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Effects of CI-1002 and CI-1017 on spontaneous synaptic activity and on the nicotinic acetylcholine receptor of *Torpedo* electric organ

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Abstract

The effect of azepino[2,1-*b*]quinazoline 1,3-dichloro-6,7,8,9,10,12-hexahydro-, mono-hydrochloride (CI-1002), a tacrine derivative, and 1-azabicyclo[2.2.1]heptan-3-one, *O*-[3-(methoxyphenyl)-2-propynyl]oxime [*R*-(*Z*)]-2-butenedioate (CI-1017), a muscarinic M_1 receptor agonist, on spontaneous synaptic activity was investigated by measuring amplitude, rise time, velocity of rising, half-width, and electrical charge of miniature endplate potentials (m.e.p.p.) recorded extracellularly in *Torpedo* electric organ fragments. The effect of CI-1002 and CI-1017 on the nicotinic acetylcholine receptor was investigated by measuring the current induced by acetylcholine in *Xenopus laevis* oocytes transplanted with membranes from *Torpedo* electric organ. CI-1002, at a concentration of 1 μM, altered the m.e.p.p. by increasing the amplitude (from 1.08 ± 0.01 to 2.76 ± 0.03 mV), rise time (from 0.700 ± 0.006 to 1.02 ± 0.01 ms), rising rate (from 1.79 ± 0.02 to 3.45 ± 0.05 mV/ms), half-width (from 0.990 ± 0.008 to 2.40 ± 0.02 ms), and electrical charge (from 304 ± 4 to 784 ± 11 mV s). CI-1017, at a concentration of 1 μM, altered the m.e.p.p. by decreasing the amplitude (from 1.08 ± 0.01 to 0.650 ± 0.007 mV), rise time (from 0.700 ± 0.006 to 0.530 ± 0.007 ms), rising rate (from 1.79 ± 0.02 to 1.53 ± 0.02 mV/ms), half-width (from 0.990 ± 0.008 to 0.670 ± 0.007 ms), and electrical charge (from 304 ± 4 to 75 ± 1 mV s). CI-1002 inhibited the acetylcholine-induced current of nicotinic acetylcholine receptors with an IC_{50} of 3.4 ± 0.3 μM. CI-1017 inhibited the acetylcholine-induced current of nicotinic acetylcholine receptors with an IC_{50} of 0.8 ± 0.1 μM. These results indicate that, although both drugs interacted negatively with nicotinic acetylcholine receptors, CI-1002 overcame this inhibition by recruiting more acetylcholine to build a quantum.

Keywords: Alzheimer's disease; Cholinergic; Exocytosis; Neuromuscular junction; Oocyte; Synaptic vesicle; Tacrine; (*Torpedo marmorata*); (*Xenopus laevis*)

1. Introduction

It is generally accepted that even though Alzheimer's disease implicates a general loss of neurotransmitters, the decrease in brain acetylcholine levels is directly related to severe cognitive dysfunction (Davies and Maloney, 1976; Bartus et al., 1982; Coyle et al., 1983). A straightforward procedure to maintain high levels of endogenous acetylcholine is intended to inhibit the acetylcholinesterases, which hydrolyse acetylcholine in the synaptic cleft. Physostigmine and tacrine, both anticholinesterasic agents, have been used in clinical trials with partial success (see Davies and Maloney, 1976; Davis et al., 1978; Weinstock, 1995; Giacobini, 1998 for review).

Another strategy for the therapy of Alzheimer's disease patients is to mimic the action of acetylcholine directly upon the receptors by using muscarinic and nicotinic acetylcholine receptor agonists. Muscarinic M_1 receptor agonists are a class of drugs that are currently being developed but their clinical use is still limited by side effects. (see Giacobini, 1997 for review). New nicotinic receptor agonists that show none of the adverse side effects of nicotine, which limit its clinical use, are likewise being investigated (see Giacobini, 1996 for review).

