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Effect of galantamine on the human α7 neuronal nicotinic acetylcholine receptor, the *Torpedo* nicotinic acetylcholine receptor and spontaneous cholinergic synaptic activity

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1 Various types of anticholinesterasic agents have been used to improve the daily activities of Alzheimer's disease patients. It was recently demonstrated that Galantamine, described as a molecule with anticholinesterasic properties, is also an allosteric enhancer of human $\alpha4\beta2$ neuronal nicotinic receptor activity. We explored its effect on the human α7 neuronal nicotinic acetylcholine receptor (nAChR) expressed in *Xenopus* oocytes.

2 Galantamine, at a concentration of 0.1 μM, increased the amplitude of acetylcholine (ACh)-induced ion currents in the human α7 nAChR expressed in *Xenopus* oocytes, but caused inhibition at higher concentrations. The maximum effect of galantamine, an increase of 22% in the amplitude of ACh-induced currents, was observed at a concentration of 250 μM Ach.

3 The same enhancing effect was obtained in oocytes transplanted with *Torpedo* nicotinic acetylcholine receptor (AChR) isolated from the electric organ, but in this case the optimal concentration of galantamine was 1 μM. In this case, the maximum effect of galantamine, an increase of 35% in the amplitude of ACh-induced currents, occurred at a concentration of 50 μM ACh.

4 Galantamine affects not only the activity of post-synaptic receptors but also the activity of nerve terminals. At a concentration of 1 μM, quantal spontaneous events, recorded in a cholinergic synapse, increased their amplitude, an effect which was independent of the anticholinesterasic activity associated with this compound. The anticholinesterasic effect was recorded in preparations treated with a galantamine concentration of 10 μM.

5 In conclusion, our results show that galantamine enhances human α7 neuronal nicotinic ACh receptor activity. It also enhances muscular AChRs and the size of spontaneous cholinergic synaptic events. However, only a very narrow range of galantamine concentrations can be used for enhancing effects.

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Abbreviations: ACh, acetylcholine; AChR, nicotinic acetylcholine receptor; MEPPs, spontaneous miniature endplate potentials; nAChR, neuronal nicotinic acetylcholine receptor

Introduction

Alzheimer's disease, the most common form of dementia, is linked to beta-amyloid protein metabolism; there is a progressive degeneration of basal forebrain cholinergic neurons innervating the hippocampus and the cortex. Although other neurotransmitters decline during Alzheimer's-associated neurodegeneration, the degree of brain acetylcholine (ACh) reduction directly correlates with deterioration of cognition and of daily activity in patients (Auld *et al*., 2002).

Since deficits in cholinergic function contribute to the pathology of Alzheimer's disease, attempts to delay the progression of the illness and improve patients' daily activities are based on pharmacological strategies to increase ACh levels by means of anticholinesterasic agents (Giacobini, 2003).

Some anticholinesterasic drugs have serious side effects on patients because they do not only act specifically on the acetylcholinesterases, but also affect other ion channels such as potassium channels (Kraliz & Singh, 1997) and neurotransmitter-associated receptors such as GABA receptors (Li *et al*., 1999). In recent years, our laboratory has been investigating the effect of various anticholinesterasic agents such as tacrine, physostigmine, bis-tacrine, huprine X, huprine Y and CI-1002 on embryonic muscle nicotinic-type receptors and on the spontaneous cholinergic synaptic activity of the *Torpedo* electric organ. Anticholinesterasic drugs increase the size and duration of spontaneous miniature endplate potentials (MEPPs), but inhibit the currents supported by the nicotinic receptors (Cantí *et al*., 1994; 1998; Ros *et al*., 2000; 2001a, b).

Recent studies have suggested that galantamine, another acetylcholinesterase inhibitor, has a beneficial effect on

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Alzheimer's patients (Dale *et al*., 2003) and improves learning deficits in rabbits (Woodruff-Pak *et al*., 2001). In this study, we investigated its effects on spontaneous synaptic transmission, on the *Torpedo* nicotinic acetylcholine receptor (AChR) and on the human α7 neuronal nicotinic acetylcholine receptor (nAChR).

