ELSEVIER

European Journal of Pharmacology 421 (2001) 77–84

ejp

www.elsevier.nl/locate/ejphar

The pharmacology of novel acetylcholinesterase inhibitors, (±)-huprines Y and X, on the *Torpedo* electric organ

Esteve Ros [a], Jordi Aleu [a], Inmaculada Gómez de Aranda [a], Diego Muñoz-Torrero [b], Pelayo Camps [b], Albert Badia [c], Jordi Marsal [a], Carles Solsona [a,*]

[a] *Laboratori de Neurobiologia Cel·lular i Molecular, Departament de Biologia Cel·lular i Anatomia Patològica, Facultat de Medicina, Hospital de Bellvitge, Universitat de Barcelona, Campus de Bellvitge, Pavelló de Govern, Feixa Llarga s/n, E-08907, L'Hospitalet de Llobregat, Spain*

[b] *Laboratori de Química Farmacèutica, Facultat de Farmàcia, Universitat de Barcelona, Av. Diagonal 643, E-08028 Barcelona, Spain*

[c] *Departament de Farmacologia i de Terapèutica, Facultat de Medicina, Universitat Autònoma de Barcelona, 08913-Bellaterra, Barcelona, Spain*

Received 23 November 2000; received in revised form 24 April 2001; accepted 27 April 2001

Abstract

The effects of the tacrine–huperzine A hybrid acetylcholinesterase inhibitors, (±)-12-amino-3-chloro-9-methyl-6,7,10,11-tetrahydro-7,11-methanocycloocta[*b*]quinoline hydrochloride ((±)-huprine Y) and (±)-12-amino-3-chloro-9-ethyl-6,7,10,11-tetrahydro-7,11-methanocycloocta[*b*]quinoline hydrochloride ((±)-huprine X), were tested on spontaneous synaptic activity by measuring the amplitude, the rise time, the rate of rise, the half-width and the area or the electrical charge of the miniature endplate potentials (m.e.p.ps) recorded extracellularly on *Torpedo* electric organ fragments. (±)-Huprine Y and (±)-huprine X at a concentration of 500 nM increased all the m.e.p.p. variables analyzed. The effect of (±)-huprine Y was smaller than that of (±)-huprine X for all the variables except for the rate of rise where there was no significant difference. The effects of these drugs were also tested on nicotinic receptors by analyzing the currents elicited by acetylcholine (100 μM) in *Xenopus laevis* oocytes, transplanted with membranes from *Torpedo* electric organ. Both drugs inhibited the currents in a reversible manner, (±)-huprine Y (IC_{50} = 452 nM) being more effective than (±)-huprine X (IC_{50} = 4865 nM). The Hill coefficient was 0.5 for both drugs. The inhibition of the nicotinic receptor was voltage-dependent and decreased at depolarizing potentials, and there was no significant difference in the effects between (±)-huprine Y and (±)-huprine X at concentrations near to their IC_{50} values. At depolarizing potentials between −20 and +15 mV, these drugs did not have any detectable effect on the blockade of the nicotinic receptor. Both huprines increased the desensitization of the nicotinic receptors since the current closed quickly in the presence of the drugs, and there was no significant difference in this effect between (±)-huprine Y (500 nM) and (±)-huprine X (5 μM). We conclude that (±)-huprine Y and (±)-huprine X increase the level of acetylcholine in the synaptic cleft more effectively than tacrine. The interaction of (±)-huprine X with nicotinic receptors is weaker than that of (±)-huprine Y, suggesting that (±)-huprine X would be more specific to maintain the extracellular acetylcholine concentration.

Keywords: Alzheimer's disease; Cholinergic; Huperzine A; Neuromuscular junction; Tacrine; *Xenopus* oocyte

1. Introduction

In the last decade, efforts have been made to synthesize new drugs to relieve the symptoms of Alzheimer's disease, namely, either muscarinic or nicotinic receptor agonists, estrogen hormones, especially 17β-estradiol, anti-inflammatory and anti-oxidant compounds and neurotrophic factors (Brinton and Yamazaki, 1998; Giacobini, 1996, 1997). Other research projects have concentrated on the mutation of genes encoding proteins involved in neuronal development and differentiation, including apolipoprotein E, presenilin 1 and 2, and amyloid precursor protein (Berezovska et al., 1999; Brinton and Yamazaki, 1998; Forsyth and Ritzline, 1998). However, the drugs most extensively studied are acetylcholinesterase inhibitors (Krall et al., 1999), which in accordance with the cholinergic hypothesis (Bartus et al., 1982) should increase the levels of brain acetylcholine and relieve cognitive deficiencies. In the European Union, the only drugs approved for clinical treatment are tacrine, donepezil and rivastigmine, all of which are acetylcholinesterase inhibitors (Francis et al., 1999). Tacrine was the first drug developed and some

* Corresponding author. Tel.: +34-93-402-4279; fax: +34-93-403-5810.

E-mail address: solsona@bellvitge.bvg.ub.es (C. Solsona).

0014-2999/01/$ - see front matter
PII: S0014-2999(01)01028-7

of its effects are well known. Doses between 80 and 160 mg/day are effective (Farlow et al., 1992; Knapp et al., 1994) but it can be hepatotoxic at these doses (Watkins et al., 1994).

For centuries, the moss *Huperzia serrata* of the Lycopodium group has been used in the Chinese herbal medicine Qian Ceng Ta. It contains an active compound with acetylcholinesterase inhibitory activity (Kozikowsky et al., 1992), known as huperzine A, and it has been used in the treatment of dementia (Skolnick, 1997). According to clinical trials performed in China, it is less toxic than tacrine or donepezil and has a longer half-life. Moreover, the acetylcholinesterase–huperzine A complex has a slower rate of dissociation than tacrine and donepezil, which may make it a more effective therapeutic agent (Skolnick, 1997). It has been also suggested that huperzine A may have some neuroprotective effects on hippocampal and cerebellum neuronal cultures (Ved et al., 1997).