The direct measurement of the quantal release in the central nervous system, and the mean life of acetylcholine molecules have not been established. An alternative experimental model is the vertebrate neuromuscular junction, in which the quantal nature of acetylcholine release can be recorded. The electric organs of Elasmobranchs are embryologically derived from the skeletal muscle and have a great number of motor nerve endings. Indeed, there are

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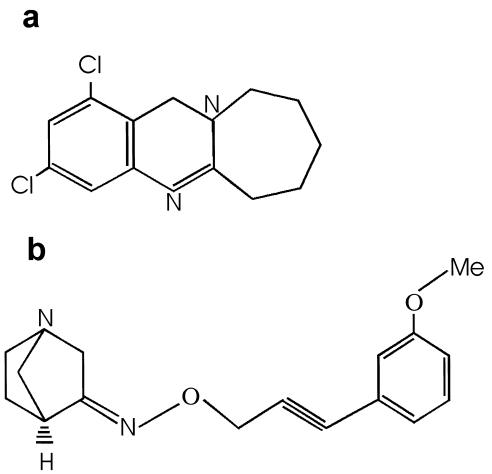

Fig. 1. Chemical structure of tested drugs. (a) CI-1002 compound: Azepino[2,1-*b*]quinazoline 1,3-dichloro-6,7,8,9,10,12-hexahydro–, mono-hydrochloride. (b) CI-1017 compound: 1-Azabicyclo[2.2.1]heptan-3-one, *O*-[3-(methoxyphenyl)-2-propynyl]oxime [*R*-(*Z*)]-2-butenedioate.

huge amounts of acetylcholinesterases and nicotinic acetylcholine receptors (Whittaker and Zimmerman, 1976). It is, therefore, a suitable preparation to explore the effects of new drugs on cholinergic synapses at the cellular and molecular levels.

To test the direct effects of drugs on nicotinic acetylcholine receptors, we used the approach of injecting nicotinic acetylcholine receptor-enriched membranes into the cytoplasm of *Xenopus* oocytes. The injection of *Torpedo* postsynaptic membrane vesicles (Marsal et al., 1995; Morales et al., 1995) leads to the incorporation of exogenous voltage and ligand-gated ion channels into the oocyte plasma membrane (essentially preserving their native properties), thus providing a fully unmodified synaptic receptor inserted into the oocyte plasma membrane.

We previously evaluated the action of tacrine and physostigmine on the peripheral nervous system in the electric organ of *Torpedo* (Cantí et al., 1994, 1998). Here, we examine the effects of new drugs (two compounds) — azepino[2,1-*b*]quinazoline 1,3-dichloro-6,7,8,9,10,12-hexahydro–, mono-hydrochloride (CI-1002), a tacrine derivative with anticholinesterase activity; and 1-azabicyclo[2.2.1]heptan-3-one, *O*-[3-(methoxyphenyl)-2-propynyl]oxime [*R*-(*Z*)]-2-butenedioate (CI-1017), which has been reported to act on muscarinic M_1 receptors (Tecle et al., 1998) on neuromuscular synaptic transmission (Fig. 1).

2. Methods

2.1. Animals and solutions

The neuromuscular junction model used was the electric organ of *Torpedo*. The electric organ was used because it is possible to assess spontaneous synaptic activity and, in addition, it is also easy to assay the effect of drugs on the nicotinic acetylcholine receptor. To record the activation of native nicotinic acetylcholine receptors, electric organ membranes were transplanted into *Xenopus laevis* oocytes.

Torpedo marmorata specimens were caught off the Catalan Mediterranean coast and kept in artificial seawater. Fish were anaesthetized with tricaine (3-aminobenzoic acid ethyl ester methanesulfonate salt) (Sigma, St Louis, MO, USA) at a concentration of 0.3 g/l of seawater, before surgical excision of electric organs.

Mature females of *Xenopus laevis* were purchased from the Centre d'Elevage des Xenopes (Montpellier, France) and were anaesthetized by immersion in water containing 0.17% tricaine. A few lobes of ovaries were removed, through a small incision in the abdomen, and oocytes at stages V and VI (Dumont, 1972) were dissected out.

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 adjusted to 7.0 with $NaHCO_3$. The same solution was used to record spontaneous synaptic activity.

Two solutions were used for *Xenopus* oocyte experiments. 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. The recording solution contained 115 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 5 mM HEPES pH 7.0, to which 1 μM atropine sulfate (Merck, Darmstadt, Germany) had been added to block any muscarinic receptor responses (Kusano et al., 1982).

2.2. Recording of spontaneous synaptic activity

All recordings were made at room temperature (20–22°C). Five to ten prisms of the electric organ were cut with a scalpel blade and 1-mm sections were incubated overnight in *Torpedo* saline solution containing one of the drugs studied. Measurements were performed in fragments fixed in a plexiglas chamber with a sylgard-coated base.

