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Pharmacological properties of rat α7 nicotinic receptors expressed in native and recombinant cell systems

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Abstract

The pharmacological properties of the rat α7 nicotinic acetylcholine receptor endogenously expressed in PC12 cells and recombinantly expressed in GH4C1 cells (α7-GH4C1 cells) were characterized and compared. Patch-clamp recordings demonstrated that activation by choline and block by methyllycaconitine and dihydro-β-erythroidine were similar, but block by mecamylamine was different. Whereas in α7-GH4C1 cells the inhibition curve for mecamylamine was monophasic (IC_{50} of 1.6 μM), it was biphasic in PC12 cells (IC_{50} values of 341 nM and 9.6 μM). The same rank order of potency was obtained for various nicotinic agonists, while acetylcholine was 3.7-fold less potent and 1.5-fold more effective in PC12 cells. Dihydro-β-erythroidine differentially blocked acetylcholine-evoked currents in both systems. Since reverse transcriptase polymerase chain reaction (RT-PCR) experiments revealed expression of α3, α4, α5, α7 and β4 subunits in PC12 cells, whereas GH4C1 cells express only the β4 subunit, our results suggest that more than one form of α7 containing heteromeric nicotinic receptors might be functionally expressed in PC12 cells.

Keywords: Nicotinic acetylcholine receptor; α7 Subunit; Perforated patch-clamp; PC12 cell; GH4C1 cell; Recombinant expression

1. Introduction

Clinical and laboratory studies indicate the involvement of nicotinic acetylcholine receptors in complex brain functions such as memory, attention and cognition and in diseases such as Alzheimer's and Parkinson's, Tourette's syndrome, schizophrenia, and epilepsy as well as nicotine addiction (Dani and Heinemann, 1996; Lindstrom, 1997; Jones et al., 1999; Weiland et al., 2000; Mihailescu and Drucker-Colín, 2000; Dani, 2001). These receptors form a large family whose members display quite distinct patterns of expression and physiological and pharmacological properties (McGehee and Role, 1995; McGehee, 1999; Picciotto et al., 2000). The subunit composition of nicotinic acetylcholine receptors in neurons is a matter of current studies. Since subunits confer distinct pharmacological and biophysical properties to the nicotinic receptors that they form (Sudweeks and Yakel, 2000; Klink et al., 2001), neuronal responses to exogenous nicotine or to endogenous acetylcholine are likely to be influenced by the particular receptor subtypes expressed. With few exceptions, subunit compositions, stoichiometries, and arrangements of naturally expressed nicotinic acetylcholine receptor types are not known with certainty (Lukas et al., 1999). Identification of the nicotinic acetylcholine receptor subtypes expressed in neuronal pathways implicated in neurological disorders and their pharmacology can be useful in the development of subtype-specific nicotinic receptor ligands for therapeutic purposes. Current studies particularly address the question of whether native α7 nicotinic acetylcholine receptor subtypes are homomeric or heteromeric assemblies of subunits. Whereas some data, based on biochemical evidence, favour a homomeric structure (Quik et al., 1996; Rakhilin et al., 1999; Drisdel and Green, 2000), others, based on biochemical, pharmacological and biophysical evidences, favour a heteromeric assembly (Anand et al., 1993; Yu and Role, 1998; Girod

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et al., 1999; Palma et al., 1999; Crabtree et al., 1997, 2000, 2001; Ferreira et al., 2001; Yakel et al., 2001).

In the present study, we used the perforated patch-clamp technique to pharmacologically characterize the rat α7 nicotinic acetylcholine receptor endogenously expressed in PC12 cells and stably expressed as a recombinant receptor in GH4C1 cells. In order to investigate the possibility that the α7 subunit might assemble with other subunits to form heteromeric populations of α7-containing receptors, we took advantage of the finding that PC12 cells express not only the α7 but also several other nicotinic acetylcholine receptor subunits, whereas GH4C1 cells express only the β4 subunit. Preliminary data have been published in abstract form (Virginio et al., 2000, 2001).

2. Materials and methods

2.1. Materials

[^{3}H]methyllycaconitine was purchased from Tocris. Culture media, Superscript II reverse transcriptase and Lipofectamine 2000 were purchased from Life Technologies, Gold Taq polymerase was obtained from Roche and pCEP4 expression vector from Invitrogen. All other compounds were obtained from Sigma. Stock solutions of all drugs were made in distilled water.

