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Blockade of nicotinic responses by physostigmine, tacrine and other cholinesterase inhibitors in rat striatum

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1 The acetylcholinesterase inhibitors physostigmine, neostigmine, tetrahydroaminoacridine (tacrine; THA) and diisopropylfluorophosphate (DFP) were tested for possible direct nicotinic actions in rat striatal synaptosomes preloaded with [^{3}H]-dopamine. In this preparation, nicotinic cholinoceptor activation evoked [^{3}H]-dopamine release.

2 Antagonist activity was examined by giving a brief nicotine (1 μM) challenge after 30 min superfusion with an acetylcholinesterase (AChE) inhibitor (0.3–300 μM). Physostigmine, neostigmine and tacrine produced a concentration-dependent blockade. Physostigmine and tacrine were particularly potent (IC_{50}s approx. 10 μM and 1 μM, respectively). DFP reduced nicotinic responses only at the highest concentration tested (300 μM).

3 Nicotinic blockade produced by superfusion with physostigmine (30 μM) was insurmountable when tested against nicotine (0.1–100 μM).

4 Physostigmine (30 μM) also reduced responses to the nicotinic agonists 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) and cytisine, but did not alter responses to high K^+ or (+)-amphetamine. A higher concentration of physostigmine (300 μM) completely blocked responses to nicotine, somewhat reduced responses to amphetamine, and did not alter responses to high K^+. Tacrine (3 μM) reduced responses to nicotine and to high K^+ but did not affect responses to amphetamine.

5 Physostigmine (0.3–300 μM), given as a brief pulse, did not produce a nicotinic agonist-like effect.

6 Physostigmine, neostigmine, tacrine and DFP (all at 30 μM) each produced near-total (>96%) inhibition of AChE activity. However, DFP at a concentration (60 μM) that produced a degree of AChE inhibition equal to that of physostigmine 30 μM, did not significantly reduce nicotine-induced dopamine release.

7 It thus appears that physostigmine blocks CNS nicotinic receptors in an insurmountable and pharmacologically selective manner, independent of its ability to inhibit acetylcholinesterase. Tacrine reduced nicotinic responses, quite possibly by an indirect mechanism. The possibility of direct or indirect blockade of nicotinic receptor-mediated actions may complicate the interpretation of preclinical studies that have employed physostigmine and tacrine.

Keywords: Physostigmine; neostigmine; diisopropylfluorophosphate (DFP); tacrine; cholinesterase inhibitors; nicotine; nicotinic receptors; striatum; dopamine

Introduction

Acetylcholinesterase inhibitors (AChEIs) continue to be widely used in studies of cholinergic pharmacology and physiology. The peripheral actions of physostigmine, the prototypical AChEI, are almost entirely attributable to an inhibition of acetylcholinesterase, and classical experimental approaches have uncovered little evidence of direct postsynaptic actions (Taylor, 1990). However, single channel patch clamp recording techniques have revealed direct agonist and antagonist actions of physostigmine on nicotinic cholinoceptors (AChRs) of frog skeletal muscle (Shaw *et al.*, 1985) and of rat hippocampal neurones (Pereira *et al.*, 1993).

AChEIs form a chemically diverse group. The quaternary ammonium compound, neostigmine, exerts additional postsynaptic actions at autonomic ganglia (Haefely, 1980) and muscle endplate (Taylor, 1990); some of these actions appear mediated directly by nicotinic AChRs (Haefely, 1980; Fiekers, 1985). Tetrahydroaminoacridine (THA; tacrine) possesses a complex pharmacological profile, with AChE-independent actions reported on muscarinic receptors and on certain enzymes and voltage-gated ion channels (Freeman & Dawson, 1991). The organophosphorous compound, diisopropylfluorophosphate (DFP; dyflos), in contrast, appears quite selective for AChE over cholinoceptors (Taylor, 1990).

The purpose of the present study was to examine these commonly used AChEIs for possible direct nicotinic actions in the mammalian CNS. This issue has received little attention (Pereira *et al.*, 1993), and is important for at least two reasons. Firstly, in experimental studies, the use of AChEIs to define possible sites of nicotinic cholinergic transmission requires that these agents have negligible direct actions on receptors. Secondly, preliminary indications suggest that stimulation of CNS nicotinic receptors may be beneficial in Alzheimer's disease (Newhouse *et al.*, 1988; Jones *et al.*, 1992), whereas CNS nicotinic receptor blockade may impair cognition (Newhouse *et al.*, 1992). It thus becomes important to determine whether physostigmine and tacrine, two centrally-active AChEIs that have been administered to patients suffering from Alzheimer's dementia or other disorders (Davis *et al.*, 1983; Thal & Fuld, 1983; Summers *et al.*, 1986; Sahakian *et al.*, 1993), exert nicotinic actions.

Methods

Male Sprague-Dawley rats (Charles River, St. Constant, Quebec), weighing 200–250 g, were maintained on a 12 h/12 h light-dark cycle. Rats were housed 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 prior to testing.