The spontaneous synaptic release of acetylcholine was recorded with focal extracellular low-resistance microelectrodes (Katz and Miledi, 1977) as adapted for the electric organ by Soria (1983) and Muller and Dunant (1987, for details) and as described elsewhere (Cantí et al., 1994). The method allows for long-term recording with minimal damage to the cells. The spontaneous miniature endplate potentials (m.e.p.p.) were amplified (2A, Axoclamp), monitored on a Tektronix 5110 oscilloscope and recorded in parallel on a VCR (Biologic, Echirolles, France). Signals were analysed using 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 3.02.

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

2.3. Oocyte preparation, microinjection and recording

The oocytes at stages V and VI (Dumont, 1972) were dissected out 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 50 min at room temperature to remove the surrounding layers (Miledi and Woodward, 1989).

Healthy oocytes were microinjected with a fresh or thawed suspension (4–10 mg/ml) of electroplaque membranes (Marsal et al., 1995; Cantí et al., 1998) by means of a pneumatic pressure injector (PLI-100 Picospritzer, Medical Systems, USA). When frozen–thawed samples were used, they were sonicated again prior to injection.

The oocytes were recorded 14–72 h after injection. They were voltage-clamped with a two-electrode system (Geneclamp 500 Amplifier, Axon Instruments, USA). 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. Membrane currents were low-pass filtered at 10 Hz and recorded on a PC using the program cited above, after sampling signals by Digidata 1200A (Axon Instruments) at twice the filter frequency. For all recordings, the potential was held at −70 mV and currents were elicited by challenges of 20 or 100 μM acetylcholine. The interval between consecutive responses was systematically set at 10 min, since we previously established that it was the 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

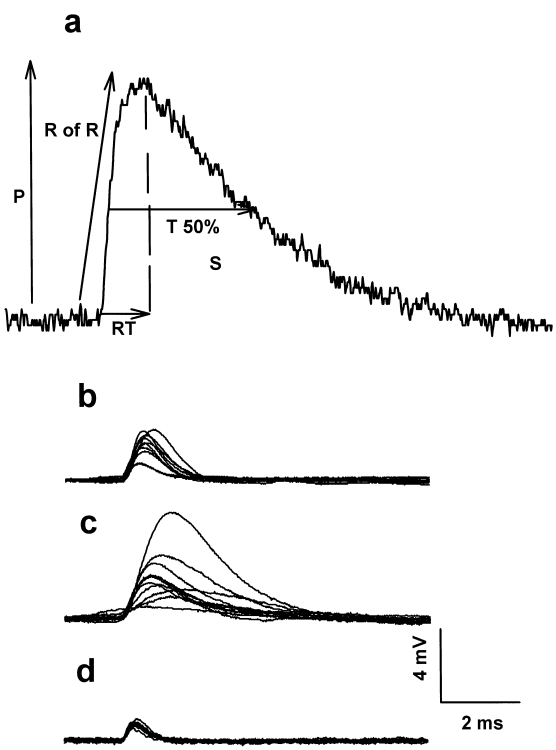

Fig. 2. (a) Parameters analysed in each miniature endplate potential (m.e.p.p.) recorded. Diagram of the studied variables in the m.e.p.p. S = total surface (equivalent to the electrical charge mobilized by an m.e.p.p.) P = Peak amplitude. RT = Rise Time. R of R = Rate of Rise. T 50% = Half-width. (b), (c) and (d) Effect of CI-1002 and CI-1017 on spontaneous m.e.p.p. Super-imposed oscilloscope traces showing spontaneous m.e.p.p. recorded in *T. marmorata* electric organ. (b) Non-treated fragments; (c) after incubation with CI-1002 (1 μM); (d) after incubation with CI-1017 (1 μM). CI-1002 more than doubled the amplitude of m.e.p.p. while CI-1017 decreased it.

2.4. Calculations and statistics

Differences between distribution functions were evaluated with Sigmastat 1.0 software (SPSS, USA) by One-Way Analysis of Variance on ranks (Kruskal–Wallis test) and by All Pairwise Multiple Comparison Procedures (Dunn's Method). In oocyte experiments, dose–response data were fitted by nonlinear regression to a sigmoidal curve using the Inplot program (GraphPad, USA). The values, as calculated by the program, are expressed as means ± S.E.M.

