M or lower) is largely $Ca^{2+}$-dependent and is reduced or blocked by classical nicotinic receptor antagonists (Giorguieff *et al.*, 1977; Giorguieff-Chesselet *et al.*, 1979; Westfall *et al.*, 1987; Rapier *et al.*, 1990; Grady *et al.*, 1992). These characteristics have been confirmed in the present study.

We have also shown that cytisine produced only moderate dopamine release. The counterbalanced experimental design permits direct comparison with the other agonists, and the results suggest that cytisine may act as a partial agonist. Such a conclusion is consistent with the results of behavioural and pharmacokinetic studies (Stolerman *et al.*, 1983; Reavill *et al.*, 1990), but would not have been predicted from previous electro-physiological and *in situ* hybridization studies. In oocytes expressing pairwise combinations of nicotinic receptor subunits, cytisine appears to require the presence of one or more beta4 subunits per receptor in order to exert appreciable agonist activity; substitution of beta4 subunits with beta2 subunits leads to a complete loss of agonist activity (Luetje & Patrick, 1991). Curiously, nigrostriatal dopamine neurones do not appear to express beta4 subunits, at least as indicated by mRNA levels detected by *in situ* hybridization (Dineley-Miller & Patrick, 1992), whereas they do appear to express beta2 subunits (Wada *et al.*, 1989). Lesion studies have identified a population of nicotinic receptors associated with nigrostriatal dopaminergic terminals (Schwartz *et al.*, 1984; Clarke & Pert, 1985), that appear to comprise combinations of alpha4 and beta2 subunits (Flores *et al.*, 1992). Several explanations could possibly account for the low efficacy of cytisine: for example, beta4 subunits may be prevalent in dopaminergic cells despite low mRNA abundance; nicotine-induced dopamine release may be mediated by a mixed population of receptor subtypes; native receptors may consist of more than pairwise combinations of alpha and beta subunits (Conroy *et al.*, 1992); and oocyte-expressed receptors may differ from native receptors in other respects. In the mouse, Marks and colleagues have recently reported evidence suggesting that cytisine acts as a full agonist in evoking [$^3$H]-dopamine release from striatal synaptosomes (Grady *et al.*, 1992), but as a partial agonist in inducing rubidium efflux from midbrain synaptosomes (Marks *et al.*, 1993).

The classical nicotinic agonist DMPP (Van Rossum, 1962a) appears to differ significantly from ACh, nicotine and cytisine in its mode of action. A significant releasing action of DMPP was demonstrated in the absence of external $Ca^{2+}$. This occurred at a concentration of DMPP as low as $10^{-5}$ M, whereas a calcium-independent action of DMPP has previously been reported only at a higher concentration of $5 \times 10^{-4}$ M (Westfall *et al.*, 1987). Also, the antagonists CHL and Mec almost completely blocked responses to DMPP in the presence of external $Ca^{2+}$; whereas only partial blockade occurred in the absence of $Ca^{2+}$. This result suggests that DMPP evoked transmitter release via separate mechanisms in these two conditions. These results also demonstrate that a 'calcium-independent' drug effect may occur by a mechanism that may to a large extent be suppressed in the presence of external calcium. In adrenal chromaffin cells, DMPP is reported to evoke transmitter release in the absence of external $Ca^{2+}$ by mobilizing calcium from internal stores (Sasakawa *et al.*, 1986). Possibly, a similar action can occur in striatal tissue.

CHL displayed an insurmountable mode of action in our CNS preparation as previously described in an autonomic ganglion (van Rossum, 1962b). We also found that brief concurrent exposure to nicotine resulted in a marked increase in the potency of CHL. To our knowledge, a 'use-dependent' action has not previously been reported in CNS tissue for any nicotinic antagonist. The use-dependent block observed with CHL is consistent with a possible channel blocking mechanism of action, and indeed, a nicotinic channel blocking action has been proposed for CHL in mammalian ganglia (Traber *et al.*, 1967; Alkadhi & McIsaac, 1974) and in non-mammalian muscle (Lingle, 1983a,b; Neely & Lingle, 1986). Nevertheless, the superfusion method used in our experiments does not permit us to rule out alternative explanations, such as the possibility that CHL enhanced nicotine-induced receptor desensitization.

Mecamylamine, which is probably the most widely used centrally-active nicotinic antagonist, acted insurmountably but did not exhibit use-dependent block. In the periphery, there are reports of either insurmountable or surmountable antagonism by Mec, and even of both components occurring together (van Rossum, 1962b). The nature of blockade by Mec has not been studied before in isolated CNS tissue. However, behavioural experiments have provided evidence for both surmountable and insurmountable actions of Mec in the CNS (Stolerman *et al.*, 1983). Thus, the mechanism of action of Mec appears complex (for review see, Martin *et al.*, 1989).

Less is known about the mode of action of DHBE. Although DHBE is active at nicotinic receptors at autonomic ganglia and skeletal muscle, it has been used principally for CNS electrophysiological studies (Krnjevic, 1975). In the present study, nicotinic blockade by DHBE was found to be surmountable and not use-dependent. These observations are both novel, in the context of the CNS.

The pattern of results obtained in our functional assay is reminiscent of previous findings from radioligand binding studies. Nicotinic receptors labelled with high affinity by [$^3$H]-nicotine and [$^3$H]-ACh represent a prevalent subtype in the brain, thought to be important for mediating the actions of low doses of nicotine (Clarke, 1987; Benwell *et al.*, 1988; for review, see Wonnacott, 1987). Most centrally-active nicotinic antagonists that have been tested do not inhibit [$^3$H]-nicotine or [$^3$H]-ACh binding, suggesting that they do not compete for agonist recognition sites (Marks & Collins, 1982; Schwartz *et al.*, 1982; Clarke *et al.*, 1984; Schwartz & Kellar, 1985). The antagonists CHL and Mec, which we have shown to act insurmountably, are two such examples. In contrast, the antagonist DHBE, which acted in a surmountable fashion, is one of the few nicotinic antagonists known that inhibit high affinity nicotinic [$^3$H]-ACh and [$^3$H]-nicotine binding (Marks & Collins, 1982; Schwartz & Kellar, 1985; Clarke *et al.*, 1984; Marks *et al.*, 1993). Whether any significance can be attached to this correlation is as yet unclear.

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