Methods

Animals and solutions

Torpedo marmorata specimens were caught off the Catalan Mediterranean coast and kept in artificial seawater. The fish were anaesthetised with tricaine (3-aminobenzoic acid ethyl ester methanesulphonate salt) (Sigma, St Louis, MO, U.S.A.) at a concentration of 0.03% in seawater, before surgical excision of electric organs. 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, 100 mM sucrose and 6.8 mM HEPES/NaOH buffer, pH adjusted to 7.0 with $NaHCO_3$. The same solution was used to record spontaneous synaptic activity.

Mature *Xenopus laevis* females (purchased from the Centre d'Élevage des Xenopes, Montpellier, France) were anaesthetised by immersion in water containing 0.17% tricaine. A few lobes of 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 $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 2.40 mM $NaHCO_3$ and 20 mM HEPES at pH 7.5, supplemented with 100 IU ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin.

Recording solution: 115 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$ and 10 mM HEPES at pH 7.4. None of the *Xenopus* female donors used in this study exhibited muscarinic ACh receptors in their oocytes. The bath solution maintains calcium at a physiologically relevant concentration because extracellular calcium is an important modulator of neuronal nAChR function (Mulle *et al*., 1992; Vernino *et al*., 1992). Calcium also has indirect effects on agonist-evoked responses *via* calcium-dependent chloride channels at this concentration. However, calcium-dependent effects produce only linear amplification of peak responses and do not distort the concentration–responses relationships over a wide range of agonist concentrations (Papke *et al*., 1997).

Recording of spontaneous synaptic activity

All recordings were performed at room temperature (20–22°C). From five to 10 prisms of the electric organ were cut with a scalpel blade and 1–5 mm sections were incubated overnight in *Torpedo* saline solution containing galantamine, to ensure complete diffusion throughout the tissue. Fragments were fixed in a plexiglass chamber with a sylgard-coated base for measurement.

The spontaneous synaptic release of ACh was recorded with focal extracellular low-resistance microelectrodes (for details, see Dunant & Muller, 1986; Muller & Dunant, 1987), as described elsewhere (Cantí *et al*., 1994; Ros *et al*., 2000; 2001a). This method allows long-term recording with little damage to the cells. MEPPs were amplified (Axoclamp-2A, Axon Instruments, U.S.A.) and monitored on a Tektronix 5110 oscilloscope and on a PC-Computer with a LabView (National Instruments, U.S.A.) program (Quantadat) written in our laboratory, using an AT-MIO16X (National Instruments, U.S.A.) digitising interface. Signals were acquired at a frequency of 100 kHz and analysed with the same Labview program and the Whole Cell Analysis program (kindly provided by Professor J. Dempster, Strathclyde University, Scotland, U.K.) with a TL-1 Labmaster digitising interface. Data in ASCII format were exported to Sigmaplot 4.01.

The following parameters of each MEPP were measured: amplitude; rise time; rate or velocity of rising; the area corresponding to the charge that generated the MEPP, measured as the integral of the contour delimited by each one; and half-width, which indicates the rate of the decay phase (see Figure 4). Results were obtained from five separate experiments and represented in a bar histogram and cumulative plots (Van der Kloot, 1991), which compared all the variables under the different experimental conditions. The number of MEPPs analysed was 2741 for the control condition, 2501 for 1 μM galantamine and 1133 for 10 μM galantamine.

Expression of human α7 nAChR

A plasmid containing the full-length cDNA for human α7 nAChR was generously supplied by Professor Jon M. Lindstrom (Department of Neuroscience, University of Pennsylvania, U.S.A.). The plasmid (10 μg) was linearised with *Xba*I (Promega) and the resulting product was used for mRNA synthesis *in vitro* using the mCAP RNA Capping Kit (Stratagene). The capped mRNA obtained was injected (50 nl, 1.5 μg μl^{-1}) into oocytes. At 12–24 h after injection, the follicular cell layer was partially removed by incubation for 30 min with 0.25 mg ml^{-1} collagenase type 1A (Sigma). Oocytes were maintained at 15–16°C in sterile Barth's solution and recordings made 2–3 days later.