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Dopamine release from superfused synaptosomes

Rats were decapitated, and both striata (combined wet weight 160–200 mg) were immediately dissected in ice-cold 0.32 M sucrose/5 mM HEPES at pH 7.5. Striata were homogenized in 20 vol of the sucrose/HEPES solution (12 up and down strokes, at 850 r.p.m., 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, in mM concentrations: NaCl 128, KCl 2.4, $CaCl_2$ 3.2, KH_2PO_4 1.2, $MgSO_4$ 1.2, HEPES 25, D-glucose 10, L-ascorbic acid 1 and pargyline 0.1 at pH 7.5. The synaptosomal preparation was incubated with [^{3}H]-dopamine (0.12 μM) for 5 min at 37°C. The synaptosomes were then centrifuged at 12000 *g* for 5 min at RT. The pellet was gently resuspended in an equal volume of SB.

Superfusion

The apparatus comprised 30 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. Drug (or SB for control channels) was introduced into the superfusate via a 1 ml syringe and 30 gauge needle (Becton Dickinson, NJ, U.S.A.) upstream to the chamber. In order to reduce the possibility of contaminants, all syringes used in these experiments were treated with bleach, followed by thorough washing with distilled water.

Each experiment comprised two or more 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. A superfusion period of 30 min followed, and AChE inhibitor was present in the buffer during this time, in the appropriate channels. Thirteen samples per channel were then collected in consecutive 1 min intervals into polypropylene minivials (Sarstedt, Montreal, Canada) containing 3 ml of scintillation fluid (BCS, Amersham, Montreal, Canada). After a 5 min baseline collection period, during which dopamine release showed little if any decline, a 1 min (0.4 ml) pulse of drug or SB (control channels) was injected. 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 30 channels. Four 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 invidual assays. 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).

Acetylcholinesterase assay

AChE activity was measured in Experiment 5. This experiment comprised three parts, each part consisting of two or four assays. In each assay, striatal tissue from one rat was used, and quadruplicate determinations were made for each drug condition. Within each assay, AChE activity was expressed as a percentage of the mean of control samples, and the normalized data were then pooled across all assays for that part.

Synaptosomes were prepared, as for dopamine release experiments, and were resuspended in approx. 1 ml of super-

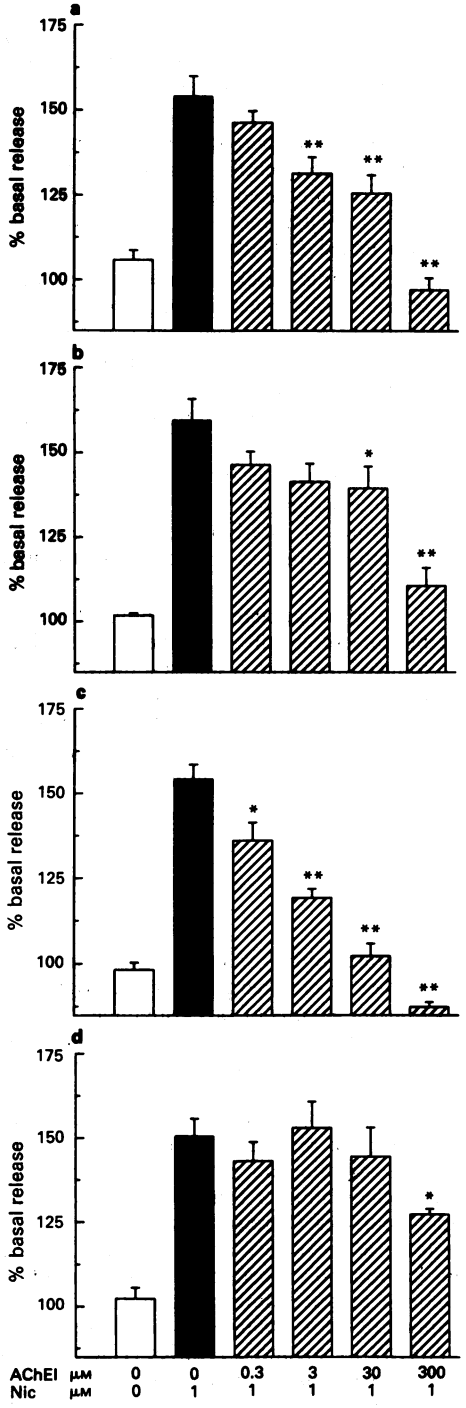


Figure 1 Effects of physostigmine (a), neostigmine (b), tacrine (c) and DFP (d) on [^{3}H]-dopamine release induced by nicotine from striatal synaptosomes (Experiment 1). Synaptosomes were superfused with buffer in the presence or absence of acetylcholinesterase inhibitor (AChEI) (0.3–300 μM) for 35 min prior to challenge with nicotine (Nic) 1 μM or buffer. The vertical axis shows mean ± s.e. mean peak release expressed as a percentage of basal release. Superfusion channels per condition: $n = 10$–20 (a), 8–11 (b), 7–10 (c), 7–14 (d). $^*P<0.05$; $^{**}P<0.01$ vs. nicotine alone (Dunnett's test).

