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Inhibition of human α7 nicotinic acetylcholine receptors by open channel blockers of *N*-methyl-D-aspartate receptors

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1 Human α7 nicotinic acetylcholine (ACh) receptors were expressed in *Xenopus* oocytes and the effects of the *N*-methyl-D-aspartate (NMDA) receptor open channel blockers memantine and cerestat on this receptor were examined using two-electrode voltage-clamp recordings and ^{125}I-α-bungarotoxin (^{125}I-α-bgtx) binding.

2 Memantine and cerestat produced complete inhibition of ACh-induced inward currents with affinities similar to that reported for native NMDA receptors. Cerestat, IC_{50} 1.7 (−1; +2) μM, was more potent than memantine, IC_{50} 5 (−3; +8) μM, and the effects of both drugs were fully and rapidly reversible.

3 Inhibition of α7 receptor function was voltage-independent, and it occurred at concentrations far lower than those needed to inhibit (never completely) binding of ^{125}I-α-bgtx to α7 receptors, suggesting that the effects of memantine or cerestat are noncompetitive.

4 These results provide evidence that human α7 receptors are inhibited by memantine and cerestat and suggest that caution should be applied when using these compounds to study systems in which NMDA and nACh receptors co-exist.
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Abbreviations: ACh, Acetylcholine; ^{125}I-α-bgtx, ^{125}I-α-bungarotoxin; NMDA, *N*-methyl-D-aspartate

Introduction

Brain nicotinic acetylcholine (ACh) receptors are considered as novel therapeutic targets in Alzheimer's and Parkinson's diseases, schizophrenia, Tourette's syndrome, attention deficit syndrome, and are also targets for analgesia, anxiolysis, neuroprotection and smoking cessation (Gotti *et al*., 1997). Nicotinic ACh receptors belong to the four-transmembrane superfamily of ligand-gated ion channels that also include the γ-aminobutyric acid type A ($GABA_A$), glycine and 5-hydroxytryptamine-3 ($5\text{-}HT_3$) receptors (Lukas *et al*., 1999). Nicotinic ACh receptors in the nervous system are assembled from at least nine subunits (α2–α10; β2–β4), which gives rise to a variety of receptors that differ with respect to their pharmacology, biophysics and localisation (Le Novère *et al*., (2002)). In native systems, the α7 and α4β2 receptors have been identified as the major nAChR subtypes in the central nervous system. However, more complex subunit combinations have been proposed (e.g., α6α5α4β2; Klink *et al*., 2001), and there is evidence that α5α4β2 (Conroy & Berg, 1998) and α6α4β2 assemble in some neurones (Zoli *et al*., 2002; Champtiaux *et al*., 2003).

Numerous studies have shown that neuronal nicotinic ACh receptors are affected by a variety of noncholinergic drugs with biological and/or clinical relevance at concentrations at which they act on their classical targets. For example, nicotinic ACh receptors are inhibited by L-type Ca^{2+} channel ligands (Lopéz *et al*., 1993; Herrero *et al*., 1999; Houlihan *et al*., 2000; Slater *et al*., 2002), antidepressants such as fluoxetine (Fryer & Lukas, 1999) and tricyclic antidepressants (Connolly *et al*., 1992; Rana *et al*., 1993), and 5-HT3 receptor ligand antiemetics such as tropisetron (Barik *et al*., 2002). These findings raise the possibility that nicotinic ACh receptors may play roles hitherto unsuspected in physiological processes as well as in drug therapy of the nervous system.