2.2. Cell culture

Rat adrenal medulla pheochromocytoma cells (PC12) were obtained from the original clonal line maintained at the American Type Culture Collection (ATCC, Rockville, MD). These cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum and a final concentration of 35 mg/l proline. Rat pituitary tumor-derived cells (GH4C1) were purchased from the European collection of cell cultures (ECACC, Salisbury, UK). These cells were grown in Ham's F10 medium supplemented with 15% heat-inactivated horse serum, 2.5% foetal bovine serum and 1 mM glutamine. PC12 and GH4C1 cells were maintained at 37 °C in a humidified atmosphere containing 10% and 5% CO_2, respectively. Trypsin-EDTA was used to detach cells during cell passages. Cells were plated on surfaces treated with poly-D-lysine (25 mg/l, MW 70,000–150,000).

2.3. RT-PCR

Total RNA was isolated from PC12 cells by LiCl/urea extraction (Auffray and Rougeon, 1980). Total RNA was purified from GH4C1 cells with the Qiagen RNeasy mini kit according to the manufacturer's instructions. RNA (5 μg) was converted into single-stranded cDNA with Superscript II reverse transcriptase in 20 μl and afterwards increased to 100 μl with water, using 1 μl in each PCR analysis. The following oligonucleotides were used:

rat α3 fwd	5′ -ATAACCATCATTGACACCAGGAC-3′
rat α3 rev	5′ -GCTTGACACTTGAAGGCTTCTTA-3′
rat α4 fwd	5′ -TGGTCTTCTATCTGCCTTCAGAG-3′
rat α4 rev	5′ -CATCTTGTGCATGGACTCAATAA-3′
rat α5 fwd	5′ -AGTGCTGGCTTACAGTTTTTGAG-3′
rat α5 rev	5′ -TGGGCCTACGATGTTTCTAGTTA-3′
rat α7 fwd	5′ -TGAAGAATGTTCGTTTTCCAGAT-3′
rat α7 rev	5′ -GCATGGTTACTGTGTAGGTGACA-3′
rat β2 fwd	5′ -CGTTCAGTGAGCACTTTAGACCT-3′
rat β2 rev	5′ -ACTCTTGTAGATGGCAGGTGGTA-3′
rat β4 fwd	5′ -GCCCTAAGTAAAGCCGTGACTAT-3′
rat β4 rev	5′ -AAGCTGGAGATTTGATGTGGTTA-3′

The reactions were carried out with 1.5 μM of each forward and reverse primer, 1.5 mM $MgCl_2$, and 1.25 units of Gold Taq polymerase in a total volume of 50 μl. The reaction mixture was heated for 12 min at 94 °C, cycled 35 times for 30 s at 94 °C, 1 min at 58 °C and 1 min at 72 °C. A final extension was carried out for 10 min at 72 °C. PCR products were analyzed on 2% agarose gels. The following numbers indicate the predicted nucleotide sizes of the amplification products for each of the nicotinic acetylcholine receptor subunits: α3, 534; α4, 347; α5, 354; α7, 390; β2 588; β4, 524. The PCR product amplified from GH4C1 cells was identified as β4 by restriction analysis with *Sac*I (Roche) and DNA sequencing.

2.4. Stable expression of the rat α7 nicotinic acetylcholine receptor subunit in GH4C1 cells

The pCEP4 vector was used to express the α7 nicotinic acetylcholine receptor in GH4C1 cells (Quik et al., 1996). A cDNA encoding the rat α7 nicotinic acetylcholine receptor, generated by PCR, was inserted into the *Bam*HI site of the pCEP4 vector. The day before transfection, 10^6 cells were plated in a 35 mm Petri dish. Transfection was performed with linearised DNA and Lipofectamine 2000 according to the manufacturer's protocol (Life Technologies). Twenty-four hours later, selection in hygromycin (200 μg/ml) was started. Fifteen days later, 63 colonies were picked and transferred to a 96-well plate. The α7 receptor expression of clones was measured by [^{3}H]methyllycaconitine binding on whole cells. Twenty-four hours prior to binding experiments, cells were plated in a 24-well plate (300,000 cells/well). At the day of experiment, cells were washed twice with phosphate buffer saline (PBS) (140 mM NaCl, 10 mM KH_2PO_4, 10 mM Na_2HPO_4, 2 mM KCl, pH = 7.4), and incubated for 30 min at 37 °C in presence of 2 nM [^{3}H]methyllycaconitine (total binding). Cells were then washed twice with PBS and lysed with 1% Triton X-100 in PBS. Samples were transferred to vials and radioactivity measured with a β-counter. Non-specific binding was meas-

ured in the presence of 1 mM nicotine. The clone that displayed the highest specific binding was further expanded in a 175 cm^2 T-flask, divided into aliquots and frozen in liquid nitrogen until further use.

