Tacrine-induced increase in the release of spontaneous high quantal content events in *Torpedo* electric organ

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1 The anticholinesterases, tacrine (100 μ M) and physostigmine (60 μ M) had different effects on the amplitude distribution and kinetics of miniature endplate currents (m.e.p.cs) recorded extracellularly from the electric organ of *Torpedo marmorata*.

2 Tacrine increased the ratio of giant miniatures (larger than 4 mV of amplitude) to more than 20% of recorded spontaneous events. In the presence of physostigmine such events represented only 4%.

3 Both tacrine and physostigmine increased the rise time and the decay phase of normal-sized m.e.p.cs when compared to control conditions. Both effects were significantly greater for tacrine.

4 We have tested the specificity of the tacrine effect on ectoenzyme activities associated with plasma membranes of these pure cholinergic nerve endings. Tacrine does not act unspecifically on every ectoenzyme, because it is not able to block the ectoaprase activity even at a concentration 100 fold greater than that required to inhibit 94% of AChE.

5 We conclude that the differential effects of tacrine and physostigmine can be explained in terms of undetermined presynaptic actions of tacrine, while comparable effects of the two compounds can be explained through a shared anticholinesterase activity.

Keywords: Tacrine; physostigmine; miniature endplate currents; *Torpedo*; acetylcholine; acetylcholinesterase; aprase; quantal transmitter release; Alzheimer's disease

Introduction

Alzheimer's disease produces a loss of cholinergic neurones in the nucleus basalis of Meynert. To increase the levels of acetylcholine, clinical trials have been carried out with acetylcholinesterase blockers. Physostigmine and tacrine (9-amino-1,2,3,4-tetrahydroacridine) block acetylcholinesterases in the central nervous system. Physostigmine produces a brief improvement in cognitive function, due to its short half-life. The use of the longer-acting tacrine has positive results, primarily in long-term trials (Kumar & Becker, 1989).

The strategy of using physostigmine or tacrine to enhance cholinergic transmission is based on the ability of acetylcholinesterases to prolong the postsynaptic action of acetylcholine (ACh). In the central nervous system the direct measurement, at the synaptic level, of ACh release and the mean life of ACh molecules is difficult. An alternative experimental model is the vertebrate neuromuscular junction with which the quantal nature of ACh release was established. Tacrine stimulates neurosecretion at mammalian motor endplates (Thesleff *et al.*, 1990) and prolongs the decay phase of miniature endplate potentials (Braga *et al.*, 1991).

Variations in clinical efficacy of tacrine and physostigmine may be related less to their acetylcholinesterase antagonism than to their interactions with other systems such as presynaptic actions or cholinergic receptors. Tacrine is in fact structurally related to the Na⁺ channel blocker, 9-aminoacridine (Yamamoto & Yeh, 1984) and to the K⁺ channel blocker, 4-aminopyridine (Yeh *et al.*, 1976; Wurtman *et al.*, 1991). Also some ligand-gated channels and receptors such as those for 5-hydroxytryptamine and adenosine are in some systems sensitive to block by tacrine (Drukarch *et al.*, 1987; Shutske *et al.*, 1989).

Electric organs of Elasmobranchs are embryologically derived from the skeletal muscle and have a great number of motor nerve endings, indeed there are huge amounts of acetylcholinesterases. Tacrine is also able to block acetylcholinesterase activity in the ray electric organ (Wu & Yang,

1989). Our goal is to explore the effects of tacrine and physostigmine on the spontaneous quantal release of acetylcholine from the electric organ of *Torpedo*.

Methods

Torpedo marmorata specimens were caught at the Catalan mediterranean coast and maintained alive in artificial sea water. Fish were anaesthetized with tricaine (Sigma, St Louis, MO, U.S.A.) before surgical excision of electric organs. Fragments were immediately immersed in *Torpedo* physiological saline solution. The composition of this solution was (in mM): NaCl 280, KCl 3, CaCl₂ 3.4, MgCl₂ 1.8, glucose 5.5, urea 300 and sucrose 100, HEPES/NaOH-buffer 6.8, pH adjusted to 7.0 with NaHCO₃. From 5 to 10 prisms of the electric organ were cross-sectioned with a scalpel blade and thin sections (1 mm) were placed in a plexiglass recording chamber coated with a sylgard bottom.

Spontaneous release of ACh was recorded with focal extracellular low-resistance microelectrodes (Katz & Miledi, 1977), as adapted to the electric organ by Soria (1983) and Muller & Dunant (1987). The method allows long-term recording with little damage to the cells.

Electrodes were made in three steps using a Mecanex horizontal puller, employing borosilicate capillaries (Clark Electromedical Instruments). Electrode tips were fire-polished in a home-made microforge. Recording electrodes having tip diameters around 10 micrometers were filled with physiological solution with a resistance of about 400 K Ω .