To further examine the sensitivity of nicotinic ACh receptors to therapeutic drugs considered as noncholinergic, we have investigated the effects of memantine and cerestat on human α7 receptors. The effects of cerestat on nicotinic ACh receptors have not been investigated previously, but there is evidence that memantine acts as open channel blocker at human α4β2 (Buisson & Bertrand, 1998) and rat α10/α9 (Oliver *et al*., 2001) nicotinic ACh receptors. Memantine is a potent open channel blocker of *N*-methyl-D-aspartate (NMDA) receptors and was developed by Merz in Europe for treatment of neuropathic pain and symptoms of dementia and Alzheimer's disease such as cognitive impairment and neuronal cell death (Parsons *et al*., 1999b). Cerestat (Aptiganel, CNS 1102), another open channel blocker of NMDA receptors, was developed by Cambridge Neuroscience as potential neuroprotective agent in the treatment of ischaemic stroke, but phase III clinical trials have been suspended due to poor therapeutic ratios and harmful side effects such as hypertension, ventricular arrhythmia, somnolence, stupor and cerebral oedema (Albers *et al*., 2001). In this study, we report that at clinically relevant

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concentrations, both memantine and cerestat inhibit the function of human α7 receptors in a noncompetitive and voltage-independent manner. Our findings suggest that caution should be applied when using these compounds to study systems in which NMDA and α7 receptors coexist.

Methods

Cloning of human α7 subunit cDNA

The cDNA encoding the human α7 subunit was obtained by PCR amplification of cDNA synthesised from polyA$^+$RNA of human SHSY5Y cell line using primers (forward: GCG TCT AGA ATG CGC TGC TCG CCG GGA GGC G; reverse: CGC GGA TCC TTA CGC AAA GTC TTT GGA), which were designed on the basis of published sequences (Groot-Kormelink and Luyten, 1997). DNA fragments of the expected size ($\sim$1.5 kb) were cloned into the *BamH*I and *Xba*I sites of the expression vector pcDNA3.1 Hygro- (Invitrogen). The putative human α7 was verified by sequencing to have a 100% identical amino-acid sequence to that of previously published human α7 sequences (accession number: Y08420, U62436).

Oocyte preparation and cDNA expression

Xenopus laevis oocytes at developmental stages V and VI were dissected out from adult *X laevis* ovaries using protocols detailed elsewhere (Houlihan *et al*., 2000; 2001). Expression of functional human α7 nicotinic ACh receptors was achieved by microinjecting into the nucleus of oocytes 18.3 nl of pCDNA3.1hα7 (1 μg μl^{-1}. Oocytes were maintained at 20°C for up to a week in Barth's solution containing (in mM): 88 NaCl, 1 KCl, 0.41 $CaCl_2$, 0.82 $MgSO_4$, 0.33 $Ca(NO_3)_2$, 2.5 $NaHCO_3$, 0.5 theophylline, 10 HEPES, pH 7.2, supplemented with 0.1 mg l^{-1} gentamicin sulphate, 0.01 mg ml^{-1} streptomycin sulphate and 0.01 mg ml^{-1} penicillin-G.

Electrophysiological recordings

Recordings were made 2–3 days following injection of cDNA. Oocytes were placed in a 100 μl bath that was gravity perfused continuously at 4 ml min^{-1} with Ba^{2+}-Ringer solution (in mM: 115 NaCl, 2.5 KCl, 10 Hepes, 1.8 $BaCl_2$, pH 7.2). This solution ($CaCl_2$ in conventional Ringer solution being replaced by $BaCl_2$) was used to record from oocytes in order to minimise the activation of Ca^{2+}-dependent chloride currents. Ba^{2+}-Ringer does not affect the pharmacology of nicotinic ACh receptors expressed in *Xenopus* oocytes (Houlihan *et al*., 2000; 2001). Oocytes were voltage-clamped at −70 mV unless otherwise mentioned, and whole-cell currents were measured as described before (Houlihan *et al*., 2000; 2001). Drugs were applied by gravity perfusion using a manually activated valve. Concentration–response curves for ACh were constructed by normalising to the maximal response of ACh (1 mM) and used to generate EC_{50} and Hill coefficient (n_H) estimates. The EC_{50} concentration was the concentration of agonist that produced 50% of the maximal agonist response. To construct antagonist concentration-inhibition curves, the responses elicited by the co-application of EC_{50} ACh concentration (100 μM; Figure 1a) and increasing concentrations of drugs were normalised to the responses evoked by ACh alone. Oocytes were first perfused

