Br. J. Pharmacol. (1994), 111, 414-418

Blockade of nicotinic receptor-mediated release of dopamine from striatal synaptosomes by chlorisondamine administered *in vivo*

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1 The chronic nicotinic blockade produced following *in vivo* administration of chlorisondamine was investigated *in vitro*. Nicotine-induced [^{3}H]-dopamine release from striatal synaptosomes was used as a measure of central nicotinic receptor function.

2 In synaptosomal preparations from rats pretreated with a single administration of chlorisondamine (10 mg kg^{-1}, s.c.), 1, 7, 21, 42, 63 or 84 days before they were killed, responses to (−)-nicotine (10^{-6} M) were blocked.

3 *In vivo* administration of chlorisondamine (10 mg kg^{-1}, s.c.), 7 days before rats were killed, produced a nicotinic blockade *in vitro* that was insurmountable even with a high concentration of (−)-nicotine (10^{-4} M).

4 Both *in vitro* and *in vivo* administration of chlorisondamine blocked nicotinic responses to acetylcholine (10^{-4} M). In contrast, neither *in vitro* nor *in vivo* administration of chlorisondamine reduced [^{3}H]-dopamine release induced by high K^+ (20×10^{-3} M) or (+)-amphetamine (10^{-6} M).

5 Nicotinic blockade resulting from *in vitro* administration of chlorisondamine (10^{-5} M) recovered partially after 60 min wash-out, and completely by 90 min. In contrast, no recovery was seen in synaptosomes prepared from rats pretreated with chlorisondamine (10 mg kg^{-1}, s.c.) *in vivo*.

6 Thus, *in vivo* treatment with chlorisondamine results in a quasi-irreversible, insurmountable block of CNS nicotinic receptors. The persistence of this block *ex vivo* indicates that physical trapping by the blood brain barrier is not solely responsible for the persistent blockade seen *in vivo*. The resistance of this blockade to prolonged *in vitro* wash-out suggests that the underlying mechanism differs from that associated with *in vitro* administration.

Keywords: Chlorisondamine; nicotine; acetylcholine; dopamine; nicotinic receptors; nicotinic; transmitter release

Introduction

Chlorisondamine (CHL) is a ganglionic nicotinic receptor antagonist (Plummer *et al.*, 1955) which has been used clinically as an antihypertensive (Boura & Green, 1984). Studies in rats have shown that central administration of CHL (5 or 10 μg kg^{-1} i.c.v.) results in a blockade of a variety of behavioural effects of nicotine that are mediated via CNS nicotinic receptors (Clarke & Kumar, 1983; Clarke, 1984; Reavill *et al.*, 1986; Fudala & Iwamoto, 1987; Kumar *et al.*, 1987; Mundy & Iwamoto, 1988; Corrigall *et al.*, 1992). This blockade is remarkably persistent, lasting at least 5 weeks after a single administration of CHL (Clarke, 1984). Although CHL is a bisquaternary amine and appears to penetrate the CNS poorly, administration of a sufficiently large systemic dose (10 mg kg^{-1}, s.c.) also results in a long-lasting central nicotinic blockade (Clarke, 1984; Clarke *et al.*, 1994), whereas ganglion blockade is only transient (Clarke *et al.*, 1994).

The mechanism underlying this persistent central antagonism is not known. The blockade is selective, insofar as administration of CHL did not reduce the behavioural effects of non-nicotinic agents such as apomorphine, midazolam, morphine, cocaine or amphetamine (Reavill *et al.*, 1986; Kumar *et al.*, 1987; Corrigall *et al.*, 1992). The apparent absence of recovery from central nicotinic blockade (Clarke, 1984) suggested the possibility of a neurotoxic mechanism, but in a companion paper (Clarke *et al.*, 1994), we present evidence that CHL does not cause neuronal degeneration. In addition, the persistent central blockade produced by CHL was not accompanied by a change in the density of [^{3}H]-nicotine binding sites in rat forebrain (Clarke *et al.*, 1994).