A series of new highly potent acetylcholinesterase inhibitor compounds, huprines, has recently been synthesized. These are molecular chimeras between tacrine and huperzine A, combining the carbobicyclic substructure of huperzine A with the 4-aminoquinoline substructure of tacrine, with the insertion of a chlorine atom at position 3 ((±)-huprine Y and (±)-huprine X) and substitution of the methyl group at position 9 ((±)-huprine Y) by an ethyl group ((±)-huprine X). They have higher affinity for human and *Torpedo* acetylcholinesterase than tacrine and (−)-huperzine A (Badia et al., 1998; Barril et al., 1999; Camps et al., 1998, 1999, 2000a). Here, we show the effect of (±)-12-amino-3-chloro-9-methyl-6,7,10,11-tetrahydro-7,11-methanocycloocta[*b*]quinoline hydrochloride ((±)-huprine Y) and (±)-12-amino-3-chloro-9-ethyl-6,7,10,11-tetrahydro-7,11-methanocycloocta[*b*]quinoline hydrochloride ((±)-huprine X) on the spontaneous synaptic activity and the current associated with the nicotinic receptor. (Fig. 1).

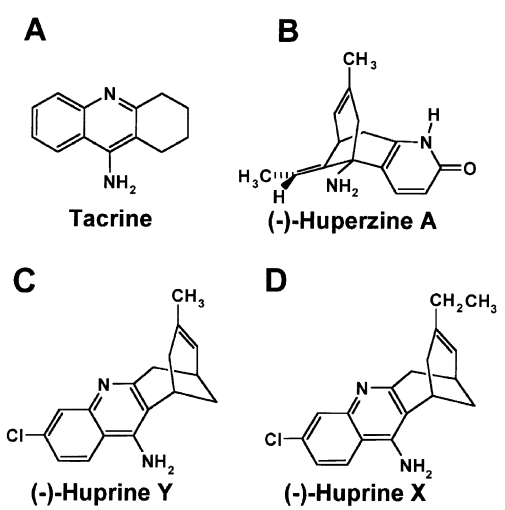

Fig. 1. (A) Chemical structure of tacrine (9-amino-1,2,3,4-tetrahydroacridine). (B) (−)-huperzine A ((−)-5R-(5α,9β,11E)-5-amino-11-ethylidene-5,6,9,1′-tetrahydro-7-methyl-5,9-methanocycloocta[*b*]pyridin-2(1H)-one). (C) (±)-Huprine Y ((±)-12-amino-3-chloro-9-methyl-6,7,10,11-tetrahydro-7,11-methanocycloocta[*b*]quinoline hydrochloride). (D) (±)-Huprine X ((±)-12-amino-3-chloro-9-ethyl-6,7,10,11-tetrahydro-7,11-methanocycloocta[*b*]quinoline hydrochloride). (±)-Huprine Y and (±)-huprine X are hybrid molecules of tacrine and huperzine A.

The experimental model was the electric organ of *Torpedo marmorata*. Synaptic activity was recorded extracellularly on slices of fresh electric organ (Cantí et al., 1994; Ros et al., 2000), while the opening of nicotinic receptors was measured in oocytes, which were transplanted with native electrocyte membranes (Marsal et al., 1995; Cantí et al., 1998; Ros et al., 2000).

2. Materials and methods

2.1. Animals and solutions

The experimental model was the electric organ of *T. marmorata*. The electric organ was used because it is possible to assess spontaneous synaptic activity and it is also easy to assay the effect of drugs on the nicotinic receptor. To record the activity of native nicotinic receptors, electric organ membranes were transplanted to *Xenopus laevis* oocytes.

T. marmorata specimens were caught off the Catalan Mediterranean coast and kept in artificial seawater. Fish were anesthetized with tricaine (3-aminobenzoic acid ethyl ester methanesulfonate salt) (Sigma, St. Louis, MO, USA), at a concentration of 0.03% in seawater, before surgical excision of the electric organs.

Torpedo electric organ fragments were kept in the following saline solution: 280 mM NaCl, 3 mM KCl, 3.4 mM $CaCl_2$, 1.8 mM $MgCl_2$, 5.5 mM glucose, 300 mM urea and 100 mM sucrose, 6.8 mM HEPES/NaOH-buffer, pH 6.8 adjusted to 7.0 with $NaHCO_3$. The same solution was used to record spontaneous synaptic activity.

Mature females of *X. laevis* were purchased from the Centre d'Elevage des Xenopes, (Montpellier, France) and were anesthetized by immersion in water containing 0.17% tricaine. A few lobes of the ovaries were removed, through a small incision in the abdomen.

Solutions for *Xenopus* oocytes: Barth's solution contained 88 mM NaCl, 1 mM KCl, 0.33 mM $Ca(NO_3)_2$, 0.82 mM $MgSO_4$, 2.40 mM $NaHCO_3$, 10 mM HEPES pH 7.4, supplemented with penicillin 100 IU/ml and streptomycin 0.1 mg/ml. Recording solution: 115 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 5 mM HEPES pH 7.0. None of the *Xenopus* female donors used in this work had muscarinic acetylcholine receptors on their oocytes.

2.2. Recording of spontaneous synaptic activity

All recordings were made at room temperature (20–22°C). Between 5 and 10 prisms of the electric organ were cut with a scalpel blade and 1–3-mm sections were incubated overnight. Because of the diffusion coefficient of molecules in the *Torpedo* electric organ, we usually incu-

bated the sections with drugs overnight in *Torpedo* saline solution containing one of the drugs studied. Measurements were performed with fragments fixed in a Plexiglas chamber with a sylgard-coated base.