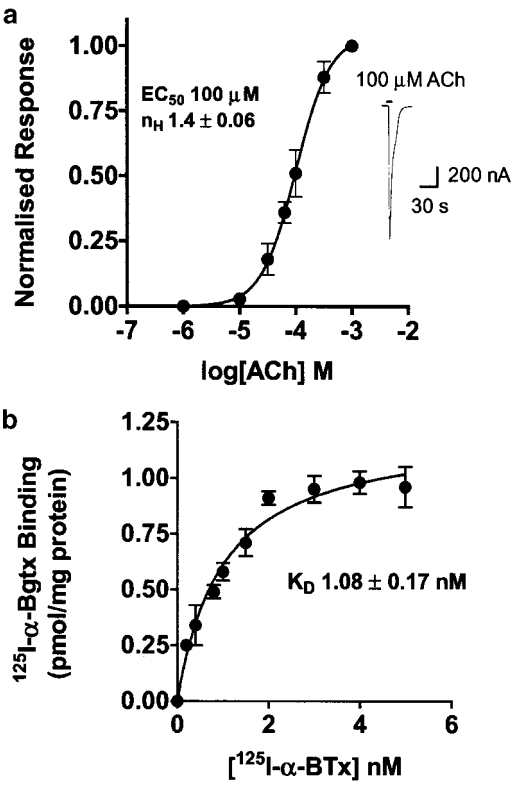

Figure 1 Pharmacological properties of human α7 receptors expressed in *Xenopus* oocytes. (a) Concentration–response curves were fitted using a modified Hill equation shown in Methods. Each point represents responses from 8–10 oocytes clamped at −70 mV. Peak currents responses for each concentration were determined. Inset: Representative trace of currents activated by application of ACh onto oocytes injected with pcDNA3.1hα7 plasmid. Oocytes were exposed to ACh for less than 10 s while continuously perfusing the recording chamber with Ba^{2+}-Ringer saline. (b) Saturation analysis of the specific binding of ^{125}I-α-bgtx to hα7 present in membrane homogenates derived from oocytes injected with pcDNA3.1hα7 plasmid. Data points are the means of triplicate samples.

with antagonists for 2 min and then challenged with a simultaneous application of ACh and antagonist, unless otherwise mentioned. At least 3 min wash time was allowed between each drug application to allow clearance of the drug and to prevent receptor desensitisation.

^{125}I-α-Bungarotoxin binding

^{125}I-α-bungarotoxin (^{125}I-α-bgtx) binding to hα7 nicotinic ACh receptors was assayed utilising membrane homogenates prepared from oocytes injected with pcDNA3.1hα7. Oocytes (100 – 150 oocytes per assay) were rinsed with ice-cold phosphate-buffered saline, mechanically disaggregated and homogenised using a Polytron homogeniser for 10 s. The homogenates were centrifuged at 40,000 × *g* at 4°C for 20 min, and the pellets resuspended in ice-cold saline to give a final protein concentration in the assay tubes of approximately 10–30 μg. The binding saline used consisted of (in mM) 140 NaCl, 1 EDTA and 50 Tris-HCl at pH 7.4. ^{125}I-α-bgtx binding was carried out as described previously (Houlihan *et al*., 2000). Briefly, incubations were carried out in a final volume of 250 μl in 1 nM ^{125}I-α-bgtx and cell membrane homogenate for 60 min at room temperature (25°C) in the presence or absence of

unlabelled drugs. The same solution and total cell membrane protein were used in saturation binding studies, but the concentration of ^{125}I-α-bgtx varied from 0.2 to 5 nM. Non-specific binding was defined using 10 μM nicotine. Incubations were terminated by vacuum filtration over GF/C glass fibre filters pre-soaked in 0.1% polyethyleneimine, and filters were washed twice with 3 ml of ice-cold binding saline. Bound radioactivity was measured using a Beta-counter.

Drugs

ACh and memantine were purchased from Sigma (U.K.) and solutions were made in Ba^{2+}-Ringer's saline. Cerestat was prepared as memantine and was supplied by Vernalis Research, Ltd.