2.5. Electrophysiological recordings

For electrophysiological experiments, cells were plated at a density of 60,000 cells/13 mm dish glass cover slips. Cells were used 2–4 days after plating. PC12 cells were treated with 10 μM nicotine 1–2 days before recording because an increase in the amplitude of the fast-desensitizing choline-activated current was observed following this treatment (data not shown). Experiments were carried out at room temperature. Whole-cell currents were recorded from cells applying the perforated patch-clamp technique (Hamill et al., 1981; Shermann-Gold, 1993) using the EPC9 patch-clamp system and the Pulse program (HEKA elektronik, Germany). Patch pipettes were pulled from thin-wall borosilicate glass capillary (1.5 mm outer diameter) using a P-97 pipette puller (Sutter Instruments) and had resistances of 4–7 MΩ. The intracellular solution contained (in mM) 140 CsCl, 2 EGTA, and 10 HEPES, pH 7.3 (with CsOH). Intracellular solution containing 240 μg/ml amphotericin B was used to backfill the pipette while intracellular solution alone was used to fill the tip. Cells were discarded unless the seal formation permitted low resistance access within 20–30 min (series resistance ≤ 20 MΩ) due to the amphotericin B. Results were not used when the access resistance changed significantly during the experiment. The external solution was (in mM) 135 NaCl, 2 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, and 10 HEPES, pH 7.3 (with NaOH). Agonists were applied using a fast flow U-tube delivery system (Fenwick et al., 1982). Except for the agonist, superfusion and U-tube solutions were always of the same composition. The holding potential was -60 mV throughout the experiment except during agonist application in which the potential was held at -80 mV. Cells were stimulated with 300–500 ms pulses of agonist delivered at intervals ≥ 2 min.

2.6. Data analysis

Peak current amplitudes were measured on-line. Concentration–response curves for agonists and antagonists were fitted to the Hill equation of the form:

$$I = I_{max}/1 + (EC_{50}/Ag)^{nH}$$

and

$$I = I_{max}/1 + (IC_{50}/An)^{-nH}$$

wherein I_{max} = maximal normalized current response (in the absence of antagonist for the inhibitory curves), Ag = agonist concentration, An = antagonist concentration, EC_{50} = agonist concentration eliciting half-maximal current, IC_{50} = antagonist concentration blocking half-maximal current, and nH = Hill coefficient.

Alternatively, antagonist concentration–response curves were fitted using the sum of two equations in the form:

$$I = I_{max}\{r/[1 + (hIC_{50}/An)^{-hnH}] + (1 - r)/[1 + (lIC_{50}/An)^{-lnH}]\}$$

where r corresponds to the percentage of high sensitive state, while hIC_{50}, hnH and lIC_{50}, lnH the midpoint of activation and Hill coefficient of the high and low-affinity states, respectively. Antagonist curves were constrained to $I_{max} = 1$. For agonist efficacy curves I_{max} was not constrained. Values stated in the text and the tables are means ± S.E.M. from individual cells, except for mecamylamine experiments. Graphs were constructed by averaging the results from all experiments and fitting a single curve to the pooled data. Results were compared for their statistical significance by one-way analysis of variance (ANOVA).

3. Results

3.1. RT-PCR

It has been reported that different PC12 cell lines express different nicotinic acetylcholine receptor subunits (Blumenthal et al., 1997). Using RT-PCR to detect the expression of various subunits, we found that the PC12 cell line used in our laboratory expresses variable amounts of α3, α4, α5, α7 and β4 nicotinic receptor subunits gene transcripts, whereas β2 subunit mRNA was undetectable (Fig. 1A). We performed the same analysis on untransfected GH4C1 cells and only the β4 subunit mRNA was detected (Fig. 1B).

