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Tacrine and physostigmine block nicotinic receptors in *Xenopus* oocytes injected with *Torpedo* electroplaque membranes

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Abstract

Tacrine and physostigmine were tested for direct nicotinic actions on *Xenopus* oocytes microinjected with *Torpedo* electroplaque membranes. In this preparation, responses to acetylcholine arise 6–8 h after microinjection, due to the incorporation of nicotinic receptors into the plasma membrane by a process not involving protein synthesis. Currents elicited by acetylcholine (100–1000 μM) were recorded by two-electrode voltage clamping. Tacrine (1–1000 μM) and physostigmine (1–100 μM) exerted a potent, reversible block of the nicotinic receptors. The concentration–dependence curves fitted simple hyperbolas, suggesting a stoichiometry of 1:1 in the drug-channel interactions. Currents elicited by the highest acetylcholine concentration were inhibited by tacrine with maximal affinity, indicating an action at a site other than the ligand-binding domain. Inhibition was reduced at depolarising potentials, which is consistent with a preferential interaction with the ligand-bound form of the receptor. Blockade by tacrine or physostigmine was accompanied by a concentration-dependent slowing of the desensitisation, resembling the action of local anaesthetics. These results could indicate a modulatory effect of these drugs on neurosecretion through nicotinic receptors.

Keywords: Tacrine; *Xenopus* oocyte; *Torpedo* nicotinic acetylcholine receptor; Cholinesterase inhibitor; Alzheimer's disease

1. Introduction

Tacrine (9-amino-1,2,3,4-tetrahydroacridine), synthesised in 1945 by Albert and Gledhill as a 4-aminoapyridine derivative, was soon reported to be a powerful antagonist of acetylcholinesterases (Shaw and Bentley, 1953). At present, it is currently being used as a therapeutic agent for memory improvement in patients with Alzheimer disease (Summers et al., 1988, 1989). It is generally accepted that its inhibitory action on acetylcholinesterase activity increases the local levels of acetylcholine, which in turn partially counteract the impairment of cholinergic function. However, tacrine actually displays a complex pharmacological profile, probably due to its relationship to 4-aminopyridine and 9-aminoacridine, but also due to other unknown structural features. Interactions with K^+ channels (Osterrieder, 1987; Drukarch et al., 1987), Na^+ channels (Rogawsky, 1987), Ca^{2+} channels (Osterrieder, 1987) and muscarinic acetylcholine receptors (Shaw and Bentley, 1953; Flynn and Mash, 1989) have been reported. While indirect effects on presynaptic nicotinic acetylcholine receptors have also been suggested, the evidence for a direct interaction with this receptor is limited to the results of non-functional binding experiments (Nilsson et al., 1987). However, in a recent work tacrine has been shown to inhibit nicotinic secretory and current responses in adrenal chromaffin cells (Sugawara et al., 1997).

The aim of the present work was to investigate the direct actions of tacrine on nicotinic receptors and the mechanisms involved at a molecular level. For such a goal, we used the novel approach of injecting nicotinic acetylcholine receptor-enriched membranes into the cytoplasm of oocytes since recently it has been reported that the injection of *Torpedo* postsynaptic membrane vesicles (Marsal et al., 1995; Morales et al., 1995) and the injection of rat brain synaptosomes (Sanna et al., 1996) leads to the incorporation of exogenous voltage and ligand-gated ion channels into the oocyte plasma membrane, with preservation of their native properties. This approach provides a fully unmodified synaptic receptor inserted into the cell membrane, and therefore may overcome any lack of fi-

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delity of translation or post-translational modifications that could occur in the process of heterologous expression.