Data analysis

Dose–response data for agonists and antagonists were fit by nonlinear regression (Prism 3.01,. GraphPad, U.S.A.) to the following equations: (a) $i=i_{max}/[1+(EC_{50}/x)^{n}H]$, or (b) $i=i_{max}/[1+(IC_{50}/x)^{n}H]$, wherein i_{max} is the maximal normalised current response (in the absence of antagonist for inhibitory currents), x the agonist or antagonist concentration, EC_{50} the concentration of agonist eliciting a half-maximal response, IC_{50} the antagonist concentration eliciting half-maximal inhibition and n_H the Hill coefficient.

The binding parameters K_d and B_{max} of ^{125}I-α-bgtx binding were determined from saturation binding isotherm data using the equation $Y=(B_{max}\times X)/(X+K_d)$, wherein B_{max} is the maximal binding, K_d the apparent equilibrium dissociation binding constant, X the concentration of radioligand and Y the specific binding.

EC_{50} or IC_{50} values are presented as geometric means (± standard error of the mean (−SEM, +SEM). B_{max}, K_d and n_H values are expressed as arithmetic means ± s.e.m. Results were derived from at least three independent experiments carried out using different batches of oocytes. The statistical significance of differences between mean values was assessed by Student's *t*-tests for unpaired or paired data or one-way analysis of variance (ANOVA), wherever appropriate. Values of $P<0.05$ were considered significant.

Results

Previous studies have shown that memantine, a classic open channel blocker of NMDA receptors, also behaves as an open channel blocker at human α4β2 (Buisson & Bertrand, 1998) and rat α10/α9 (Oliver *et al*., 2001) nicotinic ACh receptors. In order to determine whether this drug also blocks other nicotinic ACh receptor subtypes, we expressed in *Xenopus* oocytes the human α7 subunit, which forms functional homopentameric receptors. Concentration–response curves for activation of human α7 nicotinic ACh receptors by ACh established that a concentration of 100 (−95; +109) μM was the EC_{50} value (Figuere 1a), which is comparable to previously published data (Peng *et al*., 1994; Houlihan *et al*., 2000; 2001). Saturation binding studies confirmed that oocytes injected nuclearly with pcDNA3.1hα7 expressed α7 nicotinic ACh receptors. Figure 1b shows that ^{125}I-α- bgtx bound in a specific and a saturable manner to membranes from oocytes injected with pcDNA3.1-α7. Analysis of binding data indicated a K_d of 1.08 ± 0.17 nM, which is comparable to K_d values previously published for this receptor subtype (Peng *et al*., 1994; 1999; Houlihan *et al*., 2000; 2001).

Memantine applied on its own had no effects on oocytes expressing human α7 nicotinic ACh receptors, even at concentrations as high as 1 mM (not shown). In contrast, memantine fully inhibited the ACh EC_{50} responses of α7 receptors in a reversible and dose-dependent manner (Figure 2). The IC_{50} and n_H values estimated from the concentration-inhibition curve were 5 (−3; +8) μM and 1.1 ± 0.2, respectively. Cerestat, another open channel blocker of NMDA receptors (Minematsu *et al*., 1993), also blocked currents activated by EC_{50} ACh responses in oocytes expressing human α7 receptors in a reversible and dose-dependent manner (Figure 2). The IC_{50} and n_H values estimated from the cerestat concentration-inhibition curve were 1.7 (−1; +2) μM and 1.1 ± 0.16, respectively. Cerestat, like memantine, appeared to have no direct effects on membrane currents.