3.2. Pharmacological properties of choline-activated currents in PC12 cells and in GH4C1 cells stably expressing recombinant rat α7 nicotinic acetylcholine receptor

In 83.6% of the PC12 cells tested ($n = 67$), 10 mM choline activated a biphasic inward current composed of a rapidly desensitizing portion, followed by a steady-state component with an amplitude corresponding to 21.2 ± 2.4% ($n = 56$) of the fast portion, probably due to the activation of α3-subunit-containing nicotinic acetylcholine receptor to which choline acts as a partial agonist (Alkondon et al., 1997). The mean peak current amplitude and the mean steady-state current amplitude were 354.9 ± 36.4 ($n = 89$) and 38.4 ± 7.1 pA ($n = 65$), respectively (Fig. 2A). In all α7-GH4C1 cells tested, application of 10 mM choline elicited a desensitizing inward current with a mean peak current amplitude of 1405.5 ± 100.7 pA ($n = 55$) (Fig. 2B). The choline activated current in α7-GH4C1 cells and the rapidly desensitizing current elicited by this agonist in PC12 cells were almost completely and reversibly blocked by 1 nM methyllycaconitine, an α7-selective antagonist

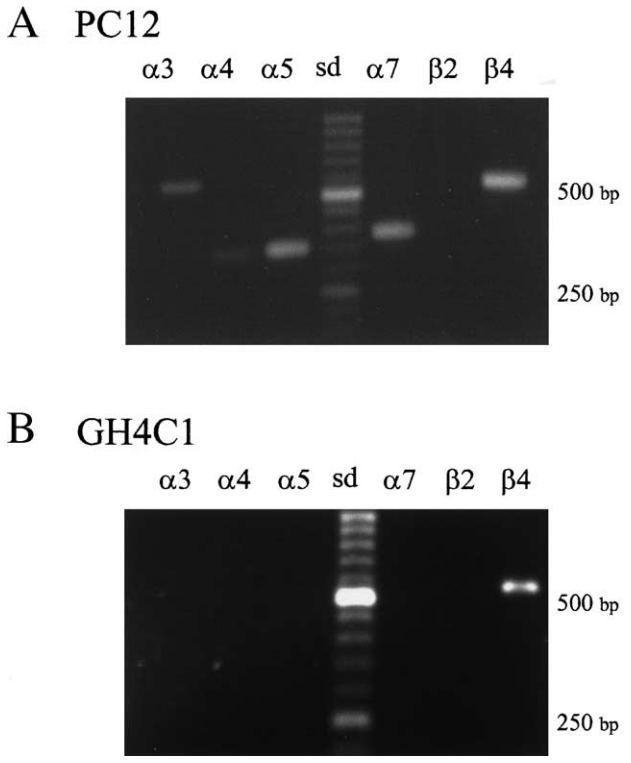

Fig. 1. RT-PCR analysis of the indicated nicotinic acetylcholine receptor subunit mRNAs from PC12 cells (A) and GH4C1 cells (B). Ethidium bromide stained agarose gel showing the amplified PCR fragments which have been obtained using subunit specific primers. The middle lane corresponds to 50 base pair ladder standards (Boehringer Mannheim).

(Fig. 2). Concentration–response curves for choline were obtained for each cell line (Fig. 3). Peak current responses to increasing concentrations of choline were normalized to

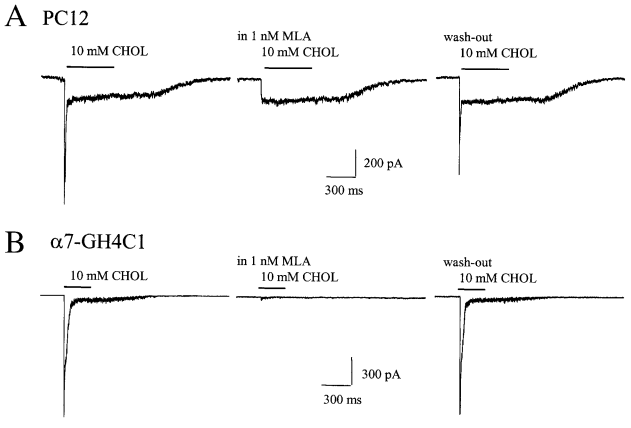

Fig. 2. Whole-cell perforated patch-clamp recording of choline-induced currents showing the block of the fast component by methyllycaconitine. (A) Sample current recorded from a PC12 cell. The slowly desensitizing response was resistant to 1 nM methyllycaconitine and therefore attributable to α3-containing nicotinic acetylcholine receptors. (B) In a GH4C1 cell stably expressing the α7 nicotinic acetylcholine receptor subunit, choline evoked a fast-desensitizing current reversibly blocked by methyllycaconitine.