Transplantation of muscular ACh receptor to oocytes

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

Healthy oocytes were micro-injected with 50 nl of a thawed suspension (2–8 mg ml^{-1}) of electroplaque membranes (Marsal *et al*., 1995; Cantí *et al*., 1998; Ros *et al*., 2000) by means of a nanolitre injector (World Precision Instruments, WPI, model A203XVZ). Samples were sonicated prior to injection.

Oocyte recording

Oocytes were voltage-clamped with a two-electrode system (Axoclamp-2A, Axon Instruments, U.S.A.). Intracellular electrodes (1–4 MΩ resistance) were filled with 3 M potassium chloride. 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 Whole Cell Analysis program v. 2.1 after sampling signals by Lab PC+ (National Instruments, U.S.A.) at twice the filter frequency. In all recordings currents were elicited by challenges of ACh chloride at the indicated

concentrations, at a flow rate of 8 ml min^{-1}. Solutions were perfused by gravidity and flow was activated or stopped by electrovalves (ALA Scientific, U.S.A.). All the oocytes were tested for consistent response amplitudes with at least three challenges of ACh prior to the application of the drug. Galantamine was co-applied with ACh for 25 s at least twice and only those responses that were constant were used for calculations. Moreover, after application of the drug, a new challenge of ACh was perfused to test that the current was still constant. Usually, the washout time between two applications of ACh was 10–15 min, to avoid desensitisation.

Galantamine purification

Galantamine was isolated from wild *Narcissus confusus* by a combination of solid-phase extraction and high-performance liquid chromatography, as described elsewhere (López *et al*., 2002).

Calculations and statistics

Differences between distribution functions were evaluated with Sigmastat 3.2 software (SPSS Inc., U.S.A.) by the Mann–Whitney rank sum test. Values are expressed as mean ± s.e.m. calculated by the program.

Results

Effect of galantamine on the human α7 nChR

ACh (500 μM) induced an inward current in *Xenopus* oocytes expressing human α7 AChRs with a mean amplitude of 0.84 ± 0.08 μA, ($n = 9$; Figure 1a). The current was transient, with a decay time constant (τ) of 1.1 ± 0.1 s. Higher concentrations of ACh did not cause any increase in current amplitude, but did increase the desensitisation of the receptor, which needed a longer period of washing before reproducing constantly the ACh response.

Co-application of 100 nM galantamine increased the amplitude of ACh-induced currents by 9.6 ± 2.7%, $n = 9$ (Figure 1b and c); concentrations above or below this level had no significant effect. The time constant of current decay (τ) with 100 nM galantamine was 0.9 ± 0.1 s. The concentration–response relationships for ACh in activating α7 nAChRs in the absence and presence of galantamine is shown in Figure 1d. In the presence of 100 nM galantamine, the EC_{50} was shifted from 305 to 189 μM, $n = 9$. The maximum effect of galantamine, a 22% increase in the amplitude of ACh-induced currents, was observed at a concentration of 250 μM ACh.

Effect of galantamine on Torpedo *AChR*

In oocytes transplanted with *Torpedo* nicotinic receptors, a kind of embryonic muscular AChR, 100 μM ACh triggered an inward current of 0.27 ± 0.12 μA ($n = 6$) that decayed with a time constant (τ) of 4.3 ± 1.7 s (Figure 2a). Galantamine also increased the response to ACh, but only at a concentration of 1 μM (0.33 ± 0.02 μA, $n = 6$; Figure 2b and c). We also explored the concentration–response relationship for ACh. The curve obtained in the presence of galantamine 1 μM was shifted to the left (Figure 2d) and the EC_{50} moved from 79 to 46 μM, $n = 6$. The maximum effect of galantamine, a 35% increase in the amplitude of currents, occurred at a concentration of 50 μM ACh.