In the preceding paper (El-Bizri & Clarke, 1994), we characterized the nicotinic blockade produced by acute, *in vitro* administration of CHL. Nicotinic receptor function was assayed by measuring nicotine-induced release of [^{3}H]-dopamine from rat striatal synaptosomes. In the present study, we use the same assay in order to examine the mechanisms underlying the persistent block of central nicotinic responses that follows *in vivo* administration of CHL. By testing nicotinic responses in synaptosomes prepared from rats that had received CHL *in vivo*, it was possible to test whether this persistent central blockade is due solely to physical trapping of the drug (or, possibly, a metabolite) by the blood brain barrier. The chronic blockade produced by *in vivo* administration of CHL was compared to the acute blockade produced by *in vitro* administration of the drug.

Methods

Full details of the procedures for synaptosomal preparation and superfusion are given in an accompanying paper (El-Bizri & Clarke, 1994).

Data analysis

Basal and drug-induced dopamine release were calculated as in the preceding paper (El-Bizri & Clarke, 1994). Statistical results refer to tests of analysis of variance, made using commercial software (Systat, Evanston, IL, U.S.A.).

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Drugs

Drugs were as described in the preceding paper (El-Bizri & Clarke, 1994), except as follows. (+)-Amphetamine sulphate was supplied by Smith, Kline and French (Canada). For *in vivo* administration, chlorisondamine chloride (CHL) was dissolved in 0.9% w/v NaCl solution (saline). Injections were given s.c. in a volume of 1 ml kg^{-1}, and the dose refers to the base. For *in vitro* administration, drugs were dissolved in superfusion buffer (SB).

Procedures

Persistence of CHL blockade ex vivo: *effect of survival duration* Animals in each of six groups were randomly allocated for pretreatment with CHL (10 mg kg^{-1}, s.c., $n = 4$ per survival time) or saline (s.c., $n = 4$ per survival time), and were killed 1, 7, 21, 42, 63 or 84 days later, depending on the group. Synaptosomes from each pretreated animal were tested with nicotine (10^{-6} M; 2 or 3 channels per rat) and SB (1 or 2 channels per rat). Ten minutes later all channels received a 1 min pulse of SB containing high KCl (20×10^{-3} M).

Surmountable vs insurmountable blockade by CHL ex vivo As above, rats were randomly allocated for pretreatment with CHL (10 mg kg^{-1} s.c., $n = 4$) or saline (Sal, $n = 4$). They were killed one week later. Synaptomes prepared from these animals were superfused with a range of nicotine concentrations (10^{-7}–10^{-4} M) (1 or 2 channels per condition per rat).

Selectivity of blockade by CHL: investigated after in vitro *or* in vivo *administration* Six rats were used: four received saline and two received CHL (10 mg kg^{-1}, s.c.) one week before they were killed. In order to test blockade following *in vitro* administration of CHL, synaptosomes from the saline-pretreated rats were allocated to two groups of channels. One group was superfused for 25 min with SB, and the other group received SB containing CHL (10^{-5} M) before administration of test drugs. In order to test blockade following *in vivo* administration of CHL, synaptosomes from rats treated with CHL *in vivo* were perfused for 25 min with SB prior to challenge. Thus there were three *in vivo*/*in vitro* pretreatment conditions: Sal/SB, Sal/CHL, and CHL/SB. Synaptosomal preparations were then challenged acutely with SB, acetylcholine (ACh; 10^{-4} M), (+)-amphetamine (10^{-6} M) or high K^+ buffer (20×10^{-3} M), in a counterbalanced set of 4 assays. Thus, there were 12 combinations of pretreatment and treatment (4–5 channels per condition). In order to inhibit hydrolysis and possible muscarinic actions of ACh, diisopropylfluorophosphate (DFP; 10^{-4} M) and atropine (10^{-6} M) were added to the superfusion buffer, and these compounds were present throughout all conditions.

Recovery from blockade by CHL after in vitro *and* in vivo *administration* First, recovery from blockade was tested following *in vitro* administration of CHL. Nicotinic responses were examined after a 30 min period of wash-out, as follows: in a set of 3 assays using 6 rats, synaptosomes were allocated to two groups of channels, receiving either SB or SB containing CHL (10^{-5} M) for a period of 35 min. Next, equal numbers of channels in each group were perfused with either SB or nicotine (10^{-6} M). A washout period of 30 min followed, during which all channels contained SB. A second dose of SB or nicotine (10^{-6} M) was then given, allocated randomly but in equal number to channels that had previously received SB or nicotine. The procedure was repeated in a second set of 3 assays, except that the wash-out time was extended to 60 min.