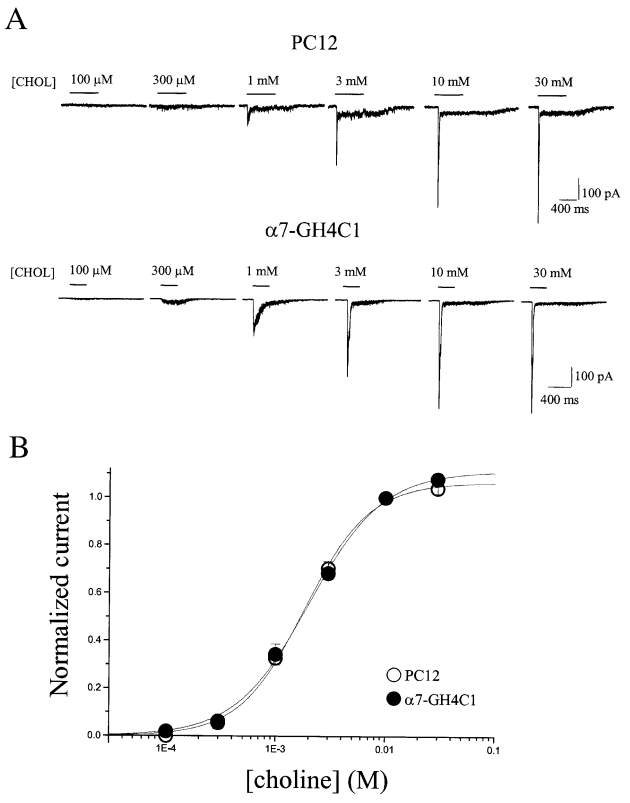

Fig. 3. Concentration–response curves for choline-evoked currents in whole-cell voltage-clamped PC12 and α7-GH4C1 cells. (A) Choline-induced currents recorded from a PC12 cell (upper traces) and an α7-GH4C1 cell (lower traces) activated with increasing concentrations of agonist. Choline was applied to and removed from the cell using a fast application system (see Materials and methods) during the time indicated by the horizontal bars. A 2–4-min wash-out in agonist-free extracellular solution was carried out between successive agonist applications. (B) Averaged concentration–response curves obtained from all PC12 (○) and all α7-GH4C1 cells (●). Peak current responses to varying concentrations of choline were normalized to the current response elicited in the same cell by the application of 10 mM choline. Data points were fitted to the Hill equation (see Materials and methods). Each data point represents the means ± S.E.M. of 7–18 cells. The EC_{50} values, Hill coefficients and values of maximum activated current were 2.1 ± 0.2, 1.5 ± 0.12 and 1.1 ± 0.02 mM ($n = 13$), respectively for PC12 cells and 2.1 ± 0.1, 1.4 ± 0.21 and 1.1 ± 0.04 mM ($n = 4$), respectively for α7-GH4C1 cells.

the current response elicited in the same cell by the application of 10 mM choline. The EC_{50}, Hill coefficient and I_{max} values for choline were 2.1 ± 0.2, 1.5 ± 0.12 and 1.1 ± 0.02 mM ($n = 13$), respectively for PC12 cells and 2.1±0.1, 1.4±0.21 and 1.1±0.04 mM ($n = 4$), respectively for α7-GH4C1 cells. These parameters are not significantly different ($P > 0.5$) for the two cell lines. Choline-activated currents in PC12 and α7-GH4C1 cells were blocked by methyllycaconitine and by the non-selective nicotinic antagonist dihydro-β-erythroidine. The IC_{50} values for methyllycaconitine and dihydro-β-erythroidine block of responses elicited by an EC_{70} concentration of choline (3 mM) are listed in Table 1. No significant differences in the sensitivity to methyllycaconitine and dihydro-β-erythroidine were observed in the two cell lines ($P > 0.5$).

Table 1
Parameter values of fitted concentration–inhibition curves for methyllycaconitine and dihydro-β-erythroidine on choline-activated current

	Methyllycaconitine		Dihydro-β-erythroidine	
	PC12 cells (3)	α7-GH4C1 cells (3)	PC12 cells (6)	α7-GH4C1 cells (3)
IC_{50}	193 ± 11.7 pM	178.9 ± 27.4 pM	4.0 ± 0.39 μM	4.6 ± 0.90 μM
nH	2.1 ± 0.10	1.4 ± 0.22	1.5 ± 0.18	1.4 ± 0.14