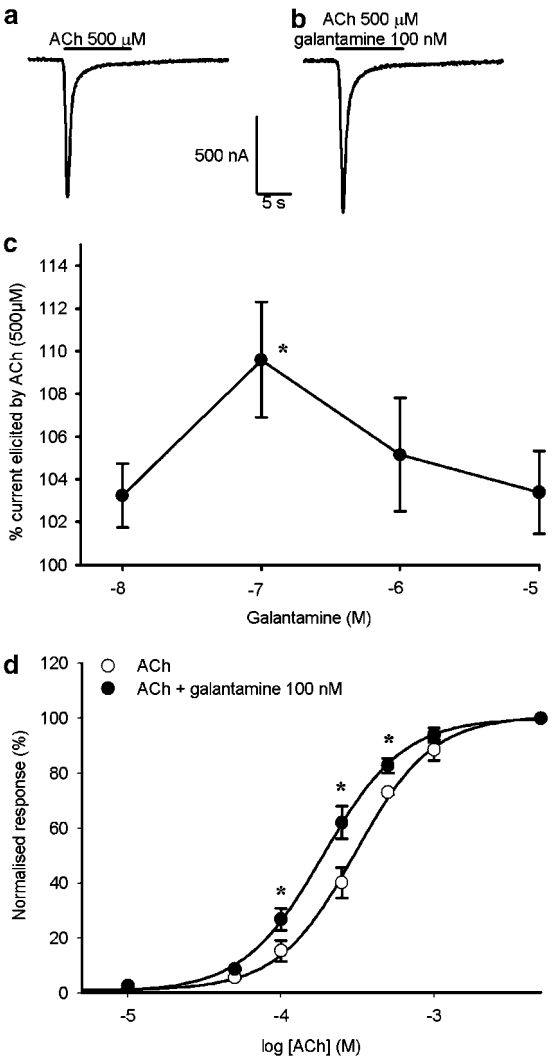

Figure 1 Effect of galantamine on ACh-induced currents supported by α7 AChRs expressed in *Xenopus* oocytes. (a) Fast inactivating inward currents were stimulated by ACh; holding potential was −70 mV. (b, c) Galantamine at very low concentration (100 nM) induced a very small but significant increase in the amplitude of ACh-activated currents. Higher concentrations did not cause a significant increase in currents. (d) Dose–response relationship for ACh obtained in the absence and presence of 100 nM galantamine. The averaged amplitudes (expressed as mean ± s.e.) of the currents recorded from 10 oocytes from different donors were plotted *versus* the respective concentration of ACh applied. *$P < 0.05$.

Effect of galantamine on spontaneous synaptic activity of Torpedo *electric organ*

Figure 3a–c shows superimposed traces of MEPPs in *Torpedo* electric organ electroplates, under resting conditions and after the application of 1 and 10 μM galantamine. In fragments incubated with 10 μM galantamine, the decay phase was prolonged, as expected with anticholinesterasic agents. The effect of galantamine on MEPP frequency is shown in Figure 3d. The mean values were 1.58 ± 0.31 MEPPs s^{-1} for