2.5. Drugs

Atropine sulfate (Sigma), CI-1002, and CI-1017 were kindly provided by Parke-Davis Pharmaceutical Research. The stock drug solutions (10 mM) were freshly prepared in deionized water and diluted to 1 μM with *Torpedo*'s saline solution.

3. Results

3.1. Effects on spontaneous synaptic activity

The spontaneous synaptic activity was recorded extracellularly through a loose patch configuration (Dunant and Muller, 1986). The drugs, CI-1002 (1 μM) and CI-1017 (1 μM), altered the size and duration of m.e.p.p. CI-1002 enhanced the size whereas CI-1017 had the opposite effect (Fig. 2b,c and d).

The mean amplitude of m.e.p.p. was 1.08 ± 0.01 mV (n = 2823, in three different experiments) in non-treated samples of electric organ. CI-1002 significantly increased the amplitude by nearly three-fold to 2.76 ± 0.03 mV

($n = 2837$, in three different experiments), $P < 0.0001$. The histogram of amplitude frequencies in CI-1002-treated samples (Fig. 3a) shows not only a shift to the right, but also a two-fold increase in the range of amplitude with respect to that of the control. The increase in the amplitude of m.e.p.p. was parallel to the increase in the electric

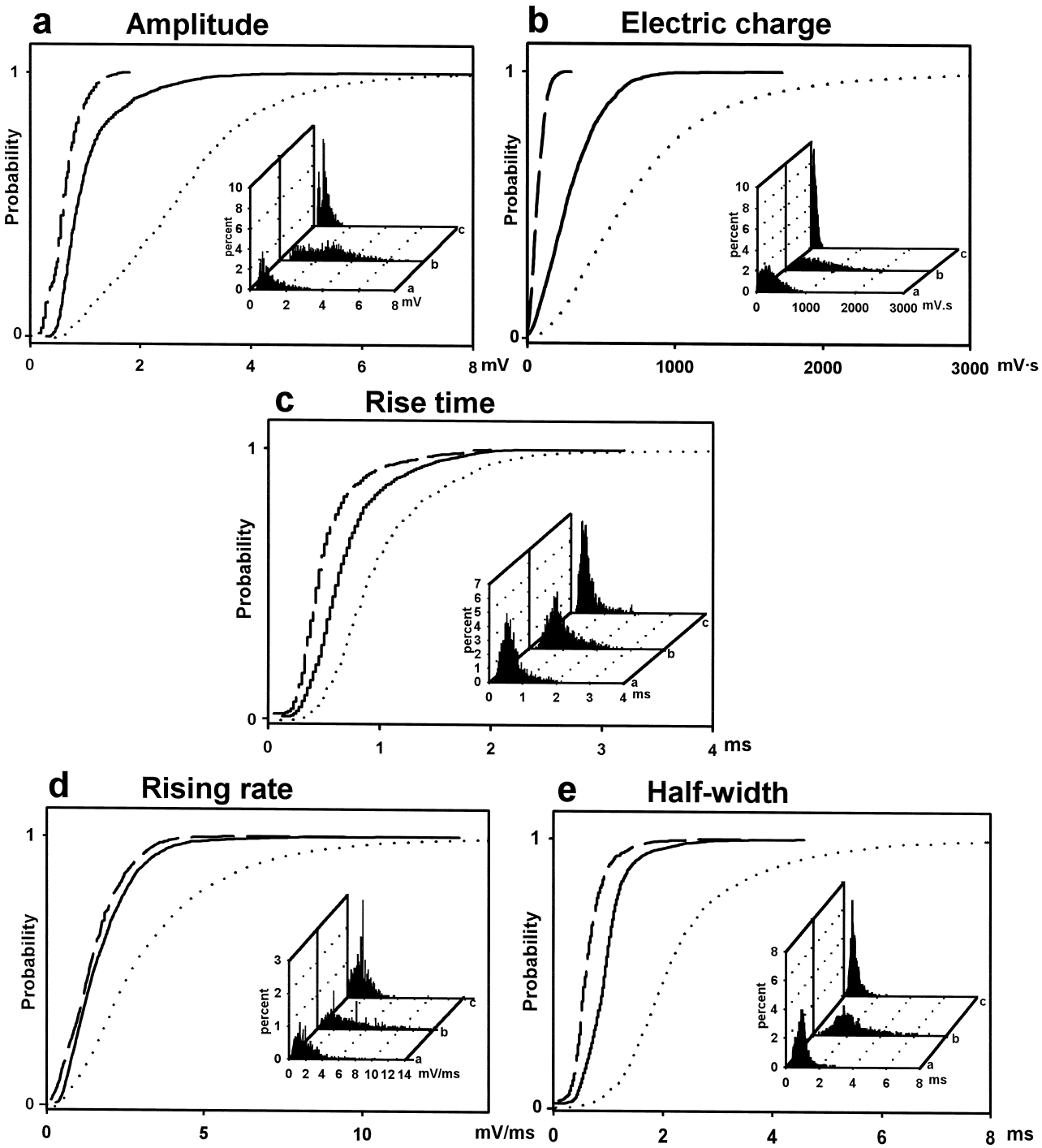

Fig. 3. Data are presented as cumulative histograms and as bar histograms (inset). In the cumulative probability histogram: non-treated tissue, continuous line; CI-1002 (1 μM), dotted line; CI-1017 (1 μM), discontinuous line. Inset bar histograms are presented in (a) non-treated tissue; (b) CI-1002 (1 μM); (c) CI-1017 (1 μM). (a) Effect of CI-1002 and CI-1017 on the amplitude of the miniature endplate potential (m.e.p.p.) (peak). (b) Electric charge mobilized by spontaneous m.e.p.p. The effect of CI-1002 and CI-1017 on 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 acetylcholine receptors as a consequence of spontaneous quanta. CI-1002 more than doubled the electrical charge mobilized by one quantum, while CI-1017 decreased it by more than three times. (c) Time rise of m.e.p.p. CI-1002 prolonged the rise time while CI-1017 shortened it. (d) Rate of rise time of m.e.p.p. CI-1002 increased the rate of rise while CI-1017 decreased it slightly. (e) Width of the m.e.p.p. at 50% of the amplitude.