Another blocker, mecamylamine, was also tested on choline-activated currents. In order to avoid a possible interference of α3-containing nicotinic acetylcholine receptors in the inhibition of α7-containing receptor by mecamylamine in PC12 cells, experiments were only performed in those cells showing a steady-state component amplitude less than 5% of the fast portion. Due to its mechanism of blockade, a single mecamylamine concentration was tested for each cell. Currents evoked by 500 ms pulses of 10 mM choline were recorded once every 2 min for several applications and only cells showing a stable peak current amplitude were used. Following this test period, the antagonist was applied continuously in the bath while the cell was activated with choline at 2-min intervals. A slow decline of the choline-evoked current was observed for concentrations of mecamylamine above 100 nM for PC12 cells and 300 nM for α7-GH4C1 cells. About 10 min after the application of mecamylamine, the decline of the current showed a progressive stabilization in which a steady-state condition was obtained within 20 min. Plots of the choline-evoked currents as a function of time for different antagonist concentrations are shown for the two cell lines in Fig. 4a. Concentration–inhibition curves were obtained by plotting the normalized peak current amplitude of the blocked steady condition as a function of the mecamylamine concentration (Fig. 4b). Data points could be fitted with a single Hill equation with an IC_{50} of 1.6 ± 0.08 μM

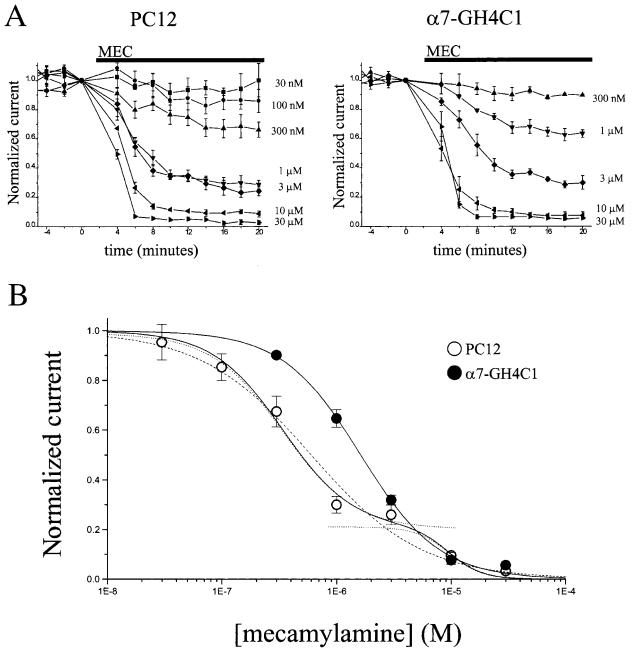

Fig. 4. Blockade of choline-activated currents in PC12 cells and α7-GH4C1 cells by mecamylamine. (A) Time course of mecamylamine blockade of current evoked by 10 mM choline in PC12 cells (left panel) and α7-GH4C1 cells (right panel). Peak currents elicited by choline were plotted as a function of time. The horizontal bar indicates the addition of mecamylamine at the indicated concentrations. The current amplitudes recorded in the presence of mecamylamine were normalized to the amplitude of the current recorded at time zero. (B) Normalized peak current responses elicited in the presence of increasing concentrations of mecamylamine were plotted as a function of mecamylamine concentration. For each mecamylamine concentration, the plotted value was the mean of the last four recorded currents (at 14, 16, 18 and 20 min). Data points for α7-GH4C1 cells (●) could be fitted to a single Hill equation yielding an IC_{50} of 1.6 μM. Data points for PC12 cells (○) could be fitted to the sum of two Hill equations (see Materials and methods). Fit by a single Hill equation is also presented (dashed line) to illustrate the relevance of the two-component fit. The single Hill equation corresponds to an IC_{50} of 602 nM, whereas the IC_{50} values of the high- and low-affinity mecamylamine components were 341 nM and 9.6 μM, respectively. Individual components of the fit are shown as dotted lines; the high- and low-affinity mecamylamine components had relative amplitudes of 80% and 20%, respectively. Each data point represents the means ± S.E.M. of three to four experiments.

Table 2
Parameter values of fitted concentration–response curves for the selected agonists

Agonist	PC12 cells	α7-GH4C1 cells
Cytisine		
(*n*)	(3)	(3)
I_{max}	1.0 ± 0.11	0.9 ± 0.04
EC_{50}	51.5 ± 13.09 μM	32.1 ± 2.33 μM
nH	1.5 ± 0.32	2.5 ± 0.52
Nicotine		
(*n*)	(4)	(4)
I_{max}	1.0 ± 0.10	1.1 ± 0.03
EC_{50}	52.3 ± 5.7 μM	41.0 ± 5.02 μM
nH	1.6 ± 0.31	1.6 ± 0.24
Acetylcholine		
(*n*)	(5)	(3)
I_{max}	1.5 ± 0.06[a]	1.0 ± 0.003
EC_{50}	389.4 ± 86.42 μM[a]	105.4 ± 14.7 μM
nH	1 ± 0.06[a]	1.6 ± 0.07
Choline		
(*n*)	(13)	(4)
I_{max}	1.1 ± 0.02	1.1 ± 0.04
EC_{50}	2.1 ± 0.2 mM	2.1 ± 0.1 mM
nH	1.5 ± 0.12	1.4 ± 0.21

[a] $P < 0.05$ compared to the corresponding I_{max}, IC_{50} and *nH* values in α7-GH4C1 cells.