Subsequently, the extent of recovery from blockade was compared following *in vivo* vs *in vitro* administration of CHL. In three counterbalanced assays, using 6 rats, synaptosomes were allocated to three groups of channels, corresponding to combinations of *in vivo* and *in vitro* pretreatment, as above: Sal/SB, Sal/CHL and CHL/SB. Following the 35 min *in vitro* pretreatment period, equal numbers of channels in each group received SB or nicotine (10^{-6} M). After a wash-out period of 90 min, a second nicotine or SB superfusion was made. Channels that had previously received a SB challenge now received nicotine (10^{-6} M), and *vice-versa*. The experiment was repeated in another set of 6 assays, using a higher *in vitro* dose of CHL (10^{-4} M), which resulted in a complete nicotinic blockade.

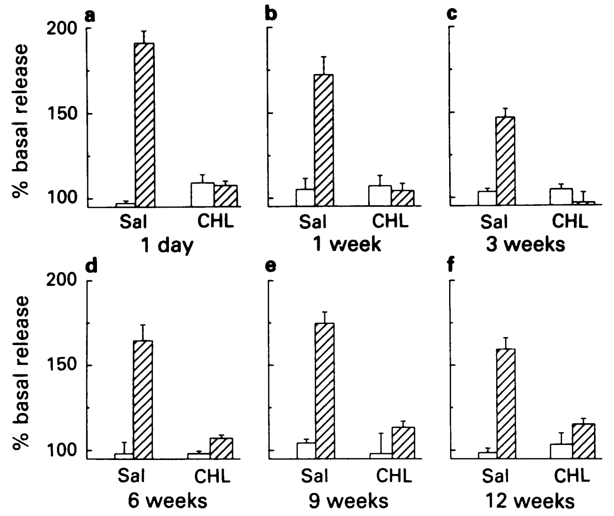

Figure 1 Effects of *in vivo* chlorisondamine (CHL) pretreatment on (−)-nicotine-induced [^{3}H]-dopamine release from rat striatal synaptosomes. Rats received a single pretreatment with CHL (10 mg kg^{-1}, s.c.) or saline and were permitted to survive for different periods before they were killed: (a) 1; (b) 7; (c) 21; (d) 42; (e) 63 or (f) 84 days. Synaptosomes were superfused with superfusion buffer (SB) for 35 min prior to administration of a 1 min pulse of (−)-nicotine (10^{-6} M) (hatched columns) or SB (open columns). The vertical axis represents the mean (± s.e.mean) peak release, calculated as a percentage of basal release ($n = 4$ rats).

Results

Persistence of CHL blockade ex vivo: *effect of survival duration*

A complete block of nicotinic responses was observed *ex vivo*, even several weeks after single administration of CHL (10 mg kg^{-1} s.c.). Thus, striatal synaptosomes prepared from rats that had been pretreated with CHL and permitted to survive for between one day and 12 weeks, failed to release [^{3}H]-dopamine when stimulated by 10^{-6} M nicotine (Figure 1). CHL pretreatment had little if any effect on K^+-induced

Table 1 High K^+-induced [^{3}H]-dopamine release* from saline (Sal) or chlorisondamine (CHL) pretreated rats

Survival time (days)	*Sal pretreatment*	*CHL† pretreatment*
1	180 ± 11	193 ± 6
7	186 ± 18	213 ± 19
21	187 ± 15	178 ± 9
42	200 ± 19	203 ± 22
63	219 ± 9	209 ± 20
84	190 ± 10	199 ± 14

*Mean (± s.e.mean) peak release, calculated as a percentage of basal release ($n = 4$), in response to K^+ 20 mM. †Dose (10 mg kg^{-1}, s.c.).

[^{3}H]-dopamine release at any of the time points tested (Table 1). CHL pretreatment did not alter the basal release ($P > 0.2$), irrespective of survival time ($P > 0.5$).

Surmountable vs insurmountable blockade by CHL ex vivo

As shown in Figure 2, blockade produced by *in vivo* CHL pretreatment was complete, and was insurmountable, even when tested with a high concentration (10^{-4} M) of nicotine.