Spontaneous synaptic release of acetylcholine was recorded with focal extracellular low-resistance microelectrodes (Katz and Miledi, 1972) as adapted for the electric organ by Soria (1983) (see Dunant and Muller, 1986; Muller and Dunant, 1987 for details), and as described elsewhere (Cantí et al., 1994; Ros et al., 2000). The method allows long-term recording with little damage to the cells. Spontaneous miniature endplate potentials (m.e.p.ps) were acquired at a frequency of 100 kHz, amplified (Axoclamp-2A, Axon Instruments, USA), monitored on a Tektronix 5110 oscilloscope and recorded in parallel on a VCR (Biologic, Echirolles, France) and on a PC-Computer with a LabView (National Instruments, USA) program (Quantadat) written in our laboratory, with an AT-MIO16X (National Instruments) digitizing interface. In some cases, signals were analyzed using the same Labview program and the Whole Cell Analysis program kindly provided by Prof. J. Dempster (Strathclyde University, Scotland, UK), and a TL-1 Labmaster digitizing interface. Data in ASCII form were exported to Sigmaplot 4.01.

The following parameters of each m.e.p.p. were measured: amplitude, rise time, rate of rise, the charge sustained by them, measured as the integral of the contour delimited by each m.e.p.p., and the half-width, which indicates the duration of the decay phase (see Fig. 2A). Results were obtained from five experiments.

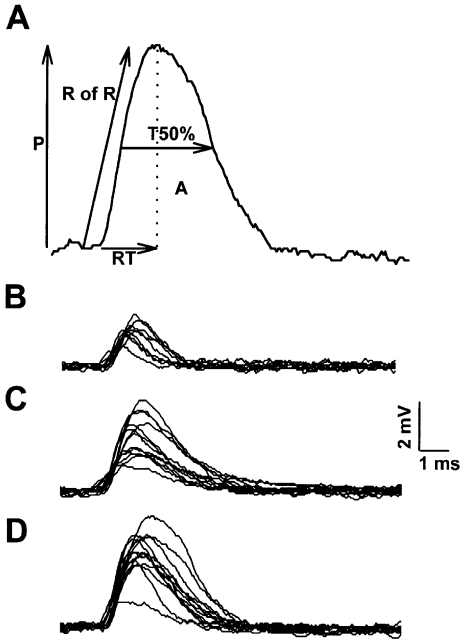

Fig. 2. Effect of (±)-huprine Y and (±)-huprine X on spontaneous cholinergic synaptic activity. (A) Variables analyzed in each miniature recorded. Diagram of the studied variables in the m.e.p.ps. A = total area (equivalent to the electrical charge mobilized by an m.e.p.p.); P = peak amplitude; RT = rise time; R of R = rate of rise; T 50% = half-width. (B, C, D) Effect of (±)-huprine Y and (±)-huprine X on spontaneous miniature endplate potentials. Superimposed oscilloscope traces showing spontaneous m.e.p.ps recorded in *T. marmorata* electric organ: (B) nontreated fragments; (C) after incubation with (±)-huprine Y (500 nM); (D) after incubation with (±)-huprine X (500 nM). Both (±)-huprine Y and X increased the amplitude and prolonged m.e.p.ps.

2.3. Oocyte preparation, microinjection and recording

Oocytes at stages V and VI (see Dumont, 1972) were dissected and kept at 15–16°C in sterile Barth's solution. One day before injection, the oocytes were treated with collagenase type 1A (Sigma) (0.5 mg/ml) for 45–50 min at room temperature to remove the surrounding layers (Miledi and Woodward, 1989).

Healthy oocytes were microinjected with 50 nl of thawed suspension (2–8 mg/ml) of electroplaque membranes (Marsal et al., 1995; Cantí et al., 1998; Ros et al., 2000) by means of an injector (WPI, model A203XVZ). Samples were sonicated prior to injection.

Oocytes were recorded 18–48 h after the injection. Oocytes were voltage-clamped with a two-electrode system (Axoclamp-2A, Axon Instruments). Intracellular electrodes (1–4-MΩ resistance) were filled with 3 M potassium chloride for voltage recording and with 3 M potassium acetate for current injection. The volume of the oocyte recording chamber was 200 μl. Membrane currents were low-pass filtered at 10 Hz and recorded on a PC, using Dempster Whole Cell Analysis v. 2.1. program (Strathclyde University, Scotland, UK), after sampling of the signals by Lab PC + (National Instruments) at twice the filter frequency. In all recordings, currents were elicited by challenges of 100 μM acetylcholine chloride. The interval between consecutive responses was systematically set to 10 min (flow rate 8 ml min^{-1}), since we had previously established that it was an appropriate time to ensure complete recovery from receptor desensitization. All the oocytes included in this study were tested for consistent response amplitudes, with at least three challenges prior to the application of the drug.

2.4. Calculations and statistics

Differences between distribution functions were evaluated with Sigmastat 3.2 software (SPSS, USA) by One-Way Analysis of Variance on Ranks (Kruskal–Wallis test) and by All Pairwise Multiple Comparison Procedures (Dunn's Method). Dose–response data were fitted by nonlinear regression to a sigmoidal curve using the Sigmaplot 4.01 (SPSS) and Inplot (GraphPad, USA) programs. Values are expressed as means ± S.E.M. calculated by the program.

2.5. Drugs

Tricaine (3-aminobenzoic acid ethyl ester methanesulfonate salt) (Sigma), (±)-huprine Y ((±)-12-amino-3-chloro-9-methyl-6,7,10,11-tetrahydro-7,11-methanocycloocta[*b*]quinoline hydrochloride) and (±)-huprine X

((±)-12-amino-3-chloro-9-ethyl-6,7,10,11-tetrahydro-7,11-methanocycloocta[*b*]quinoline hydrochloride). Drug stock solutions (1 mM) were made in deionized water and stored (at −80°C) until use. Finally, they were diluted to their final concentration in the recording solution.

3. Results

3.1. Effects on miniature endplate potentials

At 500 nM, both (±)-huprine Y and (±)-huprine X increased the size and duration of miniature endplate potentials (m.e.p.ps). (Fig. 2B, C and D). The effects of the drugs were compared with those obtained with nontreated fragments of the electric organ. Various variables of each m.e.p.p. were analyzed (see Methods and Fig. 2A). With the Labview software developed in our laboratory, we accumulated a large number of m.e.p.ps from each condition: 7011 from nontreated tissue, 5661 from fragments treated with (±)-huprine Y and 7228 from fragments treated with (±)-huprine X. All these records were made with fragments from five different animals.