charge carried (from 304 ± 4 to 784 ± 11 mV s), $P < 0.0001$, (Fig. 3b). The rise time of each m.e.p.p. was measured between 10% and 90% of the amplitude. The rise time was significantly increased by CI-1002 (from 0.700 ± 0.006 to 1.02 ± 0.01 ms), $P < 0.0001$, (Fig. 3c). The rate of rise, which is related to the velocity of release of acetylcholine, approximately doubled (from 1.79 ± 0.02 to 3.45 ± 0.05 mV/ms), $P < 0.0001$, (Fig. 3d). CI-1002 increased the decay of the m.e.p.p. (Fig. 2c) and therefore, the half-width was also significantly enlarged (from 0.990 ± 0.008 to 2.40 ± 0.02 ms), $P < 0.0001$, (Fig. 3e). In conclusion, for all the parameters of m.e.p.p. analysed, there was an increase in the mean value, which did not correspond to a net shift to the right in the histogram. All parameters showed a wider range of values in treated samples than in controls.

CI-1017 significantly decreased the amplitude of m.e.p.p. to 0.650 ± 0.007 mV ($n = 1763$, in three different experiments), $P < 0.0001$. The histogram of amplitude frequencies (Fig. 3a) shifted to the left and the smallest signals were not higher than the background. The decrease in amplitude of m.e.p.p. was parallel to the fall in the electric charge carried (75 ± 1 mV s), $P < 0.0001$, (Fig. 3b). The rise time of each m.e.p.p. was also significantly decreased by CI-1017 (0.530 ± 0.007 ms), $P < 0.0001$, (Fig. 3c). The rising rate was slightly decreased (from 1.79 ± 0.02 to 1.53 ± 0.02 mV/ms), $P < 0.0001$, (Fig. 3d). CI-1017 reduced the half-width of the signal (from 0.990 ± 0.008 to 0.670 ± 0.007 ms), $P < 0.0001$ (Figs. 2c and 3e). All these results indicated a different action of the two compounds tested. CI-1002 increased reduced the size of m.e.p.p. while CI-1017 brought an opposite reaction.

The effect of CI-1017 might be due to a decrease in each individual quantum or to a direct interaction with the nicotinic acetylcholine receptor. The latter possibility cannot be tested directly with the electric organ preparation, but it can with membranes from the electric organ transplanted into *Xenopus* oocytes.

3.2. Effects on nicotinic acetylcholine receptor

We tested the effect of CI-1017 in transplanted *Xenopus* oocytes and since we have previously demonstrated a direct interaction of tacrine with *Torpedo* nicotinic acetylcholine receptor, we also tested the effects of CI-1002 on this receptor.