2. Materials and methods

2.1. Isolation of electroplaque membranes

Torpedo marmorata specimens were caught off the Catalan coast and kept in artificial sea water until the day of use. Prior to dissection of the electric organs, fishes were anaesthetised with tricaine methane sulphonate (Sigma) at a concentration of 1 g/3 l of sea water. Electric organs (100 g) were homogenised for 1 min in a Polytron 3AR (Braun) at maximum speed with physiological saline medium containing 280 mM NaCl, 7 mM KCl, 2.8 mM $CaCl_2$, 1.3 mM $MgCl_2$, 5 mM $NaHCO_3$, 10 mM HEPES, 300 mM urea, 5.5 mM glucose. This medium was gassed with 95% O_2 and 5% CO_2, and the pH adjusted to 7.2 with NaOH. The homogenate was filtered through 100-μm pore diameter nylon tissue and subsequently centrifuged at $6000 \times g$ for 20 min. The pellet was discarded and the supernatant was concentrated by centrifugation at 40,000 $\times g$ for 40 min. The resulting pellet, containing the nicotinic acetylcholine receptor-enriched membranes, was resuspended at a concentration of 2–4 mg/ml in 10 mM HEPES, pH 7.4, and frozen at −80°C until injection. All the steps were carried out at 4°C.

2.2. Isolation of Xenopus oocytes

Mature female *Xenopus laevis* (Centre d'Elevage des Xenopes, Montpellier) were anaesthetised by immersion in water containing 0.17% tricaine. After a small incision was made in the abdomen, a number of ovarian lobes were removed and placed in physiological ND96 saline (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.6) supplemented with 100 UG/ml penicillin-100 IU/ml streptomycin (GIBCO). Stage V–VI oocytes were defolliculated by incubation in Ca^{2+}-free saline containing 2 mg/ml collagenase (Type Ia, Sigma) for 2 h at 20–22°C. Subsequently, the oocytes were injected with 150–200 ng of the membrane suspension by means of a PLI-100 Picospritzer (Medical Systems).

2.3. Electrophysiology

A standard two-electrode voltage-clamp configuration was used to record *Xenopus* oocytes that had been previously microinjected with the crude extract of electroplaque membranes. Recordings were performed under continuous perfusion of ND96 saline (flow rate of 4–6 ml/min) at 20–23°C. Currents were amplified and low-pass filtered at 100–300 Hz by using a Geneclamp 500 Amplifier (Axon Instruments), digitised through a Labmaster-DMA interface (Axon Instruments) and stored in a 08386-PC using data acquisition software pClamp5.5 (Axon Instruments).

2.4. Data and statistical analysis

Concentration–effect curves were fitted by a non-linear least-squares algorithm according to the equation $I/I(\text{drug free}) = 1/(1 + [T]/IC_{50})$, where I and $I(\text{drug free})$ are the peak currents measured in the presence and absence of the drug, respectively, $[T]$ is the drug concentration, and IC_{50} is the concentration at 50% current inhibition. Values are expressed as means ± standard error. Significant differences between means were assessed by using Student's t test.

2.5. Drugs

Tacrine (9-amino-1,2,3,4-tetrahydroacridine), physostigmine (eserine salycilate) and atropine sulphate were purchased from Sigma (St. Louis, MO, USA). The drugs were freshly dissolved in the physiological saline just before use.

3. Results

3.1. Concentration-dependent inhibition of peak currents by tacrine and physostigmine treatment

To study the putative effects of tacrine and physostigmine on nicotinic acetylcholine receptors, *Xenopus* oocytes were injected with a crude extract of *Torpedo* electroplaque membranes 12 to 48 h before two-electrode voltage-clamp recordings were made. Unless otherwise stated, membrane currents were activated from a holding potential of −60 mV by perfusion of ND96 solution containing 100 or 1000 μM acetylcholine in the presence of 1 μM atropine (as a muscarinic receptor antagonist). The interval between consecutive responses was systematically set to 15 min since we previously established that this interval ensured a complete recovery from desensitisation (data not shown). All the oocytes included in this study were tested for consistent response amplitudes, with at least three acetylcholine challenges prior to the application of the drugs. Tacrine or physostigmine at the given concentrations was continuously perfused for 15 min after a control response had been recorded. Fig. 1 illustrates the effect of the cholinesterase inhibitors on consecutive nicotinic responses. Peak inward currents elicited by acetylcholine at either 100 μM or 1000 μM were depressed by tacrine in a concentration-dependent manner (Fig. 1a,b). The inhibition of the acetylcholine (1000 μM) peak current was 69 ± 3% ($n = 10$) for 10 μM tacrine and 93 ± 4% ($n = 8$) for 100 μM tacrine. At concentrations of the drug up to 100 μM, the blockade was almost completely reversed following a 15-min wash-out (percentage recovery after washout of 100 μM tacrine of the current elicited by 100 μM acetylcholine was: 92 ± 8% ($n = 6$) and 102 ± 20% ($n = 8$) for the current elicited by 1000 μM acetylcholine). The effects of physostigmine were also analysed on the current