The amplitude of m.e.p.ps in nontreated fragments was 1.27 ± 0.03 mV, in fragments treated with (±)-huprine Y it was 1.48 ± 0.01 mV, and in fragments treated with (±)-huprine X it was 1.78 ± 0.02 mV (Fig. 3A).

The area under the profile of each m.e.p.p. reflects the electrical charge carried during the release of a quantum. Both compounds nearly doubled the original area. In nontreated fragments it was 1.05 ± 0.01 mV · ms, in fragments

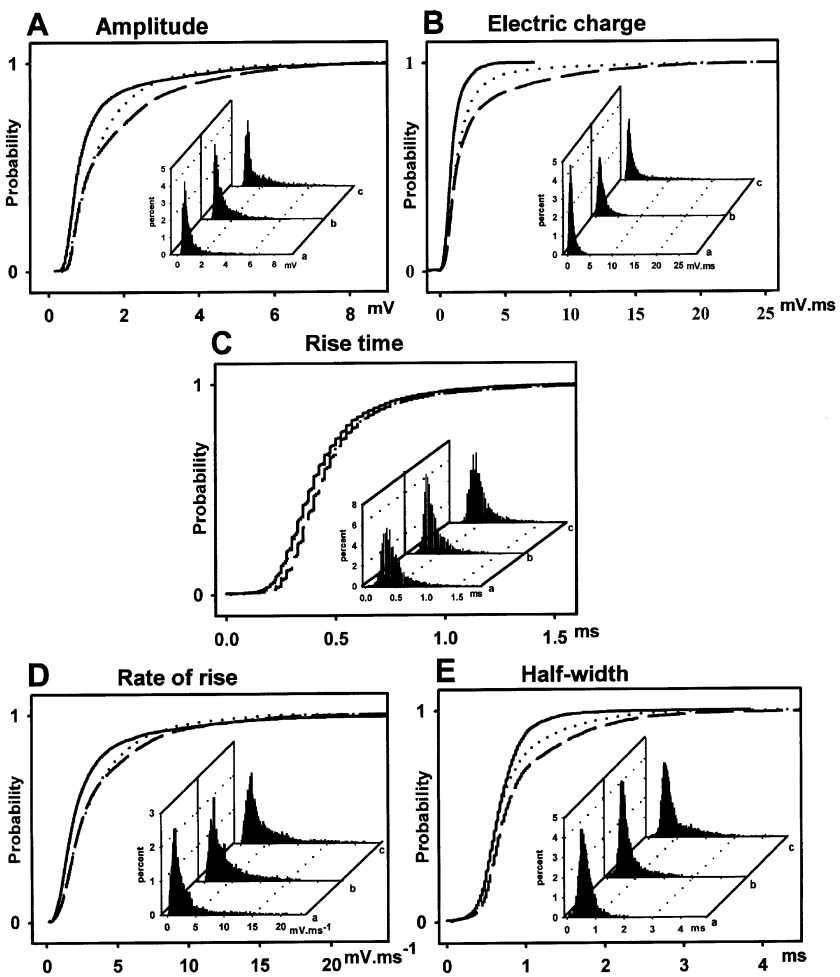

Fig. 3. Analysis of m.e.p.ps in (±)-huprine Y- and X-treated fragments of electric organ of *Torpedo*. Data are presented as cumulative plots and as bar histograms (inset). The cumulative plot shows the probability of finding an m.e.p.p. below a known amplitude, electric charge, etc. A line corresponds to a specific distribution. Differences in the distributions are easily visualized. In the cumulative probability plot: nontreated tissue, continuous line; (±)-huprine Y (500 nM), dotted line; (±)-huprine X (500 nM), discontinuous line. Inset Bar histograms are presented for (A) nontreated tissue; (B) (±)-huprine Y (500 nM); (C) (±)-huprine X (500 nM). (A) Effect of (±)-huprine Y and (±)-huprine X on the amplitude of m.e.p.p. (peak). (B) Electric charge mobilized by spontaneous m.e.p.p. Effect of (±)-huprine Y and (±)-huprine X on the electrical charge mobilized by spontaneous acetylcholine release. The surface delimited below an m.e.p.p. contour corresponds to the total electrical charge passed through the nicotinic receptors as a consequence of spontaneous quanta. (C) Rise time of m.e.p.p. (D) Rate of rise time of m.e.p.p. (±)Huprine Y and X increased the rate of rise to the same extent. (E) Half-width of the m.e.p.p. at 50% of the amplitude. Both compounds prolonged the decay of m.e.p.ps.

treated with (±)-huprine Y it was 1.95 ± 0.04 mV · ms, and in fragments treated with (±)-huprine X it was 2.83 ± 0.05 mV · ms (Fig. 3B).

The rise time of an m.e.p.p. is the result of different cellular steps: fusion of synaptic vesicles, release of acetylcholine, diffusion and opening of nicotinic receptors. In control fragments it was 0.444 ± 0.003 ms, in fragments treated with (±)-huprine Y it was 0.466 ± 0.003 ms, and in fragments treated with (±)-huprine X it was 0.490 ± 0.003 ms (Fig. 3C).

Another variable analyzed was the rate of rise between 10% and 90% of the amplitude. It was 3.29 ± 0.05 mV · ms^{-1} for control fragments, 3.55 ± 0.04 mV · ms^{-1} for fragments treated with (±)-huprine Y, and 3.87 ± 0.04 mV · ms^{-1} for fragments treated with (±)-huprine X (Fig. 3D).

The duration of an m.e.p.p. depends on the presence of acetylcholine in the synaptic cleft. Acetylcholinesterase inhibitors prolong the action of acetylcholine because they decrease the rate of hydrolysis. The inhibition of acetylcholinesterases prolongs the decay of the m.e.p.ps. Since the decay of an m.e.p.p. is an exponential function in which it is difficult to establish the end, the width of an m.e.p.p. at half-amplitude is the half-width used to measure the action of anticholinesterasic agents on synaptic activity. In nontreated fragments, the half-width of m.e.p.ps was 0.701 ± 0.003 ms, in (±)-huprine Y-treated fragments it was 0.811 ± 0.006 ms, and in (±)-huprine X-treated fragments it was 0.961 ± 0.008 ms (Fig. 3E).