fusion buffer (SB). Aliquots of 0.2 ml were incubated for 30 min at RT with or without addition of AChE inhibitor. AChE activity was determined colorimetrically by the method of Ellman *et al.* (1961). Briefly, 20 µl samples were added to 50 µl of 10 mM dithiobisnitrobenzoic acid and 1.395 ml of 0.1 M Na phosphate buffer, pH 8.0. Samples that had been incubated in the presence of AChEI were assayed in buffer containing the same concentration of the relevant AChEI. The reaction was started by the addition of 20 µl of 75 mM acetylthiocholine iodide (or Na phosphate buffer for the zeroing cuvette). The absorbance at 412 nm was measured with a spectrophotometer (Spectronic 2000, Bausch and Lomb, Rochester, NY, U.S.A.) and recorded every minute for 5 min. The rate of change of absorbance over time, determined by linear regression, was used to calculate the rate of reaction.

Data analysis

Basal release was defined as the mean radioactivity released (d.p.m. min^{-1}) over the five 1 min samples collected immediately prior to drug or SB administration. This basal [^{3}H]-dopamine release was approximately 2810 ± 210 d.p.m. min^{-1} (mean ± s.e.mean, $n = 9$ series of assays), which corresponds to approximately 3 fmol mg^{-1} of original wet tissue. Across experiments, basal release (min^{-1}) ranged from 0.7 to 2% of residual radioactivity collected on the tissue filters (304,000 ± 23,000 d.p.m. $filter^{-1}$). 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. This measure of drug effect was used since it is less likely to be affected by receptor desensitization than the time-averaged drug effect ('area under the curve'). Drug effects were examined by analysis of variance, using commercial software (Systat, Evanston, IL, U.S.A.). Multiple comparisons between all groups were made with Tukey's HSD test (Wilkinson, 1990); comparisons with a single 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). Probability values are 2-tailed.

Drugs

Chemicals and supplies were as follows: [^{3}H]-dopamine (dopamine, [8-^{3}H]-, specific activity 38.1 Ci $mmol^{-1}$; New England Nuclear, Boston, MA, U.S.A.), (−)-nicotine hydrogen tartrate, cytisine, 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), diisopropylfluorophosphate (DFP, dyflos) (Sigma Chemical Corp., St. Louis, MO, U.S.A.), physostigmine sulphate, neostigmine bromide, pargyline hydrochloride (Research Biochemicals Inc., Natick, MA, U.S.A.), 9-amino-1,2,3,4-tetrahydroacridine (tacrine, THA; gift of Pharmascience Inc., Montreal, Canada), (+)-amphetamine sulphate (gift of SmithKline Beecham Pharma, Oakville, Canada). Other chemicals and reagents were purchased from commercial sources. For *in vitro* administration, drugs were dissolved in superfusion buffer (SB).

Results

Experiment 1: Effects of physostigmine, neostigmine, tacrine and DFP on striatal [^{3}H]-dopamine release evoked by nicotine

The effects of each AChE inhibitor (0.3–300 µM) were examined in a different set of assays (Figure 1). In the absence of AChE inhibitor, nicotine increased dopamine release by 50–60% over baseline. Physostigmine (0.3–300 µM) reduced this effect in a concentration-dependent manner (main effect of concentration: $F = 19.17$, d.f. 4, 71, $P < 0.0001$). The IC_{50} of physostigmine fell between 3 and 30 µM, and complete blockade occurred at 300 µM (Figure 1a). Neostigmine (0.3–300 µM) reduced nicotinic responses in a concentration-related manner (main effect of concentration: $F = 10.36$, d.f. 4, 46, $P < 0.0001$), with an IC_{50} of approx. 100 µM (Figure 1b). Tacrine (0.3–300 µM) also produced a concentration-related inhibition of nicotine-induced dopamine release (main effect of concentration: $F = 31.68$, d.f. 4, 36, $P < 0.0001$), but appeared more potent than physostigmine or neostigmine (IC_{50} approx. 1 µM). Tacrine was effective even at 0.3 µM (Dunnett's test $P < 0.02$; Figure 1c). In the same concentration-range, DFP had no significant antagonist action as determined by ANOVA (main effect of concentration: $F = 2.33$, d.f. 4, 40, $P > 0.07$), and only at 300 µM was a significant reduction of nicotine-induced dopamine release detected (Dunnett's test: $P < 0.05$; Figure 1d).

Basal dopamine release was not significantly altered by superfusion with either physostigmine (main effect of concentration: $F = 1.92$, d.f. 4, 71, $P > 0.1$), neostigmine ($F = 0.13$, d.f. 4, 46, $P > 0.9$) or DFP ($F = 1.36$, d.f. 4, 40, $P > 0.2$). Tacrine did not significantly affect basal release at 0.3 or 3 µM (Dunnett's test, $P > 0.5$), but increased it markedly at 30 and 300 µM (Dunnett's test, $P < 0.0001$ for each). Mean (± s.e.mean) basal values of dopamine release for tacrine 0–300 µM were: 2419 ± 114, 2363 ± 134, 2759 ± 209, 5485 ± 330, and 6179 ± 326 DPM min^{-1}.