ACh concentration–response profiles were obtained on oocytes expressing human α7 nicotinic ACh receptors either alone or in the presence of memantine or cerestat at concentrations near their respective IC_{50} values to illuminate mechanisms of functional inhibition. Figure 3a shows that memantine (3 μM) reduced the maximal ACh response by 54 ± 6%, whilst cerestat (1 μM) decreased the maximal current by 48 ± 5%. The ACh EC_{50} (103 μM) derived from fitting the data to the Hill equation did not change significantly in the presence of memantine (107 μM) or cerestat (108 μM), and fractional blockade was similar at all concentrations of drugs tested (Figure 3a, inset). In both cases, n_H was 1.5 ± 0.3 and 1.2 ± 0.2, respectively, which is not significantly different from that of the control ACh curve (1.5 ± 0.1). To define more precisely the mechanism of action of cerestat or memantine, we examined the effects of these drugs on ^{125}I-α-bgtx binding to membrane homogenates derived from oocytes expressing human α7 receptors. Figure 3b inset shows that cerestat or memantine caused a partial inhibition of specific ^{125}I-α-bgtx binding, but only at concentrations that induced more than 80 – 100% of functional blockade of α7 receptors. Inhibition was dose-dependent (memantine IC_{50} 71 (−52; +95) μM; cerestat IC_{50} 55 (−38; +86) μM) giving a maximum inhibition of about 24% (memantine) and 46% (cerestat) and caused no further inhibition at concentrations greater than 1 mM. Furthermore, as shown in Figure 3b, 300 μM memantine or cerestat had no significant effect on the apparent K_d of ^{125}I-α-bgtx binding (control 1.08 ± 0.17 nM; + memantine 1.2 ± 0.04; + cerestat 1.1 ± 0.04; $n=3$) but significantly reduced B_{max} from 1.2 ± 0.07 to 0.81 ± 0.05 and 0.46 ± 0.03 pmol (mg protein)$^{-1}$ in the presence of memantine or cerestat, respectively ($n=3$; $P<0.05$, unpaired Student's *t*-test). Taken together, these studies suggest that both functional inhibition of and binding by memantine or cerestat occur *via* a non-competitive mechanism.

Voltage-dependent inhibition of ligand-gated ion channels suggests binding of the inhibitory drug to a site within or close to the mouth of the channel. To determine whether the inhibition of α7 receptors by either memantine or cerestat is voltage-dependent, we analysed the ACh current–voltage relationship in oocytes expressing α7 receptors in the presence and absence of 3 μM memantine or 1 μM cerestat. As shown in Figure 4a, the current-voltage relationship for ACh currents