In Fig. 3, the distribution of the variables studied in these experiments is represented as a histogram bar and as a cumulative probability function. The difference between control tissue and drug-treated tissue was highly significant ($P < 0.001$) for all the variables; in addition, the amplitude, area, rise time and half-width of the m.e.p.ps in fragments treated with (±)-huprine X were higher than those of fragments treated with (±)-huprine Y ($P < 0.05$). There was no difference in the rate of rise between fragments treated with (±)-huprine Y and (±)-huprine X.

3.2. Effects on acetylcholine-elicited current in transplanted Xenopus oocytes

Other acetylcholinesterase inhibitors studied in our laboratory, tacrine (Cantí et al., 1998) and the tacrine derivative CI-1002 (Ros et al., 2000), inhibited the nicotinic receptor currents recorded in *Xenopus* oocytes transplanted with membranes from the electric organ of *Torpedo*. We examined whether (±)-huprine Y and (±)-huprine X interfere with the opening of the nicotinic receptor. We analyzed the dose dependence of the effects of these drugs on the inhibition of the amplitude of currents elicited by 100 μM of acetylcholine, the voltage sensitivity of this inhibition and the possible interference of these drugs in the desensitization of the nicotinic receptors.

Fig. 4 summarizes the effect of (±)-huprine Y and (±)-huprine X on the amplitude of acetylcholine-induced currents at a holding potential of −70 mV. Currents were obtained from at least 10 oocytes from five donors and injected with membranes isolated from three animals. The effect of different concentrations of (±)-huprine Y and (±)-huprine X is shown in Fig. 4A and B, respectively. The effects of both (±)-huprine Y and (±)-huprine X were fully reversible after a 10-min wash. In Fig. 4C, a sigmoidal curve of four parameters was fitted in curves representing the inhibitory action of (±)-huprine Y and (±)-huprine X. The IC_{50} for (±)-huprine Y was 452.27 ± 1.05 nM, and for (±)-huprine X it was ten times more, 4865 ± 1.14 nM ($P < 0.001$, Fig. 4C), while the Hill coefficient was the same (−0.52 ± 0.02) for (±)-huprine Y and (±)-huprine X (−0.58 ± 0.05).

We also tested the sensitivity of drugs to membrane potential. In six experiments, currents were induced by 100 μM acetylcholine at holding potentials (from −70 to +15 mV) in the presence or absence of the drug. The concentration of the drug utilized was close to its IC_{50} for nicotinic receptor inhibition: 500 nM for (±)-huprine Y and 5 μM for (±)-huprine X. Fig. 5A shows an I/V relationship in

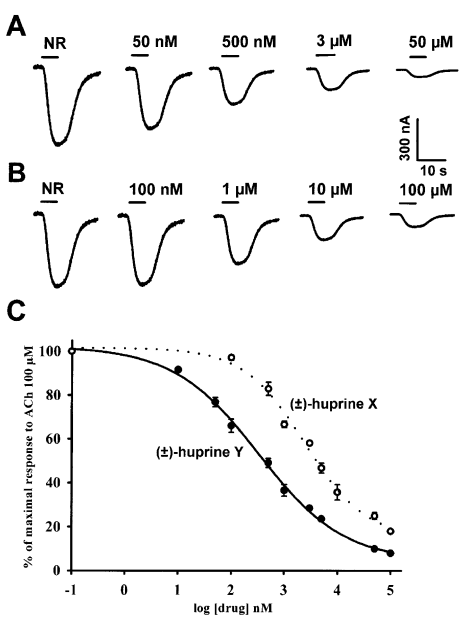

Fig. 4. Interaction of (±)-huprine Y and (±)-huprine X at the nicotinic receptor. Effects of (±)-huprine Y (A) and (±)-huprine X (B). Currents elicited by 100 μM acetylcholine in one oocyte in the presence of different concentrations of the compounds ((±)-huprine Y and (±)-huprine X). Bars indicate acetylcholine application and the holding potential was −70 mV. (C) Effects of (±)-huprine Y (filled symbol) and (±)-huprine X (hollow symbol) on the current elicited by 100 μM acetylcholine. Data are means ± S.E.M. of values obtained from 10 oocytes and are presented as percentages of the maximal current elicited by 100 μM acetylcholine at a holding potential of −70 mV.

which at negative potentials (between −70 and −40 mV) both drugs strongly inhibited the opening of nicotinic receptors ($P < 0.05$), although there was no difference between them. At more positive potentials (from −20 to +15 mV), the effect of the drugs was not detected. The reversal potential was not affected when either drug was used and was close to 0 mV as it was for acetylcholine alone, implying that the ionic selectivity of the channel was not affected.

Prolonged exposure of the nicotinic receptor to acetylcholine results in desensitization. Oocytes were exposed to solutions containing acetylcholine and acetylcholine plus drugs for 30 s. The potential was held at −70 mV; acetylcholine was applied at 100 μM, (±)-huprine Y at 500 nM, and (±)-huprine X at 5 μM, close to their IC_{50} values (Fig. 5B). The time constant (τ) of the decay phase of the acetylcholine currents was calculated by fitting to a single exponential function. In the absence of drugs, the time constant of the decay phase of the acetylcholine-induced current was $\tau = 13.3 \pm 1.9$ s ($n = 12$). The desensitization increased in a significant manner with the presence of the drugs, with (±)-huprine Y the time constant was reduced to $\tau = 5.2 \pm 0.7$ s ($n = 8$) ($P < 0.05$), which is the same as the value obtained with (±)-huprine X, ($\tau = 5.4 \pm 0.4$ s) ($n = 8$) ($P < 0.05$). Table 1 shows the percentage