Experiment 2: Surmountable vs insurmountable blockade by physostigmine

The approximate IC_{50} of physostigmine (estimated from Experiment 1) was tested against graded concentrations of nicotine (Figure 2). The procedure was otherwise identical to that of Experimental 1. In the absence of physostigmine, nicotine increased dopamine release in a concentration-related manner. A maximal effect was attained at approximately 1 µM, and the EC_{50} was between 0.1 and 1 µM. Physostigmine significantly reduced the effect of nicotine at every concentration of the agonist ($P < 0.02$ to $P < 0.01$; Figure 2). Thus, blockade by physostigmine was not surmounted, even by high concentrations of nicotine. Perfusion with physostigmine did not significantly alter basal release ($F = 0.08$, d.f. 1.82, $P > 0.7$).

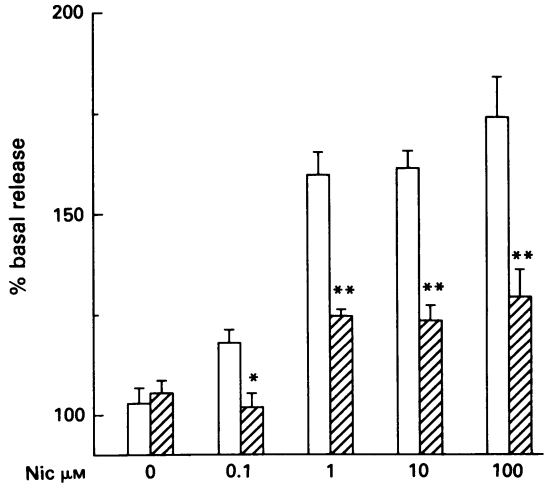
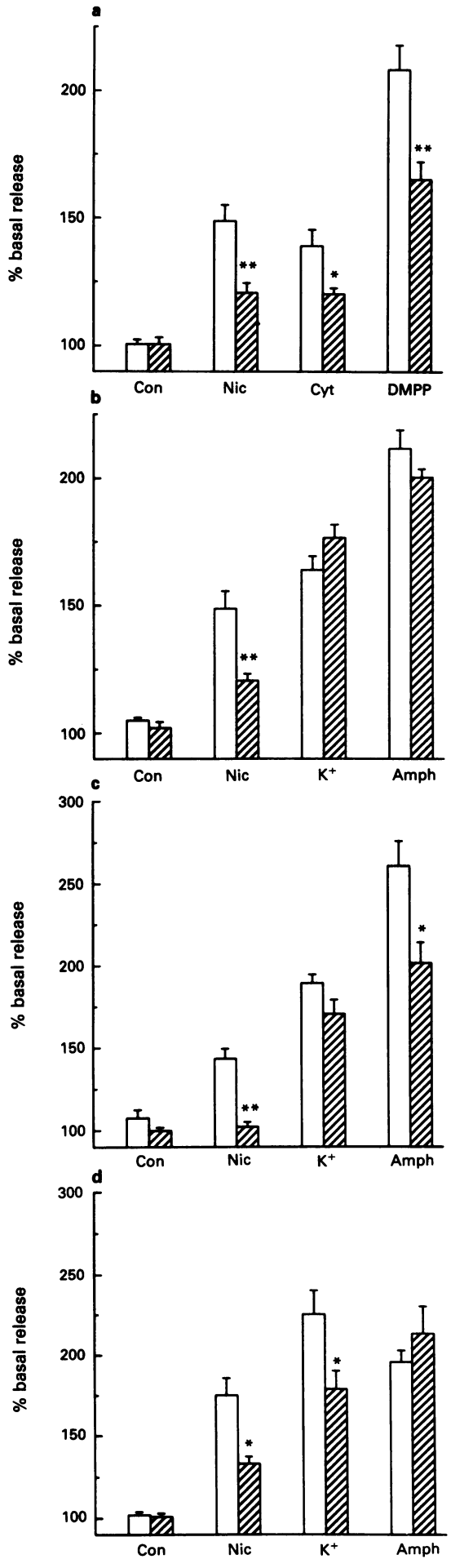


Figure 2 Insurmountable block by physostigmine (Experiment 2). Synaptosomes were challenged with nicotine (Nic, 0.1–100 µM) or with buffer alone, after a 35 min period of superfusion with (hatched columns) or without (open columns) physostigmine 30 µM. The vertical axis shows mean ± s.e.mean peak release expressed as a percentage of basal release ($n = 7$–11 superfusion channels). *$P < 0.02$, **$P < 0.01$ vs. physostigmine-free condition at same nicotine concentration (Student's *t* test with Bonferroni adjustment).



Experiment 3: Selectivity of blockade by physostigmine and tacrine

Four sets of assays were performed (Figure 3a–d). In the first set of assays (Figure 3a), physostigmine (30 μM) significantly reduced dopamine release evoked by nicotine (1 μM) ($t = 3.66$, d.f. 15, $P < 0.01$), cytisine (10 μM) ($t = 2.73$, d.f. 13.9, $P < 0.05$) and DMPP (10 μM) ($t = 3.69$, d.f. 15, $P < 0.01$). Basal release was not altered by physostigmine ($F = 0.01$, d.f. 1.76, $P > 0.8$).