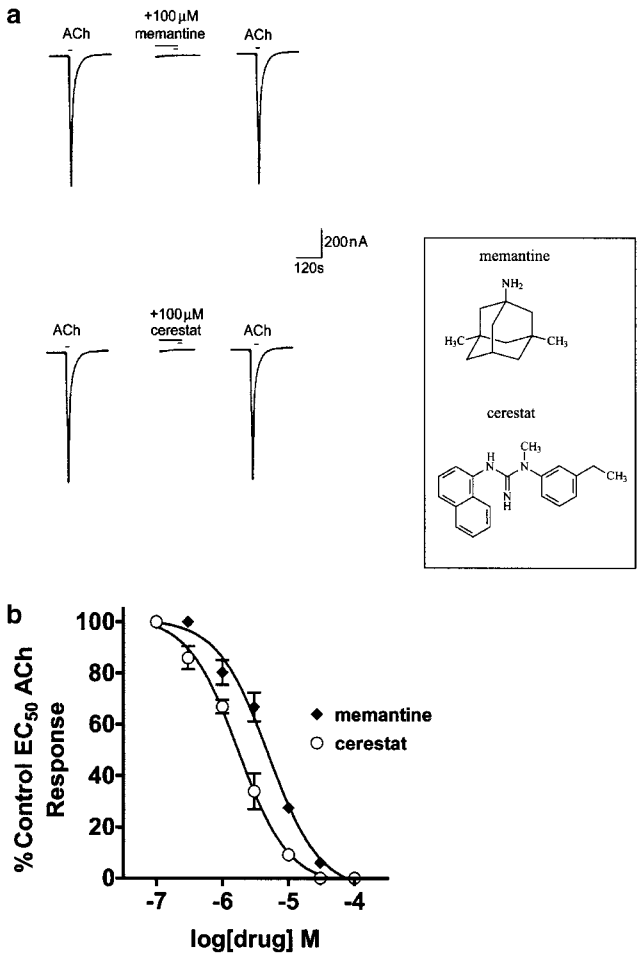

Figure 2 Effects of memantine or cerestat on human α7 nicotinic ACh receptors heterologously expressed in *Xenopus* oocytes. (a) Oocytes expressing human α7 receptors were voltage-clamped at −70 mV and exposed to EC_{50} concentration of ACh (100 μM), followed by test drug (memantine or cerestat) and then test drug and ACh, as indicated by the bars above each trace. (b) Comparison of inhibition of hα7 nicotinic ACh receptors by memantine or cerestat. Oocytes were preincubated for 2 min in cerestat or memantine, respectively, prior to simultaneous application of memantine or cerestat and EC_{50} concentration of ACh. Experimental data were averaged from 5 to 7 experiments carried out in different batches of oocytes. Data were normalised to control EC_{50} ACh responses and the curves were obtained fitting data to the inhibitory Hill equation described in Methods.

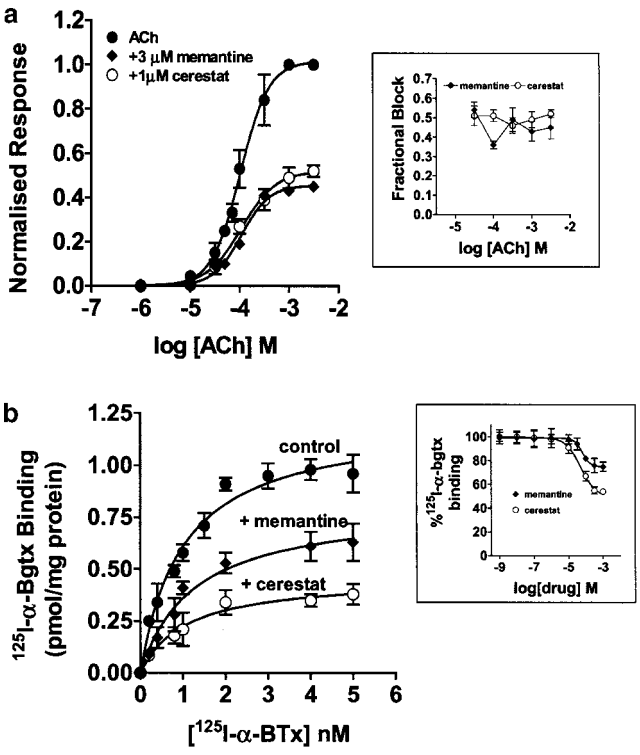

Figure 3 Effect of memantine or cerestat on the concentration–response curve of ACh at human α7 nicotinic ACh receptors. (a) Concentration-response curve for ACh responses in the absence or presence of either 3 μM memantine or 1 μM cerestat. ACh concentration–response curves were constructed as control ($n=3$) and in the presence of either 3 μM ($n=3$) memantine or 1 μM ($n=4$) cerestat. Oocytes were first exposed to ACh and then to test drug for 2 min and then to test drug and ACh. Data were normalised to responses elicited by 1 mM ACh (maximal ACh response). The inset shows fractional blockade at various ACh concentrations (note that inhibition increases with increasing concentrations of drug but then reaches a plateau). (b) Saturation analysis of the specific binding of ^{125}I-α-bgtx to human α7 receptors in the absence and presence of 300 μM memantine or cerestat. Data points are the means of three independent experiments carried out in triplicate. The inset shows the effect of memantine or cerestat on equilibrium binding of ^{125}I-α-bgtx to α7 receptors. Both memantine and cerestat displaced ^{125}I-α-bgtx binding to human α7 receptors partially but only at concentrations that produced full functional inhibition of the receptors. Data are the mean ± s.e.m. of fivc experiments.

mediated by α7 receptors had a similar inward rectification in the absence or presence of memantine or cerestat, and the percentage of inhibition by either of these drugs was equivalent at all negative holding potentials (Figure 4b). These results, therefore, show that inhibition of human α7 receptor function by memantine or cerestat appears to be voltage-independent.