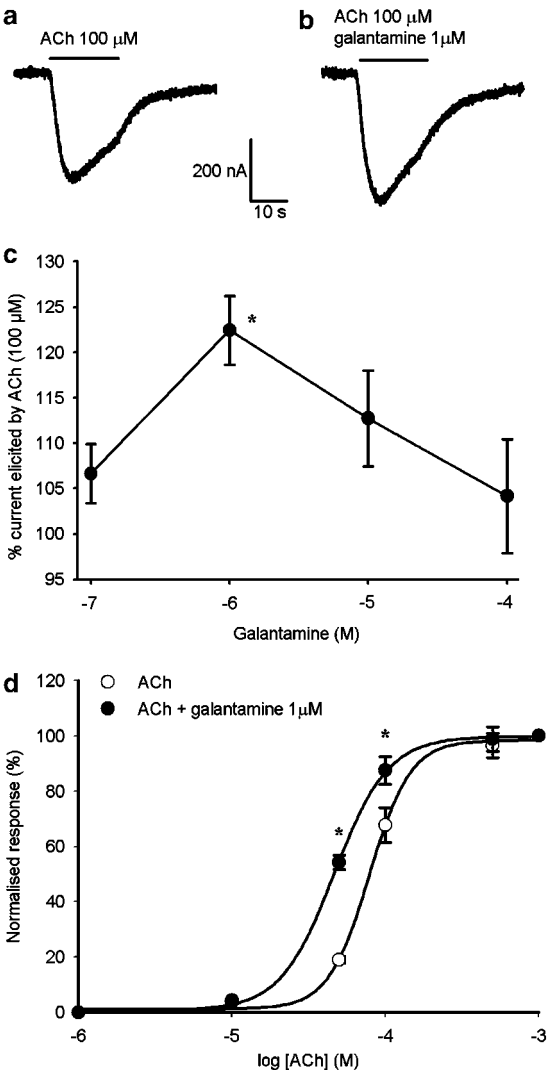

Figure 2 Effect of galantamine on ACh-induced currents supported by *Torpedo* AChRs from the electric organ. Membranes of the electric organ were transplanted to *Xenopus* oocytes. (a) Fast inactivating inward currents were stimulated by ACh; holding potential was −70 mV. (b, c) Galantamine at low concentration (1 μM) induced a significant increase in the amplitude of ACh-activated currents. Higher concentrations did not cause a significant increase in currents. (d) Dose–response relationship for ACh obtained in the absence and presence of 1 μM galantamine. The averaged amplitudes (expressed as mean ± s.e.) of the currents recorded from eight oocytes from different donors were plotted *versus* the respective concentration of ACh applied. *$P<0.05$.

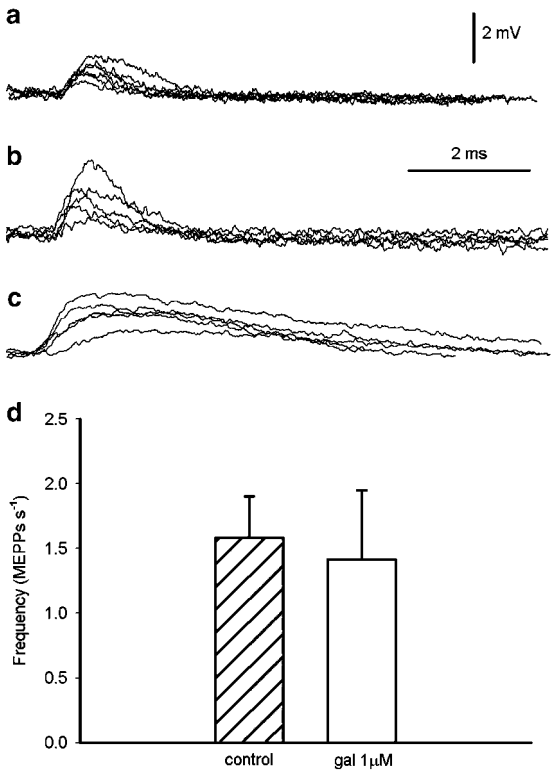

Figure 3 Effect of galantamine on spontaneous cholinergic synaptic activity of *Torpedo* electric organ. Low-resistance pipettes placed extracellularly were used for the focal recording of miniature endplate currents. Oscilloscope traces of spontaneous MEPPs were superimposed. (a) Untreated fragments. (b) 1 μM galantamine. (c) 10 μM Galantamine. The inhibition of acetylcholinesterase activity is translated into synaptic activity recording by the prolongation of the decay phase of spontaneous events, which was only observed when high concentrations of galantamine were used. (d) Effect of galantamine treatment on MEPP frequency.

untreated fragments and 1.41 ± 0.53 MEPPs s^{-1} for fragments incubated with 1 μM galantamine, $n = 12$. Galantamine caused no significant effect on MEPP frequency of the *Torpedo* electric organ.

The analysis of the traces is shown as cumulative plots and bar histograms in Figure 4. The area below the profile of each MEPP, reflecting the electrical charge carried during the release of a single quantum of Ach, was measured. The mean values were as follows: controls, 0.45 ± 0.006 mV ms^{-1}; fragments treated with 1 μM galantamine, 0.83 ± 0.02 mV ms^{-1}; fragments treated with 10 μM galantamine, 2.76 ± 0.09 mV ms^{-1} ($P<0.05$).

Under control conditions, the amplitude of MEPPs was 0.54 ± 0.004 mV. In fragments treated with 1 μM galantamine, the amplitude was increased to 0.83 ± 0.01 mV, $P<0.05$. When the concentration of galantamine was increased to 10 μM, the amplitude of MEPPs was 0.92 ± 0.01, $P<0.05$.