In the second set of assays (Figure 3b), physostigmine (30 μM) reduced nicotine-induced dopamine release ($t = 3.79$, d.f. 16.9, $P < 0.005$), but did not significantly alter release induced by either 12 mM K^+ ($t = 1.69$, d.f. 30, $P > 0.1$) or 0.3 μM amphetamine ($t = 1.44$, d.f. 22.5, $P > 0.01$). Basal release was not affected by physostigmine ($F = 0.04$, d.f. 1, 112, $P > 0.8$).

A third set of assays was conducted in identical fashion, except that a higher concentration of physostigmine was used (300 μM). Here, physostigmine completely blocked nicotine-induced release, and somewhat reduced ampetamine-induced release ($t = 2.99$, d.f. 12, $P < 0.05$), but did not alter release induced by K^+ ($t = 1.91$, d.f. 13, $P > 0.2$) (Figure 3c). This high concentration of physostigmine also increased basal release ($F = 5.94$, d.f. 1, 47, $P < 0.02$), but the effect was small (13.4% increase).

A fourth set of assays was performed likewise, except that tacrine 3 μM was tested instead of physostigmine. Tacrine 3 μM (Figure 3d) attenuated dopamine release induced by nicotine ($t = 3.70$, d.f. 9.3, $P < 0.02$) and by high K^+ ($t = 2.79$, d.f. 23, $P < 0.05$), but not by amphetamine ($t = 1.67$, d.f. 13, $P > 0.1$). Tacrine also increased basal dopamine release ($t = 2.23$, d.f. 53, $P < 0.05$), but the effect was small (14.4% increase).

Experiment 4: Effects of a brief pulse of physostigmine on striatal [^{3}H]-dopamine release

In order to test for an agonist-like effect of physostigmine, synaptosomes were challenged with a 0.4 ml pulse containing nicotine 1 μM, physostigmine (0.3, 3, 30 or 300 μM), or SB alone. Physostigmine produced no clear effect. A significant main effect of drug ($F = 12.48$, d.f. 5, 54, $P < 0.0001$) was attributable to the effect of nicotine (Figure 4). Comparisons with the control condition (SB alone) revealed no significant effect of physostigmine, even at the highest concentration (Dunnett's test: $P > 0.8$).

Experiment 5: Acetylcholinesterase inhibition by physostigmine, neostigmine, tacrine and DFP

In the first set of assays, all three drugs tested (30 μM in each case) inhibited AChE activity almost completely (Table 1). ANOVA indicated that the degree of inhibition between the

Figure 3 Pharmacological selectivity of blockade by physostigmine or tacrine (Experiment 3). In all assays, synaptosomes were superfused for 35 min with or without acetylcholinesterase inhibitor (AChEI) (hatched and open column, respectively), prior to acute drug challenge. (a) Challenge with nicotine 1 μM (Nic), cytisine 10 μM (Cyt), DMPP 10 μM or buffer alone, with or without physostigmine (30 μM). (b) Challenge with nicotine 1 μM, K^+ 12 mM, (+)-amphetamine 0.3 μM (Amph) or buffer alone, with or without physostigmine 30 μM. (c) As for (b), except physostigmine 300 μM was used. (d) As for (b), except tacrine 3 μM was used. The vertical axis shows mean ± s.e.mean peak release expressed as a percentage of basal release. Superfusion channels per condition: $n = 8–12$ (a), 13–17 (b), 5–8 (c), 6–13 (d). $^*P < 0.05$; $^{**}P < 0.01$ vs. AChEI-free condition at same agonist concentration (Student's t test with Bonferroni's adjustment).

three drugs was not the same (drug main effect: $F = 3.70$, d.f. 2, 45, $P < 0.05$), but any differences were small and *a posteriori* tests did not reveal a significant difference between physostigmine and either neostigmine or DFP (respectively: $t = 2.26$, d.f. 21.2, $P > 0.06$, and $t = 1.38$, d.f. 18.9, $P > 0.3$). In control samples exposed only to SB, the mean ± s.e.mean AChE activity was 4.26 ± 0.52 nmol min^{-1} mg^{-1} original tissue ($n = 4$ assays).

A second set of two assays was performed in order to establish a concentration of tacrine that inhibited AChE activity as effectively as physostigmine 30 μM. Tacrine 0.3 μM and 3 μM produced significantly less AChE inhibition than physostigmine 30 μM (respectively: $t = 12.9$, d.f. 7.3, $P <$ 0.0001; $t = 5.32$, d.f. 13, $P < 0.001$), whereas tacrine 30 μM and physostigmine 30 μM produced comparable effects ($t = 0.68$, d.f. 9, $P > 0.5$; Table 1). In control samples exposed only to SB, the mean ± s.e.mean AChE activity was 4.59 ± 0.14 nmol min^{-1} mg^{-1} original tissue ($n = 2$ assays).