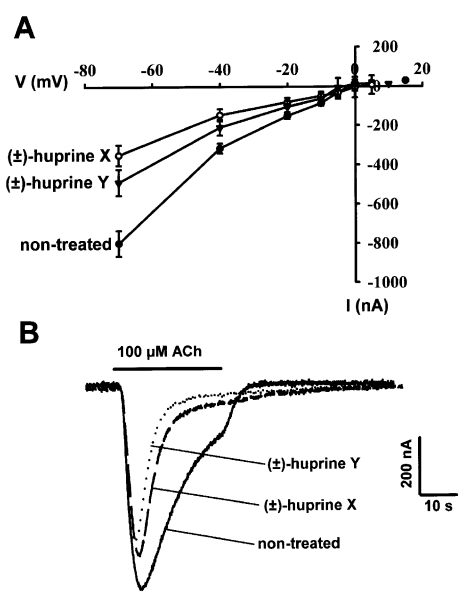

Fig. 5. Voltage dependence of the effect of (±)-huprine Y and (±)-huprine X on the nicotinic receptor and the desensitization time constant of nicotinic responses. (A) The effect of (±)-huprine Y and (±)-huprine X was reduced as the membrane became depolarized, indicating a preferential interaction with the open-channel form of the nicotinic receptor. Each point is the mean of six different oocyte measurements. (B) Currents recorded from an oocyte after sequential application of 100 μM acetylcholine and 100 μM acetylcholine plus 500 nM (±)-huprineY or 5 μM of (±)-huprine X for 30 s. The wash time between the application of solutions was 10 min at a flux of 8 ml min^{-1}. Time constant of the decay phase was calculated by fitting to a simple exponential curve. Both (±)-huprine Y and (±)-huprine X reduced the time constant to the same extent.

Table 1
Effects of (±)-huprine Y and (±)-huprine X on desensitization of nicotinic receptors
Nicotinic receptor desensitization; the desensitization of the nicotinic receptor in the presence of the drugs is shown as a percentage measured 10 and 30 s after exposure to acetylcholine or to acetylcholine with drugs.

Solution applied	%Desensitization	
	10-s perfusion	30-s perfusion
100 μM ACh	20 ± 4 ($n = 12$)	72 ± 4 ($n = 12$)
100 μM ACh + 500 nM (±)-hup Y	69 ± 10 ($n = 8$)	95 ± 2 ($n = 8$)
100 μM Ach + 5 μM (±)-hup X	65 ± 9 ($n = 8$)	91 ± 5 ($n = 8$)

of desensitization at different times after exposure to 100 μM acetylcholine in the presence or absence of (±)-huprine Y or (±)-huprine X.

4. Discussion

Alzheimer's disease is the most frequent cause of dementia in Western populations older than 65 years of age. On the basis of the cholinergic hypothesis for the cognitive symptoms of Alzheimer's disease, acetylcholinesterase inhibitors have been used as therapeutic agents for the treatment of Alzheimer's disease patients. The goal is to obtain less toxic and more potent acetylcholinesterase inhibitory agents to keep brain acetylcholine at physiological levels.

Since there is no method for directly monitoring synaptic transmission in living mammalian brain, we used the electric organ as a model of the cholinergic synapse. The electric organ of the fish is a special kind of neuromuscular junction in which there are thousands of nerve terminals, and from which it is easy to extract membrane fractions enriched in nicotinic receptors. This experimental model lets us explore the effect of acetylcholinesterase inhibitory drugs on synaptic cholinergic activity and on nicotinic receptors. Some of the mechanisms of action that we found in this experimental model might not be applicable to the mammalian brain, but it is nevertheless an approach to investigate the peripheral effects of acetylcholinesterase inhibitory drugs.

In previous studies of candidate drugs for the treatment of Alzheimer's disease, we reported that physostigmine, tacrine (Cantí et al., 1994) and the derivative of tacrine CI-1002 (Ros et al., 2000) increased the amplitude and duration of m.e.p.ps recorded in the electric organ of *Torpedo*. The action of tacrine on the neuromuscular junction was obtained over the concentration range between 10 and 100 μM (Thesleff et al., 1990; Braga et al., 1991; Cantí et al., 1994), while the second-generation acetylcholinesterase inhibitors such as CI-1002 (Ros et al., 2000) were active at 1 μM. Here, we show that (±)-huprine Y and (±)-huprine X, hybrids of huperzine A and tacrine, are effective at 500 nM. Some of the hybrids of huperzine

A and tacrine are 100 times more potent than tacrine in acetylcholinesterase inhibition. The IC_{50} for tacrine is 130 nM (Camps et al., 1999), 40 nM for CI-1002 (Emmerling et al., 1994), between 60 and 74 nM for (−)-huperzine A (Camps et al., 1999; Liu et al., 1998), 4.2 nM for (±)-huprine Y and 2.8 nM for (±)-huprine X (Camps et al., 2000b). Bis-tacrine is even more potent, with an IC_{50} of 0.4 nM (Pang et al., 1996) and (−)-huprine Y and (−)-huprine X have an IC_{50} between 0.02 and 0.04 nM (Camps et al., 2000a).

The prolongation of the decay phase of the m.e.p.ps is directly related to the acetylcholinesterase inhibitory activity. It is worth emphasizing that the concentrations of huprines used here are lower than those used previously for tacrine (Cantí et al., 1994) and CI-1002 (Ros et al., 2000). Huprines prolonged the half-width and thus the time course of a single m.e.p.p. Of the two huprines, the most effective was (±)-huprine X. Other variables analyzed are also related to acetylcholinesterase inhibitory activity: amplitude, area, rise time and rate of rise. In all cases (±)-huprine X was more effective than (±)-huprine Y, except in the rate of rise of the m.e.p.ps. It is well known that treatment with some drugs and hormones increases spontaneous and evoked quantal release (Van der Kloot and Molgó, 1994). Thus, the increase of the amplitude and the rate of rise caused by both (±)-huprine Y and X suggests that these molecules, in addition to their acetylcholinesterase inhibitory activity, could affect the recruitment of acetylcholine to build up a quantum. In any case, we wonder whether the differences between (±)-huprine Y and X were due to differences in their interaction with the nicotinic receptor. If (±)-huprine Y interferes with the nicotinic receptor more strongly than (±)-huprine X, a lower amplitude, support charge and half-width of m.e.p.ps would be expected.