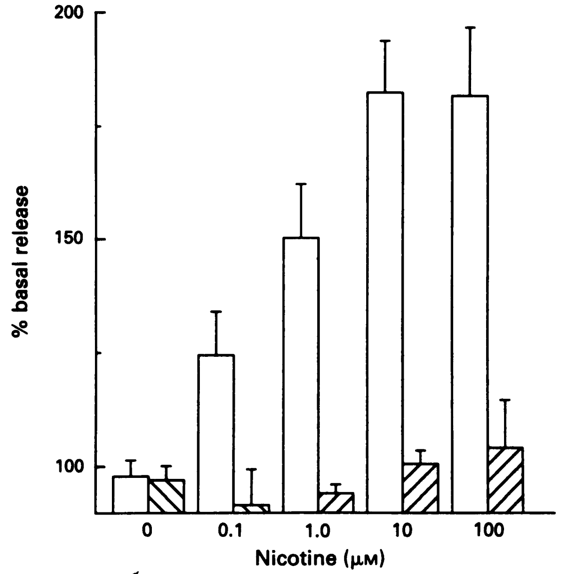

Figure 2 Effects of *in vivo* chlorisondamine (CHL) pretreatment (hatched columns) on (−)-nicotine-induced [^{3}H]-dopamine release from rat striatal synaptosomes. Rats received a single pretreatment with CHL (10 mg kg^{-1}, s.c.) (hatched columns) or saline (open columns) one week before they were killed. Synaptosomes were superfused for 35 min prior to a 1 min pulse of (−)-nicotine or superfusion buffer. The values represents the mean (± s.e.mean) peak release, calculated as a percentage of basal release ($n = 4$ rats).

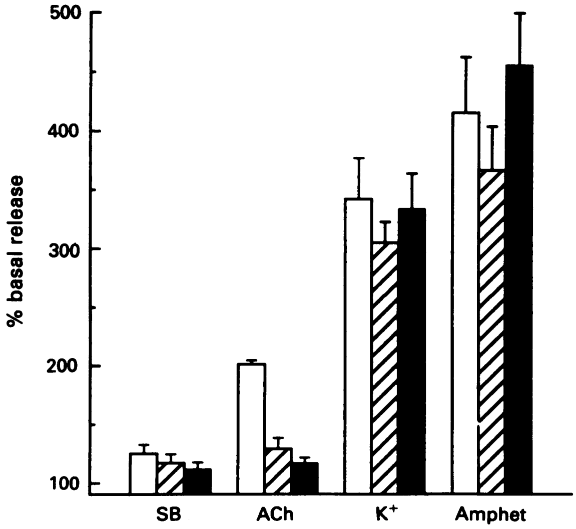

Figure 3 Effects of *in vivo* or *in vitro* chlorisondamine (CHL) pretreatment on [^{3}H]-dopamine release induced by nicotinic and non-nicotinic agents, from rat striatal synaptosomes. Rats received a single pretreatment with CHL (10 mg kg^{-1}, s.c.) or saline one week before they were killed. Synaptosomes from the saline-pretreated rats were superfused with superfusion buffer (SB) (Sal/SB, open columns) or SB containing CHL (10^{-5} M) (Sal/CHL, hatched columns). Synaptosomes from CHL-pretreated rats were superfused with SB (CHL/SB, solid columns). Synaptosomes were superfused in this way for 35 min prior to a 1 min pulse of SB alone, acetylcholine (ACh, 10^{-4} M), (+)-amphetamine (Amphet, 10^{-6} M) or high K^+ buffer (20×10^{-3} M). The vertical axis represents the mean (± s.e.mean) peak release, calculated as a percentage of basal release ($n = 6-12$).

Blockade by CHL after in vitro *or* in vivo *administration*

In control synaptosomes that had not been exposed to CHL, the three stimuli (ACh, (+)-amphetamine, high K^+) produced different peak amounts of [^{3}H]-dopamine release (Figure 3). Prior exposure to CHL, either *in vivo* or *in vitro*, did not reduce responses to high K^+ or (+)-amphetamine. However, prior exposure to CHL resulted in a complete blockade of responses to ACh, and this was the case whether CHL had been given *in vivo* or *in vitro* (Figure 3).