The rise time of a MEPP is the result of the addition of time periods from different cellular processes: the release and diffusion of ACh through the synaptic cleft and the opening time of the AChRs. The rise time was 0.48 ± 0.005 ms in the untreated fragments, 0.48 ± 0.05 ms in 1 μM galantamine-treated fragments and 0.8 ± 0.02 ms in those fragments treated with 10 μM galantamine. In this latter case, $P<0.05$.

The rate of rise between 10 and 90% of the MEPP amplitude was 1.14 ± 0.01 mV ms^{-1} in untreated fragments, 1.62 ± 0.02 in 1 μM-treated fragments ($P<0.05$) and 1.3 ± 0.08 in 10 μM-treated fragments.

The duration of width of an MEPP depends on the single or repetitive interaction of molecules of ACh with the AChR. The persistence of ACh molecules in the cleft, as a consequence of the inhibition of acetylcholinesterases, extends the decay phase of an MEPP. Since the decay phase of an MEPP fits an exponential function, it is therefore difficult to establish the final single quantal event. To compare the duration of quantal events in different pharmacological treatments, width at 50% of the amplitude was used. The half-width was 0.6 ± 0.006 ms in untreated fragments and 0.7 ± 0.008 ms in fragments treated with 1 μM galantamine, whereas it was 2.01 ± 0.04 ms in 10 μM-treated fragments ($P<0.05$).