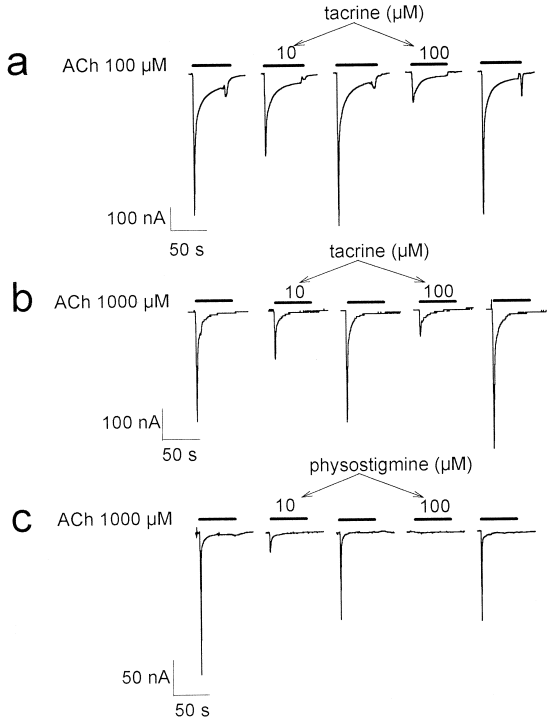

Fig. 1. Tacrine and physostigmine block acetylcholine-elicited currents in voltage-clamped oocytes injected with electroplaque membranes. Nicotinic channels were opened by a 45-s bath application of acetylcholine at either 100 μM or 1000 μM in the presence of atropine (1 μM). After superfusion with tacrine or physostigmine, the peak amplitude of the nicotinic receptor mediated inward current was reduced in a concentration-dependent manner. The blockade was fully reversible upon wash-out of the drugs.

responses to 1000 μM acetylcholine (Fig. 1c), and showed an inhibitory pattern similar to that of tacrine but with a somewhat higher affinity (percentage current reduction for physostigmine 10 μM: $80 \pm 4\%$ ($n = 4$); physostigmine 100 μM: 98 ± 1 ($n = 4$); percentage recovery after washout of 100 μM physostigmine: $78 \pm 3\%$ ($n = 3$)). In all cases the concentration–response curves were well fitted by a Hill coefficient of 1, which suggests a stoichiometry of 1:1 in the drug-channel interaction (Fig. 2a,b). At 1000 μM acetylcholine, the IC_{50} values obtained from the fits were 3.97 ± 0.32 μM ($n = 6$) for tacrine and 2.88 ± 0.16 μM ($n = 4$) for physostigmine, whilst currents obtained with 100 μM acetylcholine were reduced by tacrine with an $IC_{50} = 8.98 \pm 1.17$ μM ($n = 7$). The potency of tacrine to block currents activated by 1000 μM acetylcholine was greater than that for currents activated by 100 μM acetylcholine and this blockade was maintained over a range of tacrine concentrations (Fig. 2a).