Recovery from blockade following *in vitro* administration of CHL occurred slowly. The extent of recovery did not differ between channels that had received a pre-wash pulse of nicotine and those that had not. After 30 min wash-out no recovery was seen (Figure 4a), but after 60 min wash-out partial recovery was observed (Figure 4b). This is confirmed statistically, as follows. After a 30 min wash-out, CHL-pretreated channels failed to show a nicotinic effect (Tukey's test: $P > 0.9$). After a 60 min wash-out, CHL-pretreated channels were now stimulated by nicotine (Tukey's test: $P < 0.01$) but this nicotinic effect was less than in channels that had not received CHL (ANOVA $P < 0.05$).

When *in vivo* and *in vitro* CHL treatments were subse-

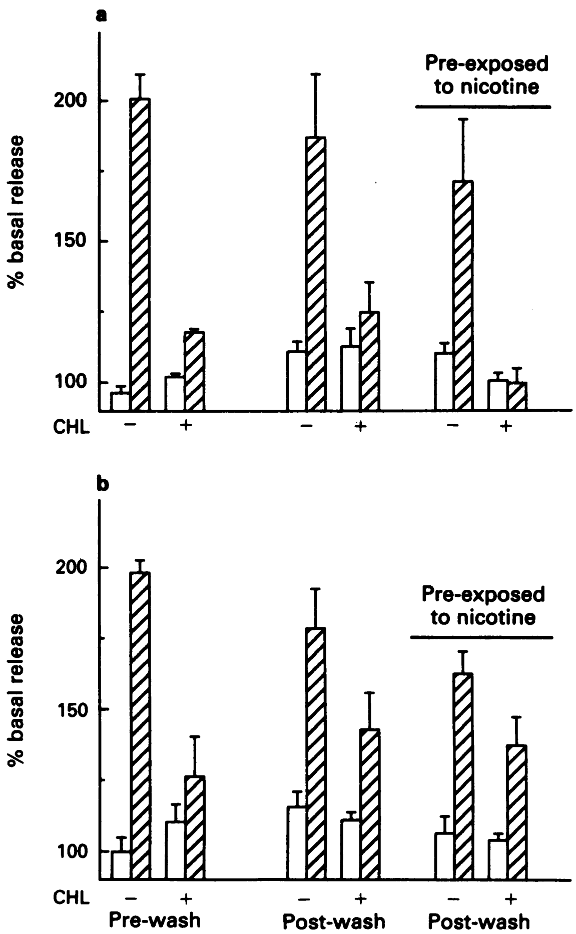

Figure 4 Effects of wash-out with superfusion buffer (SB) on nicotinic blockade produced by *in vitro* chlorisondamine (CHL) administration. Synaptosomes were either superfused with SB or SB containing CHL (10^{-5} M) for 35 min, followed by the first challenge (a 1 min pulse of (−)-nicotine 10^{-6} M or SB). All channels were then washed with SB alone for 30 min (a) or 60 min (b), followed by a second challenge with (−)-nicotine (10^{-6} M) (hatched columns) or SB (open columns), in a counterbalanced manner. Pre-exposed to nicotine, denotes the channels that were initially challenged with nicotine rather than SB. The vertical axis represents mean (± s.e.mean) peak release, calculated as a percentage of basal release ($n = 6-12$).