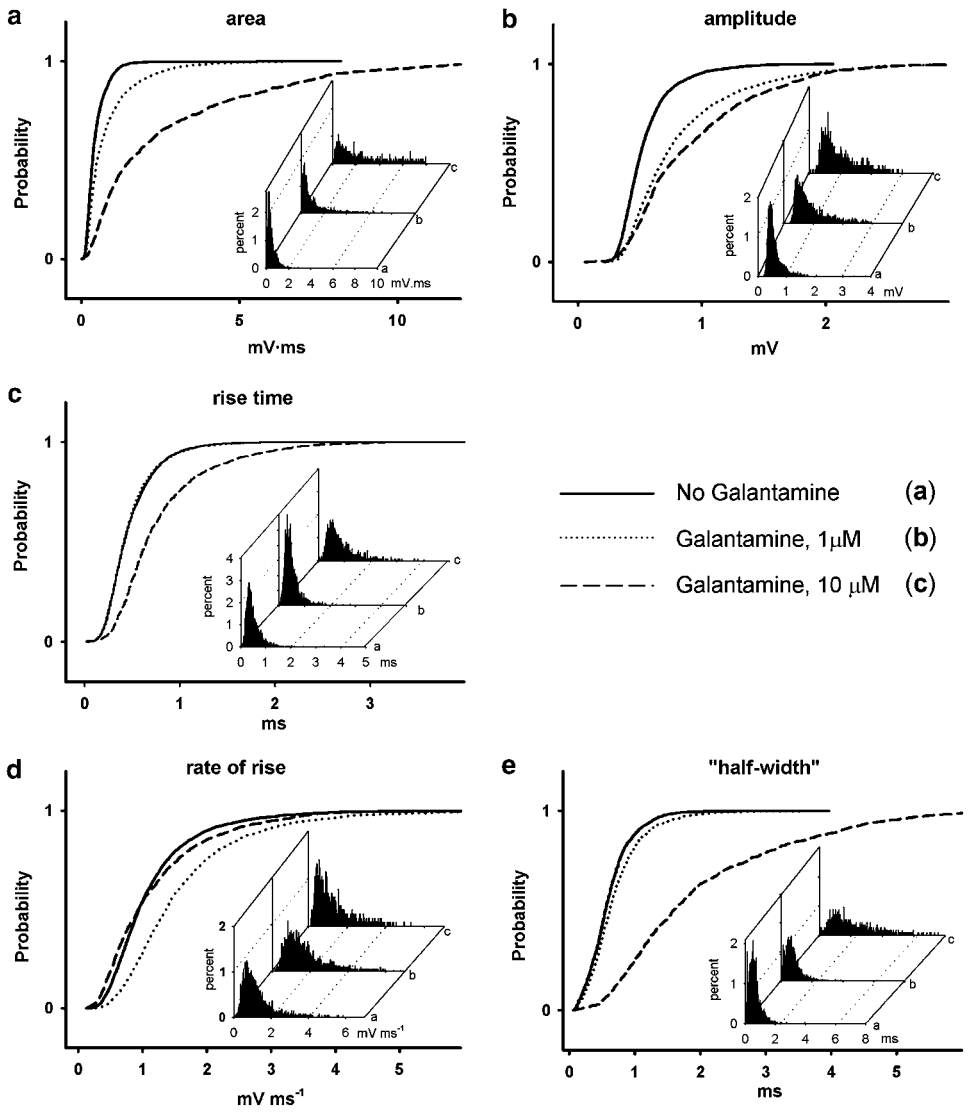

Figure 4 Analysis of MEPPs in 1 and 10 μM galantamine-treated fragments of *Torpedo* electric organ. Data are presented as cumulative plots and bar histograms (inset). (a) Electrical charge mobilised by spontaneous MEPP: effect of galantamine on electrical charge mobilised by spontaneous ACh release. The area delimited below a MEPP contour corresponds to the total electrical charge passed through the nicotinic ACh receptors as a consequence of spontaneous quanta. (b) Effect of galantamine on the amplitude of MEPP (peak). (c) Rise time of MEPP. (d) Rate of rise time of MEPP. (e) half-width of MEPP, width at 50% of amplitude. Galantamine (10 μM) prolonged the decay phase of MEPPs. The number of MEPPs analysed for control conditions was 2741, 2501 for 1 μM galantamine and 1133 for 10 μM galantamine. The data shown came from three experiments.

Discussion

Since the cholinergic hypothesis for the onset of Alzheimer's disease (Davies & Maloney, 1976; Bartus *et al*., 1982; Coyle *et al*., 1983), various efforts have been made to increase the cerebral levels of ACh. The most obvious method was to use anticholinesterasic agents, but the side effects of these were very severe. Only some drugs were well tolerated by a small group of patients. In these cases, however, the cognitive deficiencies associated with the illness were delayed. It is likely that these side effects are related to the nonspecific interaction of anticholinesterasic agents with other membrane receptors and ion channels.

We tested the effect of various anticholinesterasic agents on the ionic currents conducted by *Torpedo* AChRs in earlier research (Cantí *et al*., 1998; Ros *et al*., 2000; 2001a, b). There is a close correlation between the potency of inhibitory action of the anticholinesterasic agent and the degree of inhibition of muscular nicotinic currents.

Galantamine has been described as an anticholinesterasic agent, but it increases $\alpha4\beta2$ AChR-activated currents at concentrations between 10^{-7} and 10^{-6} M (Maelicke *et al*., 2001), which are far from the IC_{50} (30 μM) for human brain acetylcholinesterase activity (Thomsen *et al*., 1991). Nonetheless, galantamine at very low concentrations (10^{-7} M) increased the currents activated by the human $\alpha7$ neuronal nicotinic receptor. The galantamine-induced potentiation effect has been described in human $\alpha4\beta2$ nAChR (Maelicke *et al*., 2001), human $\alpha3\beta4$ and $\alpha6\beta4$ nAChR (Samochocki *et al*., 2003) as an allosteric potentiation. With single-channel recording configuration, galantamine alone activated the opening of the nAChR channel by acting as a weak

noncompetitive agonist in rat hippocampal cultured neurons (Pereira *et al*., 1993) and mouse transfected fibroblasts (Pereira *et al*., 1994). Similar results were obtained in frog skeletal muscle AChR with physostigmine at a concentration of 0.5 μM: this effect correlated with an increase of nerve-elicited endplate currents (Shaw *et al*., 1985). Physostigmine and galantamine bind on a specific site of the α subunit, which is recognised by the monoclonal antibody FK1 (Pereira *et al*., 1994).