Spontaneous miniature endplate currents (m.e.p.cs), recorded as potential changes of a focal electrode plugged into a high impedance amplifier (P16, Grass), were monitored on a Tektronix 5110 oscilloscope and recorded in parallel on a VCR tape recorder (Biologic). Analysis of signals was done by means of the pClamp 5.5.1 software, Axon Instruments Inc., and a TL-1 labmaster digitizing interface and event detector (Axon Ins.). Data in ASCII form were exported to Sigmaplot 4.0.

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Results are presented in mean $\pm$ s.e.mean. Differences between means were tested by Student's *t* test. Differences between distribution functions were evaluated by the Kolmogorov-Smirnov (K-S) test.

Torpedo electric organ synaptosomes were obtained by differential centrifugation following the method described by Morel *et al.* (1977). The acetylcholinesterase activity was measured according to Ellman *et al.* (1961). Ectoaprase activity was measured as described by Sarkis & Saltó (1991), and phosphate formation was determined according to Lanzetta *et al.* (1979).

All recordings were made at room temperature (20–23°C).

1,2,3,4-Tetrahydro-9-aminoacridine (tacrine), and physostigmine (eserine-salicylate) were purchased from Sigma, St. Louis, MO, U.S.A. All other reagents used and tricaine were of synthesis grade.

Results

In the present work we show that tacrine (100 μ M) efficiently inhibited the acetylcholinesterase (AChE) activity associated with isolated presynaptic plasma membranes of cholinergic nerve terminals of *Torpedo marmorata* electric organ (Mas-soulie *et al.*, 1991). As in the same preparation, Sarkis & Saltó (1991) reported the presence of another ectoenzyme which catalyzed the hydrolysis of ATP that is co-released with ACh during the stimulation of cholinergic synaptic vesicles, it was of interest to determine if tacrine at high concentration (100 μ M) was able to modify the ectonucleotidase activity associated with nerve terminals. Tacrine does not act unspecifically on every ectoenzyme, because whereas at the concentration of 1 μ M it blocks AChE activity fully (remaining activity is $2.4 \pm 3.0\%$, $n = 4$), it is not able to block the apyrase activity even at a concentration 100 fold higher (remaining activity after application of tacrine 100 μ M is $93.9 \pm 4.8\%$, $n = 4$).

We focused the rest of our study on the spontaneous electrical activity of cholinergic nerve terminals in slices of *Torpedo* electric organ. Because we were looking for effects other than purely anticholinesterase action, the effects of tacrine on m.e.p.cs have been compared with the effects induced by a strong AChE inhibitor, physostigmine, at a similar concentration (60 μ M). The amplitude and kinetics of the spontaneous events were modified by the action of these compounds (Figure 1).

The mean amplitude of miniature endplate currents in control conditions was 1.66 ± 0.04 mV (449 m.e.p.cs, number of experiments (n) = 3). Tacrine (100 μ M) increased this value to 2.54 ± 0.10 mV (420 m.e.p.cs, $P < 0.05$, $n = 3$) and physostigmine (60 μ M) to 1.80 ± 0.05 mV (455 m.e.p.cs, $P < 0.05$, $n = 3$).

Histogram plots comparing the amplitude distribution of m.e.p.cs (see Van der Kloot, 1991) in the three experimental conditions, are shown in Figure 2a. With tacrine the appearance of a population of large size m.e.p.cs can be noted clearly. In control conditions only 0.7% of the m.e.p.cs had amplitudes greater than 4 mV. Tacrine resulted in a big increase in this kind of spontaneous event which represented 21% of the total recorded. In contrast, after physostigmine treatment such big m.e.p.cs only represented 4.5%. Because these giant m.e.p.cs had no brakes on their rising phases, we discarded the possibility that they arose from the simultaneous release of two or more quanta.

As tested by the K-S statistics, the distribution function of m.e.p.c. amplitudes recorded in presence of tacrine 100 μ M was different from controls ($P < 0.001$) and from m.e.p.cs recorded in the presence of physostigmine 60 μ M ($P < 0.001$). In contrast, m.e.p.c. amplitude distribution recorded after application of physostigmine is not different from that in control conditions.

The mean value of the rise time of m.e.p.cs in a non-treated preparation was 0.42 ± 0.06 ms, while in physostig-

Figure 1 Superimposed oscilloscope tracings showing spontaneous m.e.p.cs recorded in *Torpedo marmorata* electric organ: (a) an untreated control electric organ shows normal-sized, fast-rising and fast-decaying m.e.p.cs; (b) application of tacrine (100 μ M) leads to the appearance of giant m.e.p.cs, and to a general increase of rise time and decay time constants; (c) in the presence of physostigmine (60 μ M) spontaneous events have prolonged rising times and decaying time constants when compared with controls, but the frequency of slow-rising and giant m.e.p.cs is low in comparison with (b).

mine solution it was 0.79 ± 0.02 ms and in the presence of tacrine, 1.28 ± 0.03 ms. The differences are significant in all cases at the $P < 0.05$ level. Histogram plots are shown in Figure 2b. Tacrine and physostigmine caused an increase in the frequency of slow-rising m.e.p.cs. In the distribution functions, differences between control and tacrine or physostigmine data are significant at the $P < 0.001$ level. The effect was more potent for tacrine, because m.e.p.cs distribution after addition of tacrine shifts significantly to prolonged rise times when compared with physostigmine ($P < 0.001$, K-S statistics).