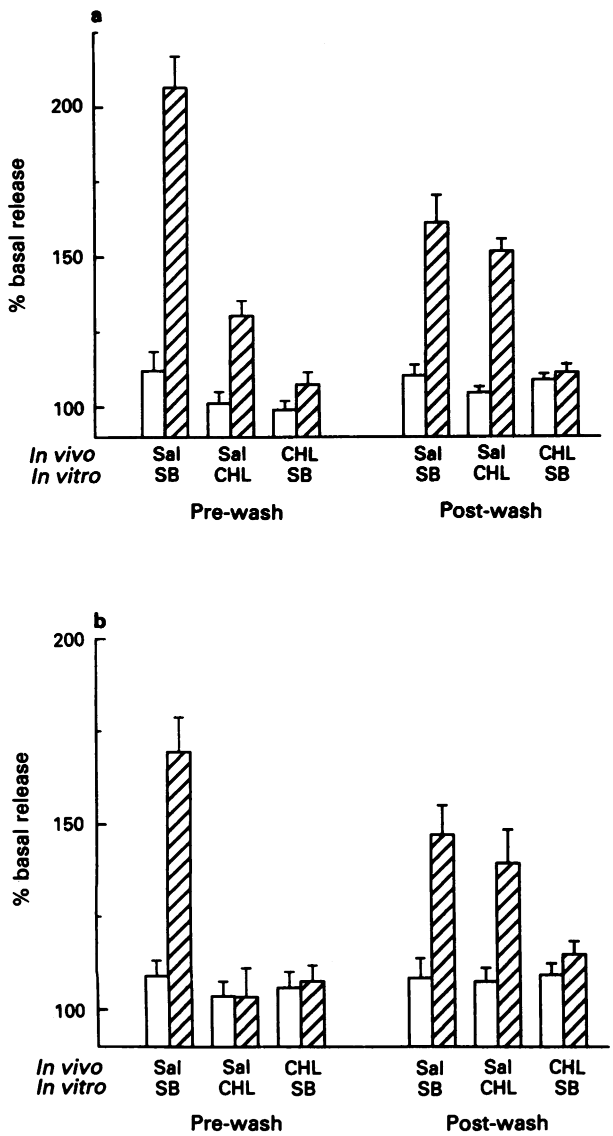

Figure 5 Effects of 90 min wash-out with superfusion buffer (SB) on nicotonic blockade, produced by *in vitro* or *in vivo* chlorisondamine (CHL) administration. Rats received a single pretreatment with CHL ($10\ mg\ kg^{-1}$, s.c.) or saline one week before they were killed. Synaptosomes from the saline-pretreated rats were superfused with SB or SB containing CHL: (a) 10^{-5} M; (b) 10^{-4} M. Synaptosomes from CHL-pretreated rats were superfused with SB. After a 35 min superfusion period, all synaptosomes received 2 pulses, separated by a 90 min wash period with SB alone, one pulse with (−)-nicotine (10^{-6} M) (hatched columns) and the other with SB (open columns). The order of the pulses was counterbalanced within each pretreatment group. The vertical axis represents mean (± s.e.mean) peak release, calculated as a percentage of basal release (a: $n = 5-6$; b: $n = 8-10$).

quently compared, both treatments resulted in nicotinic blockade prior to the wash-out period, as expected. The nicotinic blockade following *in vitro* administration of CHL was complete at 10^{-4} M but not at 10^{-5} M (Figure 5). Following the 90 min wash-out period, synaptosomes that had been exposed *in vitro* to either of the CHL concentrations showed complete recovery of the nicotinic response (Figure 5). Thus, the effect of the post-wash nicotine challenge was not significantly reduced by pretreatment with either the low ($P > 0.1$) or high ($P > 0.2$) concentration of CHL. In marked contrast, synaptosomes prepared from rats that had received CHL *in vivo* showed no signs of recovery from nicotinic blockade (Figure 5).

Discussion

The long-lasting central blockade by CHL is well documented in *in vivo* studies using behavioural testing (Clarke & Kumar, 1983; Clarke, 1984; Reavill *et al.*, 1986; Fudala & Iwamoto, 1987; Kumar *et al.*, 1987; Mundy & Iwamoto, 1988; Corrigall *et al.*, 1992; Clarke *et al.*, 1994). Here, we demonstrate for the first time that *in vivo* administration of CHL results in *ex vivo* blockade, using an *in vitro* assay of brain nicotinic receptor function. Remarkably, this blockade persisted even 12 weeks after *in vivo* pretreatment with CHL, and resisted extensive washing *in vitro*. We further demonstrate that CHL exerts a selective action, in so far as neither *in vivo* nor *in vitro* administration of CHL altered the responses to high K^+ buffer or (+)-amphetamine. We are not aware of any other selective nicotinic antagonist that produces such a persistent central blockade.

Is the long-lasting block in vivo *due to persistence of chlorisondamine in the CNS?*

At physiological pH, CHL is positively charged and does not readily cross the blood brain barrier (Clarke, 1984). Presumably, therefore, any CHL that reaches the CNS following systemic administration of a high dose may be retained for some time. Physical trapping of this sort suggested itself as a possible cause for the long-lasting blockade by CHL. However, the blood brain barrier clearly cannot contribute in synaptosomal experiments, where CHL block was persistent.