The effects of CI-1002 and CI-1017 on the nicotinic acetylcholine receptor are illustrated in Fig. 4. There was a dose-dependent inhibition of acetylcholine (20 μM)-induced currents. At each concentration of the drug, the blockade was reversed by 83% of the initial current after a wash-out of 10 min. Plots of currents were obtained from 12 oocytes (from five donors) treated with CI-1002 and from nine oocytes (from four donors) treated with CI-1017. Sigmoidal curves showed a satisfactory fit to the data. The Hill coefficients estimated from the dose–response curves

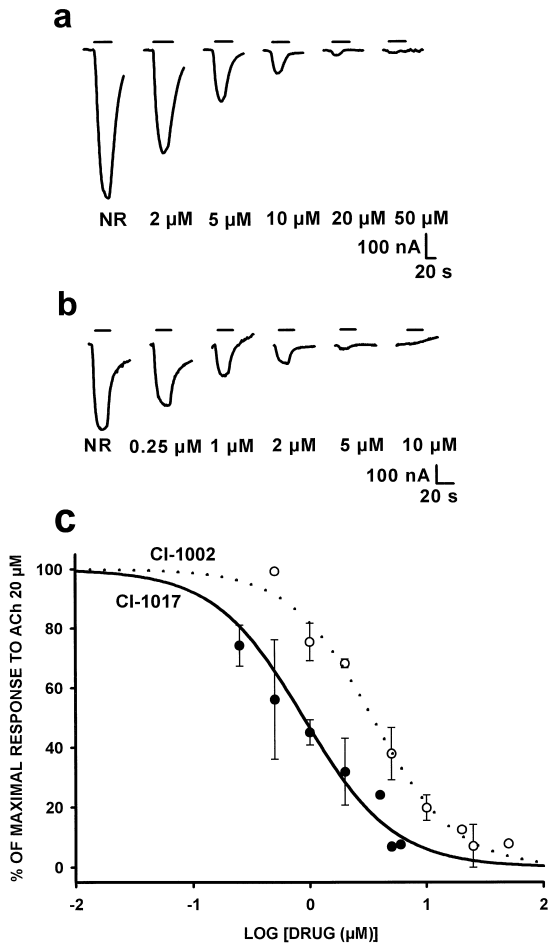

Fig. 4. The effects of CI-1002 (a) and CI-1017 (b). Currents elicited by 20 μM acetylcholine in one oocyte in the presence of different concentrations of the compounds (CI-1002 and CI-1017). Bars indicate acetylcholine application and the holding potential was −70 mV. (c) Effects of CI-1017 (filled symbol) and CI-1002 (hollow symbol) on the current elicited by 20 μM acetylcholine. Data are the means ± S.E.M. of values obtained from 9 and 12 oocytes, respectively, and are represented as percentages of the maximal current elicited by 20 μM acetylcholine at a holding potential of −70 mV.

were not significantly different: -1.2 ± 0.1 and -0.9 ± 0.1 for CI-1002 and CI-1017, respectively. In addition, the IC_{50} for CI-1017 (0.8 ± 0.1 μM) was significantly lower than that of CI-1002 (3.4 ± 0.3 μM) ($P < 0.001$) and CI-1017 exerted a stronger inhibition of nicotinic acetylcholine receptor currents.

4. Discussion

So far, there is no experimental model to monitor the cholinergic synaptic quantal activity in the mammalian brain. The *Torpedo* electric organ is an alternative model for screening active cholinergic molecules because it is suitable for measuring their action on synaptic secretion and their interaction with the nicotinic acetylcholine receptor or acetylcholinesterases. We have previously reported

the interaction of tacrine with cholinergic nerve terminals of *Torpedo* electric organ (Cantí et al., 1994; 1998). In the present communication, we report that CI-1002 (a tacrine derivative) increased the amplitude, charge, half-width, and rise time of m.e.p.p.

Compounds with an azabicyclo oxime have muscarinic receptor binding properties (Plate et al., 1996). CI-1017 is an M_1-selective muscarinic receptor agonist (Tecle et al., 1998) but it induced the opposite effect of CI-1002 in that it induced a general reduction in m.e.p.p.