3.2. Voltage-dependence of the blockade

The effect of tacrine on the amplitude of acetylcholine currents activated in oocytes was voltage-dependent. In a series of experiments, currents were elicited by 1000 μM acetylcholine at different holding potentials (from −60 to

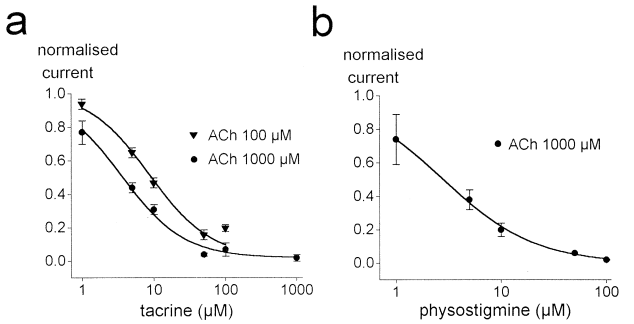

Fig. 2. Concentration–effect curves for tacrine (a) and physostigmine (b) at nicotinic acetylcholine receptors. The data are described by a Hill coefficient of 1. The tacrine-induced decrease of peak currents was more effective when currents were induced by 1000 μM acetylcholine (a, filled circles) than by 100 μM acetylcholine (a, filled triangles), which suggests a non-competitive blocking action. Points represent the mean ± S.E. of 4 to 7 determinations in different oocytes.

+10 mV) in each oocyte. The I–V curve in Fig. 3a, obtained by plotting the peak currents against voltage values, shows that at negative membrane potentials the slope conductance of acetylcholine currents in the presence of tacrine was clearly lower than that recorded under control conditions, but this difference decreased as the membrane was depolarised (peak current reduction of $75 \pm 6\%$ at −60 mV compared with $48 \pm 4\%$ at −20 mV; $n = 5$). However, the reversal potential of the acetylcholine current was not modified by the treatment with tacrine (Fig. 3b), showing that the ion selectivity was maintained.

3.3. Effects on nicotinic acetylcholine receptor desensitisation

Tacrine and physostigmine slowed the time course of desensitisation of the membrane currents elicited by perfu-

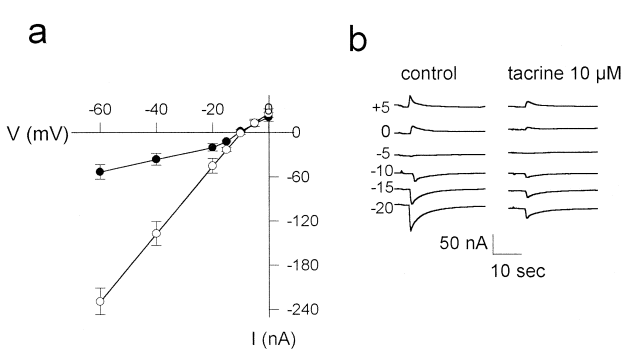

Fig. 3. (a) Current–voltage relationships of the peak currents elicited by 1000 μM acetylcholine before (open circles) and after (filled circles) treatment with 10 μM tacrine. The effect of tacrine on the acetylcholine current was reduced as the membrane was depolarised, indicating a preferential interaction with the open-channel form of the nicotinic receptor. Every point is the mean ± S.E. for 5 different oocyte measurements. (b) The reversal potential for currents induced by 1000 μM acetylcholine. The average reversal potential for the acetylcholine-induced currents obtained from 5 oocytes was -9 ± 4 mV and was not changed by tacrine treatment (reversal potential of -11 ± 4 mV).

sion of acetylcholine at 100 μM or 1000 μM (Fig. 4a,b). Desensitisation time constants were calculated by fitting the decay phase of the acetylcholine currents to a single exponential function, and the values obtained in the presence of the drugs were normalised to the controls. Fig. 4c displays the mean time constants for the different experimental conditions, expressed as percentage of control values. A correlation between the concentration of tacrine and the slowing of the decay time constant was also observed. For currents elicited by 100 or 1000 μM acetylcholine the slowing of decay produced by 10 μM tacrine was lower than that induced by 100 μM tacrine (see Fig. 4c). Conversely, the effect of equal concentrations of tacrine on desensitisation was of the same magnitude for currents elicited by either 100 μM or 1000 μM acetylcholine (Fig. 4c).