($n=3$) for α7-GH4C1 cells while a dual Hill equation was used for PC12 cells. The high and low-affinity components had IC_{50} values of 341±116 nM and 9.6±5.4 μM ($n=3-4$), respectively. The high-affinity component represented 79.9 ± 12.5%.

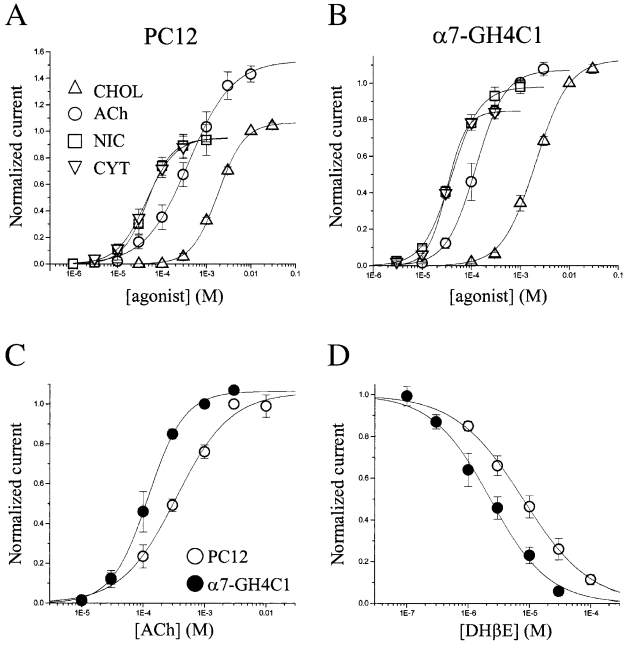

Fig. 5. Pharmacological characterization of the low affinity mecamylamine component in PC12 cells and recombinant α7 nicotinic acetylcholine receptor expressed in GH4C1 cells. (A), (B) Concentration–response curves for the agonists cytisine (CYT ▽), nicotine (NIC □), acetylcholine (ACh ○) and choline (CHOL △). Responses to the various agonists were normalized to the amplitude of the response elicited by 10 mM choline in the same cell. In PC12 cells, experiments were performed in the presence of 3 μM mecamylamine (A), whereas in α7-GH4C1 cells mecamylamine was omitted (B). Concentration–response curves were fitted to the Hill equation (see Materials and methods). Each data point represents the means ± S.E.M. of 2–18 experiments. The EC_{50} values, Hill coefficients and values of maximum activated current are summarized in Table 2. (C) Concentration–response curves for acetylcholine obtained with PC12 cells (in the presence of 3 μM mecamylamine) (○) and with α7-GH4C1 cells (●). Peak current responses to varying concentrations of acetylcholine were normalized to the current response elicited in the same cell by the application of 3 and 1 mM acetylcholine in PC12 and α7-GH4C1 cells, respectively. Data points were fitted to the Hill equation (see Materials and methods). The EC_{50} values and Hill coefficients were 389.4 μM and 1 for the PC12 cells, and 105.4 μM and 1.6 for α7-GH4C1 cells, respectively. Each data point represents the means ± S.E.M. of three to six cells. (D) Blockade of acetylcholine activated current by dihydro-β-erythroidine in PC12 cells (in the presence of 3 μM mecamylamine) (○) and in α7-GH4C1 cells (●). Cells were incubated with increasing concentrations of dihydro-β-erythroidine for 4–8 min prior to the application of acetylcholine. Peak current responses evoked in the presence of increasing concentrations of antagonists were normalized to the current response elicited in the same cell by the application of an EC_{50} concentration of acetylcholine (300 μM for PC12 cells and 100 μM for α7-GH4C1 cells). Data points were fitted to the Hill equation (see Materials and methods). Each data point represents the means ± S.E.M. of two to four experiments. The IC_{50} values were 8.6 μM for PC12 cells, and 2.5 μM for α7-GH4C1 cells, respectively.