4.1. Anticholinesterasic activity

It is well known that tacrine has anti-acetylcholinesterase activity (see Freeman and Dawson, 1991, for review). The inhibition of acetylcholinesterases prolongs the half-life of acetylcholine molecules in the synaptic cleft, and thus increases the probability that they will interact with nicotinic acetylcholine receptors several times. These events prolong the decay and half-width of the m.e.p.p. profile. CI-1002 is a tacrine derivative in which chlorine atoms are added in positions 1 and 3 and one carbon atom is added to the carbocyclic ring. The insertion of chlorine enhances the binding of tacrine to *Torpedo* acetylcholinesterase (Wlodek et al., 1996), whereas changes in the size of the carbocyclic ring of tacrine produce modest potency against cholinesterases (McKenna et al., 1997). In the present work, we have used much lower concentrations (1 μM) than those reported for tacrine (Thesleff et al., 1990, 20 μM; Braga et al., 1991, 10–30 μM; Cantí et al., 1994, 100 μM). The IC_{50} of tacrine to inhibit cholinesterase was 30 nM, which is close to the value reported for CI-1002 ($IC_{50} = 40$ nM) (Emmerling et al., 1994), but CI-1002 induced a larger increment in the quantal size (79%); tacrine only induced an increment of 11% (one experiment, not shown). These results suggest that CI-1002 may be more effective in maintaining acetylcholine in the synaptic cleft.

4.2. Blockade of nicotinic acetylcholine receptors

The general inhibitory effect of CI-1017 on m.e.p.p. may be due to a decrease in the number of molecules of acetylcholine released or to a direct interaction with the nicotinic acetylcholine receptor. Here, we show that a muscarinic M_1 receptor agonist inhibited muscle-type nicotinic acetylcholine receptors. The IC_{50} at the nicotinic acetylcholine receptor was 0.8 ± 0.1 μM, which is close to ED_{50} for muscarinic M_1 receptors (1 ± 0.3 μM) measured in transfected CHO (Chinese hamster ovary) cells (Tecle et al., 1998). We can not ignore that CI-1017 may have decreased the number of acetylcholine molecules released by acting on the presynaptic muscarinic receptors of *Torpedo* electric organ, because muscarinic M_1 acetylcholine-like receptors have been described and it has been suggested that these receptors may play a retroinhibitory role in acetylcholine release (Michaelson et al., 1979; Green and Dowdall, 1992).

Atropine, the classic muscarinic receptor antagonist, inhibits ionic currents mediated by neuronal nicotinic acetylcholine receptors (Zwart and Vijverberg, 1997), but does not affect the muscle-type nicotinic acetylcholine receptor (Miledi and Sumikawa, 1982). It has been reported that CI-1002 has a muscarinic receptor antagonist activity with an $IC_{50} = 1.6$ μM (Emmerling et al., 1994). CI-1002 also inhibited currents mediated by the nicotinic acetylcholine receptor. CI-1002 had an IC_{50} (3.4 μM) four times higher than CI-1017, suggesting that muscarinic M_1 receptor agonists may interfere more strongly with nicotinic acetylcholine receptors than do anticholinesterasic agents.

In this paper, we show that CI-1002 and CI-1017 interfere with muscle nicotinic acetylcholine receptors, and thus, may modify motor activity. However, neuronal nicotinic acetylcholine receptors have a different subunit composition and are presumably located on presynaptic terminals. We wonder whether these drugs will also interfere with ion channel opening.

4.3. Does the tacrine derivative interfere with synaptic vesicle exocytosis?

During continuous recording of spontaneous synaptic activity, the concentration of CI-1002 was sufficient to inhibit nicotinic acetylcholine receptors, but otherwise increased the size of m.e.p.p. Under these conditions, the release of acetylcholine must have increased more to obtain this increment in m.e.p.p. size. However, the increase in m.e.p.p. size may reflect an imbalance between an inhibitory action on postsynaptic nicotinic acetylcholine receptors and an increase in nerve terminal activity mediated by the inhibition of the presynaptic muscarinic acetylcholine receptors.

An increase in quantal size has been reported after treatment with acetylcholinesterase inhibitors (Thesleff et al., 1990; Cantí et al., 1994) or after the action of calcitonin gene-related peptide (Van der Kloot et al., 1998) and corresponds to large amounts of acetylcholine released from a single synaptic vesicle. More experiments are need to elucidate the action of CI-1002 at the subcellular level.

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