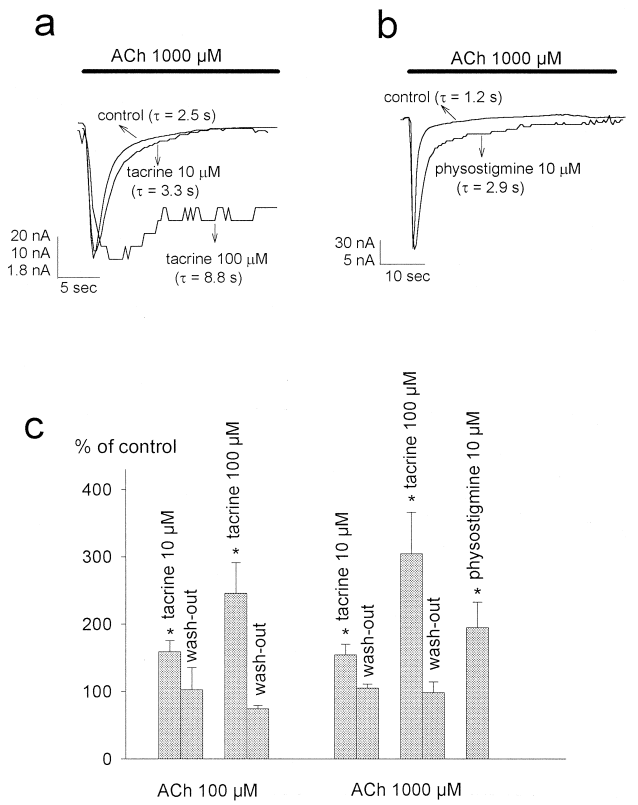

Fig. 4. Effects of tacrine and physostigmine on the desensitisation time constant of nicotinic responses. (a) Acetylcholine currents recorded from an oocyte before and after sequential application of 10 μM or 100 μM tacrine. Calibration: 20 nA for control, 10 nA for 10 μM tacrine and 1.8 nA for 100 μM tacrine. (b) In a different oocyte, effect of 10 μM physostigmine on the decay time constant. Physostigmine at 100 μM completely abolished the currents. Calibration: 30 nA for control and 5 nA for 10 μM physostigmine. Currents are scaled to superimpose their peak amplitudes, allowing comparison of desensitisation kinetics. The time constants given for each trace were obtained by fitting the decay phase to a single exponential function. (c) Desensitisation time constant histograms showing the effects of tacrine (10 μM or 100 μM) and physostigmine (10 μM) on acetylcholine (100 μM or 1000 μM)-elicited currents, including after wash-out. Normalised data are mean ± S.E. for at least 5 oocytes. $* P < 0.05$, significantly different from respective controls by Student's t test.

4. Discussion

Xenopus oocytes have been used widely for the heterologous expression of many membrane receptors. Their ability to translate foreign RNAs (Gundersen et al., 1984) and to perform post-translational modifications as in native cells has been demonstrated extensively, and particularly for nicotinic acetylcholine receptors (Kushner et al., 1989 for review). Nevertheless, an altered pattern of *N*-glycosylation of the *Torpedo* nicotinic acetylcholine receptors was found when these were expressed in oocytes (Buller and White, 1990). Furthermore, different single channel properties between native nicotinic acetylcholine receptors recorded in rat superior cervical ganglion cells and recombinant nicotinic acetylcholine receptors expressed in amphibian oocytes have been recently reported (Sivilotti et al., 1997), suggesting the occurrence of changes in the composition or stoichiometry of the subunits. In this work, we took advantage of the novel approach of injecting synaptic membranes into the oocyte cytoplasm, which ensures the complete integrity of the receptors inserted into the oocyte membrane, to study the interactions of tacrine and physostigmine with *Torpedo* nicotinic receptors.