A third set of four assays was performed in order to establish a concentration of DFP that inhibited AChE activity as effectively as physostigmine 30 μM. Physostigmine and all three concentrations of DFP almost completely inhibited AChE activity (Table 1). The degree of inhibition differed to a small extent between drug conditions (drug main effect: $F = 14.98$, d.f. 3, 60, $P < 0.0001$); the lowest concentration of DFP (30 μM) gave significantly less inhibition than physostigmine 30 μM ($t = 7.66$, d.f. 25, $P < 0.0001$), whereas DFP 60 and 100 μM did not differ from physostigmine ($P > 0.6$ for each). In control samples exposed only to SB, the mean ± s.e.mean AChE activity was 5.49 ± 0.15 nmol min^{-1} mg^{-1} original tissue ($n = 4$ assays).

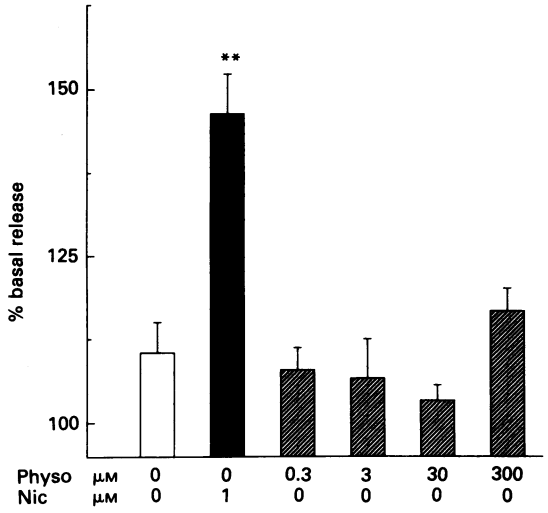


Figure 4 Test of physostigmine (Physo) as a nicotinic agonist (Experiment 4). Synaptosomes were challenged with brief (0.4 ml) pulses of nicotine 1 μM (Nic) (solid column), physostigmine 0.3–300 μM (hatched column), or buffer alone (open column). The vertical axis shows mean ± s.e.mean peak release expressed as a percentage of basal release ($n = 9$–13). **$P < 0.001$ vs. no drug control (Dunnett's test).

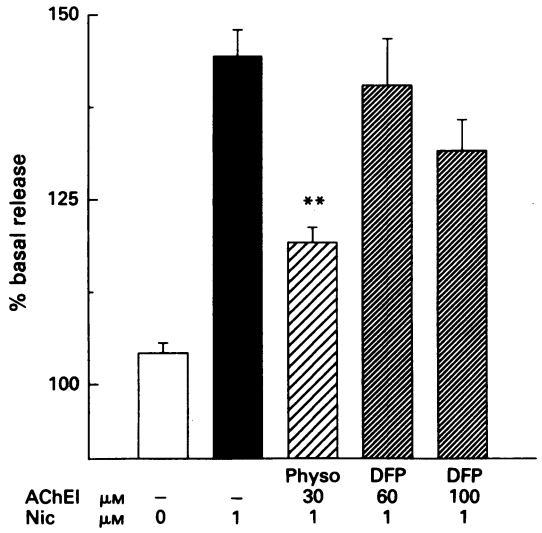


Figure 5 Comparison of physostigmine and DFP at concentrations giving equivalent degrees of acetylcholinesterase inhibition (AChEI) (Experiment 6). Synaptosomes were superfused with buffer in the presence or absence of physostigmine 30 μM or DFP 60 or 100 μM for 35 min, prior to challenge with nicotine 1 μM (Nic) or buffer. The vertical axis shows mean ± s.e.mean peak release expressed as a percentage of basal release ($n = 10$–22). **$P < 0.0005$ vs. nicotine alone and $P < 0.005$ vs. nicotine/DFP 60 μM (Tukey HSD test).

Table 1 Residual acetylcholinesterase (AChE) activity of synaptosomal preparations treated with AChE inhibitors (Experiment 5)

Drug	*Concentration* (μM)	*AChE activity**	n
Assay set No. 1			
Control		100.0 ± 3.0	16
Physostigmine	30	2.5 ± 0.4	16
Neostigmine	30	1.6 ± 0.2	16
DFP	30	3.9 ± 1.0	16
Assay set No. 2			
Control		100.0 ± 3.1	8
Physostigmine	30	2.2 ± 0.3	8
Tacrine	0.3	27.0 ± 1.9	8
Tacrine	3	4.6 ± 0.3	8
Tacrine	30	2.0 ± 0.2	4
Assay set No. 3			
Control		100.0 ± 2.0	16
Physostigmine	30	1.5 ± 0.1	16
DFP	30	2.6 ± 0.1	16
DFP	60	1.7 ± 0.2	16
DFP	100	1.6 ± 0.1	16

*Residual AChE activity (mean ± s.e.mean) expressed as a percentage of control samples not treated with AChE inhibitor.