Discussion

Memantine antagonises NMDA receptor-mediated inward currents *in vitro* with an IC_{50} of 1 – 3 μM (Chen *et al.* , 1992; Parsons *et al.*, 1996). Electrophysiological studies on NMDA receptors expressed in *Xenopus* oocytes indicate that memantine has similar potency at NR1a/2B, 2C and 2D subtypes ($IC_{50} \approx 0.4$ μM) and two times lower potency at NR1a/2A subtype (Parsons *et al.*, 1999a). Memantine also acts as an open channel blocker at human α4β2 nicotinic ACh receptors but its potency (IC_{50}) at this receptor is about five times lower than at NMDA receptors (Buisson & Bertrand, 1998). Similar voltage-dependent effects on α9/α10 nicotinic ACh receptors have been reported previously for memantine (Oliver *et al.*, 2001). Memantine also inhibits human α7 receptors (this study) but it does so in a voltage-independent and predominantly non-competitive manner, which is comparable to the effect of memantine on 5-HT3 receptors (Rammes *et al.*, 2001). These studies, together with others showing blockade of NMDA receptors by mecamylamine (O'Dell & Christensen, 1988) and tubocurarine (Halliwell *et al.*, 1989), provide further evidence of the pharmacological similarity between ligand-gated ion channels and the need to be cautious in the interpretation of data from preparation where several types of ligand-gated ion channels are coexpressed.

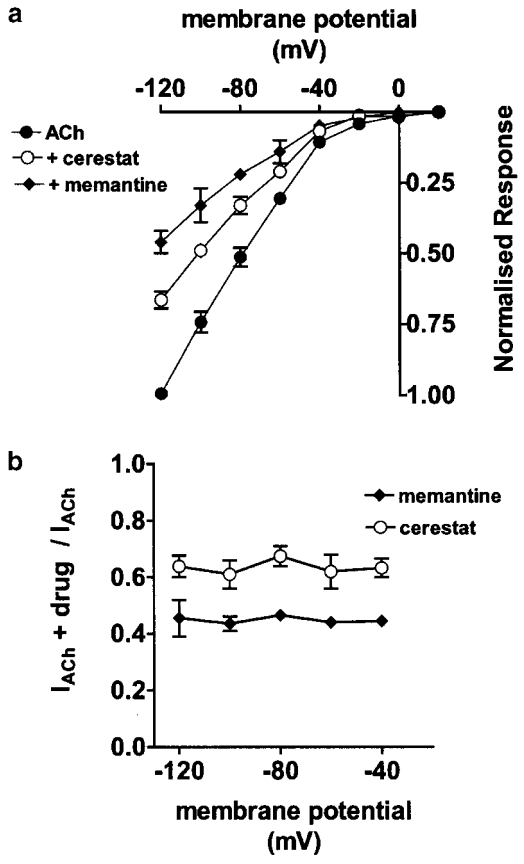

Figure 4 Effect of memantine or cerestat on the inward rectification of α7 homomers–mediated currents. (a) The current–voltage relationship for ACh Currents mediated by α7 receptors is shown in the absence and presence of 3 μM memantine or 1 μM cerestat. Oocytes were initially held at −60 mV, and then brief voltage steps were applied from −120 mV to 20 mV. Oocytes were exposed to test drugs for 2 min before simultaneous application with an EC_{50} concentration of ACh (100 μM). Responses were normalised to the ACh currents obtained at −120 mV (ACh I_{max}). (b) Inhibition of α7 receptor function by 3 μM memantine or 1 μM cerestat at a range of membrane potentials. Inhibition was equivalent at all potentials at which ACh responses could be elicited (Anova test).