However, we found no ionic current activated by galantamine alone in *Xenopus* oocytes expressing α7 nAChRs, perhaps because it sank into the amplifier noise. The concentration–response relationships for ACh, in the presence of galantamine, gave us direct evidence that galantamine was effective in enhancing the current amplitude at a range between 10^{-5} and 10^{-3} M ACh, which is the same range of concentrations previously described in $\alpha4\beta2$ receptors (Maelicke *et al*., 2001) that also increased ACh-induced currents. The fact that we obtained significant results using high concentrations of ACh may reflect a physiological condition, because these concentrations of ACh may be closer to the concentration actually reached in the synaptic cleft.

The allosteric potentiating effect was specific for nAChR because galantamine did not enhance the cholinergic response mediated by muscarinic ACh receptors (Samochocki *et al*., 2003). However, *Torpedo* nicotinic receptors (an embryonic receptor) were also sensitive to the effect of galantamine and, as shown in the present study, galantamine enhanced the currents mediated by ACh. However, the galantamine concentration needed for this enhancement was higher in the case of muscular receptor than in the α7 receptor. Galantamine is also an allosteric potentiatior of muscular nicotinic receptors and should act as described in adult frog neuromuscular junction (Shaw *et al*., 1985).

Both our results and those of others suggest that galantamine acts specifically on all AChRs, because it enhances cholinergic synaptic transmission in hippocampal slices (Santos *et al*., 2002). α7/5-HT3 chimeras of serotonin-ACh receptors are also sensitive to galantamine (Samochocki *et al*., 2003). Probably, a conserved region in the different types of alpha subunit is related to activation activity (Pereira *et al*., 1993). It is also possible that galantamine acts like lynx, an endogenous protein that enhances the currents activated by ACh in nicotinic receptors expressed in *Xenopus* oocytes (Miwa *et al*., 1999). Moreover, it has been described that galantamine enhances nicotine-induced catecholamine release from mouse striatal brain slices (Zhang *et al*., 2004) and from the hippocampus of freely moving rats (Sharp *et al*., 2004).

We also explored the effect of galantamine on spontaneous synaptic activity. Galantamine did not change the frequency of MEPPs. Each MEPP is the result of the amount of ACh released during synaptic vesicle exocytosis, its diffusion, its interaction with the AChR and the enzymatic activity of the acetylcholinesterases present in the synaptic cleft. Inhibition of acetylcholinesterases increases the amplitude and time course of a single miniature endplate potential. Our results clearly distinguish the effects related to anticholinesterasic activity (10 μM galantamine) from other more subtle effects observed at low galantamine concentration (1 μM). Galantamine at a concentration of 10 μM increased the amplitude, area, half-width and rise time of MEPPs; all of these effects are the consequence of anticholinesterasic activity. However, at concentrations with an increase in muscular nicotinic response (1 μM), we observed a significant increase in the amplitude, but no significant increase in the half-width of the miniature population. Apparently, galantamine increased the quantal size of individual spontaneous events. In principle, this could be due to an increase in the rate of ACh transport into the synaptic vesicle, an increase in the rate of synthesis or an increase in the rate of precursor transport. In other experimental conditions, such as IP_3 mobilisation (Brailoiu & Miyamoto, 2000) or the action of frog urotensin (Brailoiu *et al*., 2003), an increase of quantal size has been reported. There are no reports in the literature of galantamine effects on presynaptic release machinery. It was recently suggested that exocytosis of neurotransmitters may be due to a very rapid and transient fusion of synaptic vesicles in the hippocampus (Aravanis *et al*., 2003; Gandhi & Stevens, 2003). Galantamine may prolong the time during which the fusion pore connects the interior of the vesicle with the extracellular milieu. The effect of galantamine on quantal size may be due to the direct activation of nicotinic receptors (Maelicke *et al*., 1997). Finally, we cannot rule out the possibility that galantamine activates all of these mechanisms.

In conclusion, our research demonstrates that galantamine has beneficial potentiation effects on nicotinic receptors only over a very narrow range of concentrations. Concentrations that approach IC_{50} as an anticholinesterasic agent may increase the side effects in Alzheimer's disease patients. The low concentrations that enhance the nicotinic currents also increase the size of quanta, contributing to an increase in the levels of Ach, which may be related to an improvement in the daily life of patients. Our results reinforce the view that unconventional ligands on nAChR may benefit patients with impaired nAChR function (Pereira *et al*., 2002).

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