Experiment 6: Effects of selected concentrations of physostigmine and DFP on striatal [^{3}H]-dopamine release evoked by nicotine

Nicotine-induced dopamine release was again studied, in order to test whether concentrations of physostigmine and DFP that had been found in Experiment 5 to give equivalent degrees of AChE inhibition would also produce equivalent degrees of nicotinic block (Figure 5). Dopamine release induced by nicotine challenge was significantly altered by treatment with AChE inhibitor (AChE inhibitor main effect: $F = 8.31$, d.f. 3, 62, $P < 0.001$). Multiple comparisons between nicotine-challenged drug conditions revealed no significant differences between control (SB alone), DFP 60 μM and DFP 100 μM conditions (Tukey's HSD test: $P > 0.1$ for each; Figure 5). In contrast, the nicotine response after treatment with physostigmine was significantly less than the nicotine response after perfusion with either SB alone ($P < 0.005$) or DFP 60 μM ($P < 0.005$). Basal release was not altered by treatment with AChE inhibitor ($F = 0.09$, d.f. 3, 72, $P < 0.9$).

Discussion

CNS nicotinic receptors are genetically diverse, but two main subfamilies can be distinguished, based on sensitivity to the antagonist α-bungarotoxin (Deneris *et al.*, 1991; Clarke, 1992). Evidence was recently provided for direct actions of physostigmine on CNS nicotinic receptors that are susceptible to blockade by α-bungarotoxin (Pereira *et al.*, 1993). Here, we describe effects of physostigmine and other AChEIs on nicotine-induced striatal dopamine release, a response mediated by nicotinic receptors that are not sensitive to α-bungarotoxin (Rapier *et al.*, 1990; Grady *et al.*, 1992).

In the present study, physostigmine antagonized nicotinic responses in a pharmacologically selective manner, insofar as dopamine release evoked by the nicotinic agonists DMPP and cytisine was reduced, whereas that evoked by high K^+ was not. Amphetamine-induced dopamine release was reduced, but only at a high concentration of physostigmine (300 μM). This suggests that physostigmine, at least at moderate concentrations, acts at nicotinic receptors and not on release mechanisms *per se*.

Physostigmine is reported to exert agonist actions when applied at submicromolar concentrations to frog skeletal muscle and to rat cultured hippocampal cells that express α-bungarotoxin-sensitive nicotinic receptors (Shaw *et al.*, 1985; Pereira *et al.*, 1993). These observations suggested that the nicotinic blockade seen following 30 min perfusion with physostigmine might reflect desensitization secondary to an initial receptor activation. However, when physostigmine was tested in a short pulse in the same manner as nicotine, no agonist activity was seen over a wide concentration range (Experiment 4). Consistent with its lack of agonist activity and with its insurmountable blocking action (observed in Experiment 2), physostigmine inhibits only weakly [^{3}H]-nicotine and [^{3}H]-ACh binding to brain tissue (Nilsson *et al.*, 1987; Perry *et al.*, 1988). Thus, it appears possible that physostigmine may act as an open channel blocker in the CNS, as proposed for nicotinic receptors in *Torpedo* electroplaque (Sherby *et al.*, 1985).

Physostigmine inhibited nicotinic responses at concentrations that inhibit AChE activity *in vitro*, and the three other AChEIs tested (neostigmine, tacrine, DFP) also inhibited nicotinic responses to some extent, as shown in Experiment 1. Conceivably, then, the nicotinic antagonist action of physostigmine could have been due to esterase inhibition, perhaps mediated via receptor desensitization resulting from increased concentrations of ACh in the superperfusate. This possibility appears unlikely in view of subsequent comparisons with DFP and tacrine. Thus, in Experiment 5, a concentration of DFP (60 μM) was determined that inhibited AChE to the same degree as physostigmine 30 μM; at these concentrations, physostigmine markedly inhibited nicotine-induced dopamine release, whereas DFP was inactive. Contrariwise, tacrine 3 μM was as effective as physostigmine 30 μM in inhibiting dopamine release (Experiment 1), but produced significantly less inhibition of AChE (Experiment 5).

The receptor subtype(s) mediating nicotine-induced dopamine release have not been classified with certainty. The association of [^{3}H]-nicotine binding sites with dopaminergic afferents (Clarke & Pert, 1985) suggests mediation by receptors containing alpha4 and beta2 subunits (Whiting *et al.*, 1991; Flores *et al.*, 1992), but other receptor subtypes are also expressed by dopaminergic neurones (Wada *et al.*, 1989) and may thus contribute. *In situ* hybridization experiments (Dineley-Miller & Patrick, 1992) indicate that receptors containing beta4 subunits, which appear to be prevalent at autonomic ganglia (Listerud *et al.*, 1991; Tarroni *et al.*, 1992), are probably not expressed by nigrostriatal dopaminergic neurones. Nevertheless, these are indications that physostigmine may exert an antagonist action in the periphery as well as in the CNS. Thus, in the myenteric plexus, physostigmine (10 μM) inhibited [^{3}H]-ACh release evoked by the direct nicotinic agonist DMPP (Briggs & Cooper, 1982), and physostigmine has also been shown to block carbachol-induced $^{86}Rb^+$ efflux from cultured cells derived from a peripheral neuroblastoma with an IC_{50} of 40 μM (Lukas *et al.*, 1993).