Using *Xenopus* oocytes transplanted with membranes from the electric organ of *Torpedo*, we tested the effect of these drugs on the native form of the nicotinic receptor. Membrane transplant preserves the activity of native receptors better than when receptors are expressed in oocytes by mRNA translation (Morales et al., 1995). Very large inward currents were obtained when transplanted oocytes were perfused with acetylcholine. We determined the IC_{50} for (±)-huprine Y and for (±)-huprine X to be 0.45 and 4.8 μM, respectively. This difference may explain the differences in the effect on the m.e.p.ps recorded. The IC_{50} of (±)-huprine X was similar to that reported for CI-1002 (3.4 μM) (Ros et al., 2000) and tacrine (8.9 μM) (Cantí et al.,1998). The reduction in the size of the acetylcholine currents in the presence of the drugs may be due to different factors, such as open channel block, competitive block or stabilization of the receptor in the closed state.

The action of (±)-huprine Y and (±)-huprine X was sensitive to membrane voltage, since the inhibitory effect was reduced at depolarizing potentials, suggesting, as in the case of tacrine, the preference of the drugs for the open-channel form of the receptor (Cantí et al., 1998). According to the recordings obtained during long periods of acetylcholine perfusion, the receptor desensitized more quickly in the presence of the drugs, suggesting that the drug–channel complex closed for a short time. Both drugs inhibited the current induced by acetylcholine with a Hill coefficient of 0.5, indicating that one molecule of the drug interacts with two receptors. In fact, dimers of the nicotinic receptor are the most abundant form in native *Torpedo* membranes (Schindler et al., 1984); however, we cannot rule out that the blocking of one receptor decreases the affinity of the second receptor.

In summary, (±)-huprine Y and (±)-huprine X produced effects similar to those of tacrine on spontaneous synaptic activity but at a 20–200-fold lower concentration. These low concentrations of huprines may overcome the side effects elicited by tacrine and would help to reach higher levels of brain acetylcholine.

Acknowledgements

We thank Dr. J. Dempster for kindly providing “Whole Cell Analysis Program 2.1.” We are also indebted to Jordi Segura, Universitat Politècnica de Catalunya, for writing the Labview software (Quantadat) to record and analyze m.e.p.ps. DGESIC from the Spanish Government and CIRIT from the Generalitat de Catalunya funded this work.

References

Badia, A., Baños, J.E., Camps, P., Contreras, J., Görbig, D.M., Muñoz-Torrero, D., Simon, M., Vivas, N.M., 1998. Synthesis and evaluation of tacrine–huperzine A hybrids as acetylcholinesterase inhibitors of potential interest for the treatment of Alzheimer’s disease. Bioorg. Med. Chem. 6, 427–440.

Barril, X., Orozco, M., Luque, F.J., 1999. Predicting relative binding free energies of tacrine–huperzine A hybrids as inhibitors of acetylcholinesterase. J. Med. Chem. 42, 5110–5119.

Bartus, R.T., Dean, R.L., Beer, B., Lippa, A.S., 1982. The cholinergic hypothesis of geriatric memory dysfunction. Science 217, 408–414.

Berezovska, O., Frosch, M., McLean, P., Knowles, R., Koo, E., Kang, D., Shen, J., Lu, F.M., Lux, S.E., Tonegawa, S., Hyman, B.T., 1999. The Alzheimer-related gene presenilin 1 facilitates notch 1 primary mammalian neurons. Mol. Brain Res. 69, 273–280.

Braga, M.F., Harvey, A.L., Rowan, E.G., 1991. Effects of tacrine, velnacrine (HP029), suronacrine (HP128), and 3,4 diaminopyridine on skeletal neuromuscular transmission in vitro. Br. J. Pharmacol. 102, 909–915.

Brinton, R.D., Yamazaki, R.S., 1998. Advances and challenges in the prevention and treatment of Alzheimer disease. Pharmacol. Res. 15, 386–398.

Camps, P., Contreras, J., Font-Bardia, M., Morral, J., Muñoz-Torrero, D., Solans, X., 1998. Enantioselective synthesis of tacrine–huperzine A hybrids: preparative chiral MPLC separation of their racemic mixtures and absolute configuration assignments by X-ray diffraction analysis. Tetrahedron: Asymmetry 9, 835–849.

Camps, P., El Achab, R., Görbig, D.M., Morral, J., Muñoz-Torrero, D., Badia, A., Baños, J.E., Vivas, N.M., Barril, X., Orozco, M., Luque, F.J., 1999. Synthesis, in vitro pharmacology, and molecular modeling

of very potent tacrine–huperzine A hybrids as acetylcholinesterase inhibitors of potential interest for the treatment of Alzheimer's disease. J. Med. Chem. 42, 3227–3242.

Camps, P., Cusack, B., Mallender, W.D., El Achab, R., Morral, J., Muñoz-Torrero, D., Rosenberry, T.L., 2000a. Huprine X is a novel high-affinity inhibitor of acetylcholinesterase that is of interest for the treatment of Alzheimer's disease. Mol. Pharmacol. 57, 409–417.

Camps, P., El Achab, R., Morral, J., Muñoz-Torrero, D., Badia, A., Baños, J.E., Vivas, N.M., Barril, X., Orozco, M., Luque, F.J., 2000b. New tacrine–huperzine A hybrids (huprines): highly potent tight-binding acetylcholinesterase inhibitors of interest for the treatment of Alzheimer's disease. J. Med. Chem. 43, 4657–4666.

Cantí, C., Martí, E., Marsal, J., Solsona, C., 1994. Tacrine-induced increase in the release of spontaneous high quantal content events in *Torpedo* electric organ. Br. J. Pharmacol. 102, 909–915.