What mechanism(s) would underlie the persistent central nicotinic block following in vivo *administration of CHL?*

In the present study, we have demonstrated that extended washing did not effect recovery in synaptosomes prepared from rats pretreated with CHL *in vivo*. In contrast, a 60 min wash-out period produced a partial recovery from blockade following *in vitro* administration of CHL, and this recovery became complete by 90 min wash.

This final experiment therefore demonstrates that the mechanism underlying the acute blockade following *in vitro* administration of CHL differs from that associated with the chronic blockade occurring after *in vivo* administration of the drug. The basis for this difference is not clear. Possibly, CHL is transformed *in vivo* but not *in vitro*, forming an active metabolite which is more slowly reversible than CHL itself. It is also conceivable that *in vivo*, the receptors undergo a conformational change, assuming a stable state in which CHL (or a metabolite) is captured. In non-mammalian tissue, Lingle and Neely (Lingle, 1983a,b; Neely & Lingle, 1986) proposed that, following use-dependent block by CHL, the receptor assumes a stable-blocked state in which CHL would be trapped within the closed ion channel. Whether this proposal, derived from acute *in vitro* studies, relates to the chronic *in vivo* blockade produced by CHL is not clear. Moreover, whereas in the experiments of Lingle and colleagues (Lingle, 1983a,b; Neely & Lingle, 1986), recovery from blockade by CHL required re-exposure to agonist, such a requirement was not observed in the present study (Figure 4).

Is the chronic blocking action of CHL reversible?

The turnover rate of CNS nicotinic receptors is unknown. However, nicotinic receptor turnover, where studied in other tissues, has typically been found to occur over a period of hours to a few days at most (Kemp & Edge, 1987; Higgins & Berg, 1988; Avila *et al.*, 1989; Fumagalli *et al.*, 1990). A possible effect of CHL on nicotinic receptor synthesis or on the assembly of the receptor subunits is unlikely, since high affinity [^{3}H]-nicotine binding appears to be unaltered during persistent blockade by CHL (Clarke *et al.*, 1994). Thus, it is likely that over the course of this extended period of blockade, several generations of nicotinic receptors are produced and then degraded. This suggests that an extremely slow dissociation of the active compound from its binding sites cannot by itself explain the persistent central blockade; even if CHL were to bind irreversibly to CNS nicotinic receptors, persistent *in vivo* blockade would require either efficient recycling of the antagonist, or a reserve of antagonist retained in the brain. Possibly, the long-lasting *in vivo* blockade by CHL results from a combination of a slow dissociation of the active compound, with some form of physical trapping provided either by the synaptic environment or by the blood brain barrier.

The nicotinic blockade obtained after a single *in vivo* administration of CHL was not overcome by a high concentration of acetylcholine (10^{-4} M) or even by concentrations of nicotine that would be acutely toxic or lethal *in vivo* (10^{-4} M). Circulating concentrations of nicotine in habitual cigarette smokers are typically in the range of $0.1-0.5 \times 10^{-6}$ M, and during active cigarette smoking, transient concentrations several-fold higher may be achieved in the brain (Benowitz *et al.*, 1990; Russell, 1990). In this concentration-range, nicotine can induce receptor-mediated dopamine release (Giorguieff *et al.*, 1977; Giorguieff Chesselet *et al.*, 1979; Westfall *et al.*, 1987; Rapier *et al.*, 1988; Grady *et al.*, 1992; El-Bizri & Clarke, 1994). Thus, it is likely that the receptor subtype(s) that were blocked by CHL in our experiments would mediate some of the central effects of nicotine encountered during smoking. The persisent and insurmountable antagonism seen following administration of CHL may be of relevance in the context of developing a pharmacological treatment for nicotine dependence.

We wish to thank Drs Sharon Grady, Michael Marks and Allan Collins for generously sharing their expertise, which was indispensable in setting up the release assay. We thank Dr Brian Collier for his valuable comments. We also thank Miss Melanie Reuben for excellent technical assistance. Ciba-Geigy generously donated samples of chlorisondamine. Supported by the Medical Research Council of Canada. H.E.-B. was a Hydro-Quebec Fellow and is an MRC (Canada) Student. P.B.S.C. holds a Senior I Career Award from the FRSQ.

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(Received May 4, 1993
Revised September 21, 1993
Accepted September 29, 1993)