Physostigmine is commonly employed in concentrations of 10–50 μM in order to inhibit AChE *in vitro* (Murrin *et al.*, 1977; Lapchak & Collier, 1988; Suzuki *et al.*, 1993). Within this concentration-range, nicotinic actions were clearly inhibited in our assay. Moreover, the insurmountable nature of the block produced by physostigmine implies that this antagonist action would be apparent even at high agonist concentrations. The demonstration that moderate concentrations of physostigmine can block nicotinic response raises the possibility that the prevalence of nicotinic actions of ACh has been underestimated in the CNS: (1) The possibility of presynaptic nicotinic modulation of the ascending cholinergic innervation to cerebral cortex has been examined in synaptosomal preparations; in cerebral cortex, release of cortical ACh was enhanced by activation of nicotinic receptors in mouse (Rowell & Winkler, 1984) but not in rat (Meyer *et al.*, 1987). In the latter study, the use of physostigmine (50 μM) may have prevented a nicotinic action. (2) In another study, a lower concentration of physostigmine (10 μM) reduced nicotine-induced depolarization in rat cerebral cortical synaptosomes (Hillard & Pounds, 1991); the possibility of a direct blocking action on nicotinic receptors was not discussed. (3) In the substantia nigra pars compacta, a nicotinic cholinergic innervation has been described (Clarke *et al.*, 1987; Blaha & Winn, 1993). However, studies in which physostigmine has been applied directly to this area have emphasized a muscarinic component (Winn *et al.*, 1983; Smelik & Ernst, 1966). (4) Use of physostigmine perhaps also helps to account for the surprising absence of demonstrable nicotinic cholinergic transmission reported in the interpeduncular nucleus (Brown *et al.*, 1983).

A further issue relates to the interpretation of radioligand binding data obtained after chronic AChEI treatment. Brain nicotinic receptors can be identified by radioligand binding techniques, but the extent to which these receptors are cholinergically innervated remains uncertain. Initial reports of [^{3}H]-nicotine and [^{3}H]-ACh binding site downregulation following chronic AChEI treatment suggested that the nicotinic AChRs so identified are indeed targets for endogenous ACh in a number of brain regions (Schwartz & Kellar, 1983; 1985; Costa & Murphy, 1985). Subsequent reports indicate that AChEI-induced upregulation of nicotinic binding sites can also occur (De Sarno & Giacobini, 1989; Bhat *et al.*, 1990). The source of this variability has not been adequately explained. Hence, it may be significant that chronic treatment with physostigmine, which might be expected to exert an insurmountable nicotinic antagonism at the doses administered in these studies, upregulated nicotinic binding (De Sarno & Giacobini, 1989; Bhat *et al.*, 1990), whereas DFP, which appears to be at best a weak nicotinic antagonist, decreased binding (Schwartz & Kellar, 1983; 1985; Costa & Murphy, 1985; van de Kamp & Collins, 1992).

A nicotinic blocking effect of tacrine has not been identified previously (Experiment 1). The potency of this action is noteworthy; tacrine significantly reduced nicotinic responses even at the lowest concentration tested (0.3 μM), and the IC_{50} was around 1 μM. These values compare quite closely with IC_{50} values reported for AChE inhibition in brain (Freeman & Dawson, 1991), whereas the therapeutic dose-range of tacrine is associated with serum levels between 0.02 and 0.3 μM (Park *et al.*, 1986).

Tacrine also reduced dopamine release associated with the administration of buffer containing high K^+ (Experiment 3), and thus it is not clear whether this drug exerted a direct antagonist action at nicotinic receptors. Published attempts to demonstrate an interaction of tacrine with CNS nicotinic receptors have yielded mixed results. Tacrine has been reported to inhibit [^{3}H]-nicotine or [^{3}H]-ACh binding to rat or human brain tissue with IC_{50} or K_i values ranging from 1 μM

to 300 μM (Nilsson *et al.*, 1987; Perry *et al.*, 1988; Xiao *et al.*, 1993). However, in functional experiments designed to test nicotinic agonist activity, detectable activity was only reported at high micromolar concentrations (Nilsson *et al.*, 1987).

Certain reports have indicated that some of the cognitive impairments experienced in Alzheimer's disease may be alleviated by treatment with physostigmine (Thal & Fuld, 1983; Davis *et al.*, 1983) and tacrine (Summers *et al.*, 1986). However, other studies have shown little or no benefit with either drug (Ashford *et al.*, 1981; Caltagirone *et al.*, 1982; Sahakian *et al.*, 1983; Chatellier & Lacomblez, 1990; Gauthier *et al.*, 1990). In the light of recent evidence indicating that cognitive functioning can be enhanced by nicotinic receptor agonists and impaired by nicotinic blockers (Newhouse *et al.*, 1988; 1992; Jones *et al.*, 1992), it must be emphasized that the concentrations of physostigmine and tacrine shown to antagonize nicotinic responses in the present study, also inhibited AChE activity almost completely, and thus would not normally be achieved in the clinic.

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