4.1. Tacrine blockade of nicotinic acetylcholine receptors

It was already known that the cholinesterase inhibitors neostigmine, pyridostigmine or edrophonium (Sherby et al., 1985; Wachtel, 1990; Yost and Maestrone, 1994) but also physostigmine (Shaw et al., 1985; Wachtel, 1993) at micromolar concentrations may interact with the activated form of nicotinic receptors. However, data about the actions of tacrine on nicotinic acetylcholine receptors is so far limited to indirect measurements of nicotinic function such as interference with ligand binding sites (Nilsson et al., 1987) or inhibition of neurotransmitter release evoked by nicotinic receptor agonists (Clarke et al., 1994). Sugawara et al. (1997) also observed that tacrine and physostigmine inhibited both cathecolamine secretion and inward currents evoked by nicotine in chromaffin cells, although it was not determined whether these effects were due to an action on agonist binding or on the channel itself. In this article we report that tacrine and physostigmine at clinically relevant concentrations blocked *Torpedo* nicotinic receptors in a direct, voltage-dependent manner. The effect was reduced at depolarising potentials, indicating the preference of the drugs for the open-channel form of the receptor. The fact that tacrine blocked with slightly higher affinity currents activated by 1000 μM acetylcholine rather than by 100 μM acetylcholine argues against a competitive interaction with the ligand binding site of the receptor, but further supports an open channel blocking action. The concentration–effect curves for tacrine and physostigmine fitted simple hyperbolas, suggesting a single-site interaction with the receptor. In addition, we found

that these compounds slowed the desensitisation of the nicotinic acetylcholine receptors in a dose-dependent relation. This feature fits a blocking reaction in which the drug binds to the open-channel form, such that in order to become inactivated the channel must pass back through the open state upon drug dissociation. This contrasts with the enhancement of desensitisation shown for edrophonium (Yost and Maestrone, 1994). It could account for different clinical efficacy of the different members of the broad family of cholinesterase inhibitors.

4.2. Possible physiological consequences

It is well established that stimulation of presynaptic nicotinic receptors increases transmitter release, and this modulatory action has been claimed to be the predominant role of nicotinic acetylcholine receptors in the brain (reviewed in Wonnacott, 1997). In cholinergic systems this positive modulation opposes the negative feedback exerted by muscarinic autoreceptors. Thus, it is feasible that discrepancies about the effects of tacrine on neurotransmitter release are due to a different distribution of cholinoceptors among the models studied. For example, tacrine has been reported to increase neurosecretion from *Torpedo* electric organ (Cantí et al., 1994), mammalian motor endplates (Thesleff et al., 1990) or rat hippocampal slices (Suzuki et al., 1994), but to inhibit acetylcholine or GABA release in rat cerebral cortical slices (Locaiono and Mitchelson, 1990; Gardiner and De Belleroche, 1990). Interestingly, Nilsson et al. (1987) observed that tacrine or physostigmine reduced in vitro acetylcholine release from control postmorten brain, but the effect was opposite for tissue from patients with Alzheimer's disease. It has also been reported that nicotinic autoceptors mediating enhancement of acetylcholine release from rat cortical synaptosomes become operative under conditions of impaired cholinergic function (Marchi and Raitieri, 1996). Altogether, these results indicate that the effects of anticholinesterases on neurosecretion are related to the prevalence of either presynaptic nicotinic-positive or muscarinic-negative feedback. Nevertheless, the different results also could be due to the heterogeneity of nicotinic acetylcholine receptors. In this regard, nicotinic antagonists enhance quantal release at the rat neuromuscular junction depending on the stimulation frequency (Tian et al., 1994; Domet et al., 1995), which suggests the existence of a distinct population of presynaptic inhibitory nicotinic receptors. Progress in our knowledge of the composition and distribution of nicotinic acetylcholine receptors will provide a better understanding of the mechanisms by which tacrine increases cholinergic activity.

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