Cantí, C., Bodas, E., Marsal, J., Solsona, C., 1998. Tacrine and physostigmine block nicotinic receptors in *Xenopus* oocytes injected with *Torpedo* electroplaque membranes. Eur. J. Pharmacol. 363, 197–202.

Dumont, J.N., 1972. Oogenesis in *Xenopus laevis* (Daudin): stages of oocyte development in laboratory maintained animals. J. Morphol. 136, 153–179.

Dunant, Y., Muller, D., 1986. Quantal release of acetylcholine evoked by focal depolarization at the *Torpedo* nerve-electroplaque junction. J. Physiol. 379, 461–478.

Emmerling, M.R., Gregor, V.E., Schwarz, R.D., Scholten, J.D., Callahan, M.J., Lee, C., Moore, C.J., Raby, C., Lipinsky, W.J., Davis, R.E., 1994. PD 142676 (CI 1002) a novel anticholinesterase and muscarinic antagonist. Mol. Neurobiol. 9, 93–106.

Farlow, M., Gracon, S.I., Hershey, L.A., Lewis, K.W., Sadowsky, C.H., Dolan-Ureno, J., 1992. A controlled trial of tacrine in Alzheimer's disease. J. Am. Med. Assoc. 268, 2523–2529.

Forsyth, E., Ritzline, P.D., 1998. An overview of the etiology, diagnosis and treatment of Alzheimer disease. Phys. Ther. 78, 1325–1331.

Francis, P.T., Palmer, A.M., Snape, M., Wilcock, G.K., 1999. The cholinergic hypothesis of Alzheimer's disease: a review of progress. J. Neurol., Neurosurg. Psychiatry 66, 137–147.

Giacobini, E., 1996. New trends in cholinergic therapy for Alzheimer disease: nicotinic agonists or cholinesterase inhibitors? Prog. Brain Res. 109, 311–323.

Giacobini, E., 1997. From molecular structure to Alzheimer therapy. Jpn. J. Pharmacol. 74, 225–241.

Katz, B., Miledi, R., 1972. The statistical nature of acetylcholine potential and its molecular components. J. Physiol. 224, 665–699.

Knapp, M.J., Knopman, D.S., Solomon, P.R., Pendlebury, W.W., Davis, C.S., Gracon, S.I., 1994. A 30-week randomized controlled trial of high-dose tacrine in patients with Alzheimer's disease. J. Am. Med. Assoc. 271, 985–991.

Kozikowsky, A.P., Thiels, E., Tang, X.-C., Hanin, I., 1992. Huperzine A —a possible lead structure in the treatment of Alzheimer's disease. Adv. Med. Chem. 1, 175–205.

Krall, W.J., Sramek, J.J., Cutler, N.R., 1999. Cholinesterase inhibitors: a therapeutic strategy for Alzheimer disease. Ann. Pharmacother. 33, 441–450.

Liu, J., Zhang, H.Y., Tang, X.C., Wang, B., He, H.C., Bai, D.L., 1998. Effects of synthetic (−)-huperzine A on cholinesterases activities and water maze performance. Acta Pharmacol. Sin. 19, 413–416.

Marsal, J., Tigyi, G., Miledi, R., 1995. Incorporation of acetylcholine receptors and Cl^- channels in *Xenopus* oocytes injected with *Torpedo* electroplaque membranes. Proc. Natl. Acad. Sci. U. S. A. 92, 5224–5228.

Miledi, R., Woodward, R.M., 1989. Effects of defolliculation on membrane current responses of *Xenopus* oocytes. J. Physiol. 416, 601–621.

Morales, A., Aleu, J., Ivorra, I., Ferragut, J.A., Gonzalez, J.M., Miledi, R., 1995. Incorporation of reconstituted acetylcholine receptors from *Torpedo* into the *Xenopus* oocyte membrane. Proc. Natl. Acad. Sci. U. S. A. 92, 8468–8472.

Muller, D., Dunant, Y., 1987. Spontaneous quantal and subquantal transmitter release at the Torpedo nerve–electroplaque junction. Neuroscience 20, 911–921.

Pang, Y.P., Quiram, P., Jelacic, T., Hong, F., Brimijoin, S., 1996. Highly potent, selective, and low cost bis-tetrahydroaminacrine inhibitors of acetylcholinesterase. J. Biol. Chem. 271, 23646–23649.

Ros, E., Aleu, J., Marsal, J., Solsona, C., 2000. Effects of CI-1002 and CI-1017 on spontaneous synaptic activity and on the nicotinic acetylcholine receptor of *Torpedo* electric organ. Eur. J. Pharmacol. 390, 7–13.

Schindler, H., Spillecke, F., Neumann, E., 1984. Different channel properties of *Torpedo* acetylcholine receptor monomers and dimers reconstituted in planar membranes. Proc. Natl. Acad. Sci. U. S. A. 81, 6222–6226.

Skolnick, A.A., 1997. Old Chinese herbal medicine used for fever yields possible new Alzheimer disease therapy. J. Am. Med. Assoc. 227, 776.

Soria, B., 1983. Properties of miniature post-synaptic currents at the *Torpedo marmorata* nerve–electroplaque junction. Q. J. Exp. Physiol. 68, 189–202.

Thesleff, S., Sellin, L.C., Tagerud, S., 1990. Tetrahydroaminoacridine (tacrine) stimulates neurosecretion at mammalian motor endplates. Br. J. Pharmacol. 100, 487–490.

Van der Kloot, W., Molgó, J., 1994. Quantal acetylcholine release at vertebrate neuromuscular junction. Physiol. Rev. 74, 899–991.

Ved, H.S., Koenig, M.L., Dave, J.R., Doctor, B.P., 1997. Huperzine A, a potential therapeutic agent for dementia, reduces neuronal cell death caused by glutamate. NeuroReport 8, 963–968.

Watkins, P.B., Zimmerman, H.J., Knapp, J.M., Gracon, S.I., Lewis, K.W., 1994. Hepatotoxic effects of tacrine administration in patients with Alzheimer's disease. J. Am. Med. Assoc. 271, 992–998.