The mean half decay of m.e.p.cs was 0.36 ± 0.01 ms in control m.e.p.cs, while after physostigmine treatment it was 1.67 ± 0.05 ms, after tacrine it was 2.24 ± 0.05 ms. These differences are significant ($P < 0.05$). Control and tacrine data are different ($P < 0.001$) when comparing the frequency distribution of the half decay time constants of m.e.p.cs. Control and physostigmine are also different at $P < 0.001$. Differences between tacrine and physostigmine are significant at a lower level ($P < 0.05$, K-S statistics). Half decay histograms are shown in Figure 2c.

The increases in both time constants affected apparently normal-sized m.e.p.cs, because when we excluded giant

Figure 2 Histograms showing the effects of tacrine and physostigmine on m.e.p.cs size and kinetics. (a) M.e.p.cs amplitudes; (b) m.e.p.cs rise time constants and (c) m.e.p.cs half decay time constants. Non-treated condition: filled histogram bars. Tacrine (100 μ M): thick histogram outline. Physostigmine (60 μ M): discontinuous histogram outline. Tacrine treatment induces clearly an increase in the number of m.e.p.cs larger than 4 mV and also a shift towards prolonged time constants. All histogram plots have a Normal distribution as tested by the Kolmogorov-Smirnov test. $n = 3$ different experiments.

Table 1 Mean values ($\pm$ s.e.mean) of m.e.p.cs measurements and the correlation coefficients between paired measurements

	Control	Tacrine (100 μ M)	Physostigmine (60 μ M)
Mean amplitude (mV)	1.66 ± 0.04^2	$2.54 \pm 0.10^{1,2}$	1.80 ± 0.05^1
Mean rise time (ms)	0.42 ± 0.06^2	$1.28 \pm 0.03^{1,2}$	0.79 ± 0.02^1
Mean half decay (ms)	0.36 ± 0.01^2	$2.24 \pm 0.05^{1,2}$	1.67 ± 0.05^1
Amplitude vs. rise time	$r = 0.185$	$r = 0.271$	$r = -0.148$
Amplitude vs. half decay	$r = 0.153$	$r = 0.142$	$r = -0.168$

¹Different from control at $P < 0.05$

²Different from physostigmine at $P < 0.05$

Note negative r values in physostigmine treatment, that could account for a molecular action different from tacrine. $n = 3$ different experiments.

m.e.p.cs (larger than 4 mV) from the measurements the given mean values did not change significantly.

Correlation coefficients between the parameters, amplitude and rise time or amplitude and half-decay, were slightly positive for m.e.p.cs recorded in untreated tissue or after bathing with tacrine. On the contrary, physostigmine treatment led to a slightly negative correlation between the paired measurements (Table 1).

Discussion

It is well known that tacrine has anticholinesterase activity (Freeman & Dawson, 1991, for review). We have confirmed this property in the electric organ by means of enzymatic activity determination and through the observation of the slower decay phase of m.e.p.cs. Prolonged rise times and decay phases of m.e.p.cs have been reported in relation to different anticholinesterase agents in the neuromuscular junction (Magleby & Stevens, 1972; Kordas, 1977; Fiekers, 1985). We have determined that the action of tacrine is not unspecific upon any ectoenzyme, since apyrase activity is not modified.

Tacrine, in contrast to physostigmine, has another effect on quantal ACh release, increasing the proportion of giant and slow-rising m.e.p.cs. Diverse treatments like 4-aminoquinoline (Molgó & Thesleff, 1982), clostridial toxins (Sellin

& Thesleff, 1981; Dunant *et al.*, 1987) and chloride replacement by isethionate (Ashford & Wann, 1983) are able to increase the frequency of giant and slow-rising m.e.p.cs. Recently it has been shown in the neuromuscular junction that tacrine induces the appearance of giant m.e.p.cs. Thesleff *et al.* (1990) suggested that they may reflect the fusion of dense-cored granules present in the nerve endings. In the electric organ of *Torpedo*, large dense-cored vasoactive intestinal polypeptide containing vesicles have been described (Whittaker, 1990) which could be homologous with the dense-cored vesicles of the neuromuscular junctions. However, there is no electrophysiological correlation between the release of dense-core vesicles and m.e.p.cs. Alternatively, it has been proposed that under resting conditions a m.e.p.c. could represent the concerted release of several vesicles (Stevens, 1993); in this case the action of tacrine should be to increase the number of vesicles cooperating for a quantum, that would lead to an eventual increase of acetylcholine in the synaptic cleft.

Further work has to be done to determine the molecular actions of tacrine on the increase of the release of high quantal spontaneous events and on the possible relation to its effectiveness on patients with Alzheimer's disease.

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