Our findings further highlight the importance of subunit composition in the pharmacology of nicotinic ACh receptors. Memantine inhibits α7 receptors in a voltage-independent manner, while its actions on α4β2 (Buisson & Bertrand, 1998) receptors and α9/α10 (Oliver *et al*., 2001) nicotinic ACh receptors are voltage-dependent. Other examples of drugs whose effects are influenced by receptor subunit composition are tetrandrine and strychnine. Tetrandrine, an antihypertensive alkaloid that inhibits both L-type Ca^{2+} channels and nicotinic ACh receptors (Slater *et al*., 2002), inhibits human α4β2 nicotinic ACh receptors in a voltage-independent noncompetitive manner but is a mixed competitive/noncompetitive inhibitor of human α7 nicotinic ACh receptors (Slater *et al*., 2002). Strychnine acts as an open channel blocker at α4β2 and α4β4 nicotinic ACh receptors (García-Colunga & Miledi, 1999) but as a competitive inhibitor at α7 nicotinic ACh receptors (Peng *et al*., 1994).

Cerestat, which blocks active NMDA receptors at nanomolar concentrations (Albers *et al*., 2001), inhibits human α7 receptors (this study) at micromolar concentrations. To our knowledge, this is the first time cerestat has been shown to interact with non-NMDA receptors. It is interesting to note that despite the different structural properties of memantine and cerestat, both drugs exert similar effects on α7 receptors. The effects of either memantine or cerestat on both the ACh-response curve in oocytes expressing the human α7 receptors and the binding of ^{125}I-α-bgtx to membrane homogenates derived from oocytes expressing hα7 receptors are consistent with a noncompetitive mode of inhibition. Moreover, in both cases, the degree of functional blockade was similar and voltage-independent. These findings suggest that these drugs may interact with an identical, structurally tolerant site or overlapping sites of the α7 receptor. More work to illuminate this point is needed, particularly on whether the effects of memantine and cerestat are additive.

Memantine and cerestat are thought to have neuroprotective, analgesic and cognitive enhancement efficacy because of their open channel inhibitory effects at NMDA receptors. Our findings raise the possibility that inhibition of α7 receptors may also contribute to the overall biological and/or therapeutic actions of memantine or cerestat. The therapeutic concentration of these drugs in the blood plasma is around 0.4 – 1 μM (Danysz *et al*., 1997; Parsons *et al*., 1999b) and 1 μM cerestat or memantine inhibits α7 receptor function by 35 and 20%, respectively. It is thus entirely possible that memantine-or-cerestat-based therapies lead to a reduction in brain α7 receptor function. Because α7 receptors facilitate synaptic release of glutamate from several types of brain neurones, including hippocampal (Radcliffe & Dani, 1998; Radcliffe *et al*., 1999) and cortical (Mansvelder & McGehee, 2000) neurones, memantine- or cerestat-mediated inhibition of NMDA and α7 receptors may result in positive synergistic effects, which could contribute to the neuroprotective effects of these drugs. Such scenario would be consistent with studies showing that activation of α4β2 nicotinic ACh receptors, rather than activation of α7 receptors, is required for nicotine-mediated neuroprotection in brain neurones. Thus, mecamylamine or dihydro-β-erythroidine, antagonists relatively selective for α4β2 nicotinic ACh receptors, reverses nicotine-mediated neuroprotection in cortical and striatal neurones (Kaneko *et al*., 1997; Ryan *et al*., 2001; Laudenbach *et al*., 2002). Moreover, knockout mice lacking β2 subunit, unlike α7 knockout mice, show neocortical hypothrophy, loss of pyramidal neurones in the CA3 field, and astro- and micro-gliosis in neocortex and CA1-3 hippocampal fields (Zoli *et al*., 1999). However, there are studies showing that activation of α7 receptors reduces NMDA excitotoxicity in cortical (Kaneko *et al*., 1997; Carlson *et al*., 1998) and hippocampal cultures (Dajas-Bailador *et al*., 2000), suggesting that activation of this receptor may also contribute to the neuroprotective effects of nicotine. More research is needed to establish the relative importance of activation of α7 receptors in neuroprotection, although knockout mice studies clearly indicate that β2-containing receptors but not α7 receptors are essential for nicotine ACh receptor-mediated neuroprotection.

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