3.3. Pharmacological characterization of the α7 nicotinic acetylcholine receptor stably expressed in GH4C1 cells and of the mecamylamine low-affinity component in PC12 cells

Further pharmacological characterization was carried out to analyze the relative potencies and efficacies of various cholinergic agonists at α7 nicotinic acetylcholine receptors heterologously and endogenously expressed in GH4C1 and PC12 cells. PC12 cells express different nicotinic acetylcholine receptor subunits that are activated by non-selective nicotinic agonists. Therefore, the presence of a blocker of the non-α7 nicotinic receptors was required in order to study the agonist profile of the α7 nicotinic acetylcholine receptors in these cells. Since antagonists that block all the nicotinic acetylcholine receptors without affecting α7 receptors are not yet available, the study in PC12 cells was limited to the α7-mediated low-affinity mecamylamine component. Accordingly, all further experiments performed with PC12 cells were carried out in the presence of 1–3 μM mecamylamine, a concentration able to block the high-affinity mecamylamine α7 component and all other non-α7 receptors (Giniatullin et al., 2000).

Concentration–response curves were obtained for acetylcholine, nicotine and cytisine (Fig. 5a and b). Peak current responses to increasing concentrations of agonist were normalized to the current response elicited in the same cell by 10 mM choline. Data are summarized in Table 2. The rank order of EC_{50} values in both cell lines was cytisine = nicotine>acetylcholine>choline. Acetylcholine was 3.7-fold more potent in α7-GH4C1 cells ($P<0.05$) (Fig. 5c) and had an efficacy which was 1.5-fold higher in PC12 cells ($P<0.01$) (Fig. 5a and b). Acetylcholine activated currents in PC12 cells (in the presence of 3 μM mecamylamine) and in α7-GH4C1 cells were blocked by dihydro-β-erythroidine (Fig. 5d). The IC_{50} values for dihydro-β-erythroidine block of responses elicited by an acetylcholine concentration close to its EC_{50} (300 μM for PC12 and 100 μM for α7-GH4C1 cells) were 8.6 ± 2.5 μM ($n=3$) for PC12 cells and 2.5 ± 0.6 μM ($n=4$) for α7-GH4C1 cells. These differences which are statistically significant ($P<0.05$) show that dihydro-β-erythroidine was 3.4-fold more potent on α7-GH4C1 cells. On the other hand, block of nicotine-evoked currents by dihydro-β-erythroidine was not significantly different in the two cell lines (data not shown).

4. Discussion

The purpose of the present study was to compare the pharmacology of the α7 nicotinic acetylcholine receptors endogenously expressed in PC12 cells with the recombinant α7 receptor expressed in GH4C1 cells. PC12 cells express the α7 gene product along with other nicotinic receptor subunit mRNAs (α3, α4, α5 and β4) (present work and Blumenthal et al., 1997), whereas GH4C1 cells express only β4 mRNA. Previous results showed that GH4C1 cells do

not contain detectable mRNA for α2, α3, α4, α5, α7, β2 and β3 subunits (Quik et al., 1996; Sweileh et al., 2000).

The main functional nicotinic acetylcholine receptors in PC12 cells are the α3-containing and the α7-containing nicotinic acetylcholine receptors. In order to study only the latter, the activated current had to meet the following properties: (i) activation by the selective agonist choline, (ii) inhibition by the selective antagonist methyllycaconitine (Alkondon et al., 1997, 1992; Palma et al., 1996) and (iii) fast kinetic properties (Klink et al., 2001).

The pharmacological properties of the recombinant α7 nicotinic acetylcholine receptor are very similar to those of the α7-containing nicotinic acetylcholine receptors natively expressed in PC12 cells. The receptors expressed in the two cell lines showed the same affinity for choline and the same sensitivity to methyllycaconitine and dihydro-β-erythroidine of the choline-activated currents.

Mecamylamine, an open channel blocker, revealed different populations of α7-containing receptors in PC12 cells and α7-GH4C1 cells. Inhibition of choline-activated currents by mecamylamine yielded a monophasic concentration–response curve in α7-GH4C1 cells and a biphasic concentration–response curve that was best fitted using the sum of two Hill equations in PC12 cells. The biphasic nature of the concentration–response curve suggests the presence of at least two receptor populations displaying high affinity and low affinity for mecamylamine. Since PC12 cells express other nicotinic acetylcholine receptor subunits, the two receptor populations may be composed of heteromeric α7 receptors. Furthermore, the high- and low-affinity mecamylamine components displayed IC_{50} values different from that obtained with α7-GH4C1 cells, which is consistent with this hypothesis.

The comparison of concentration–response curves for various nicotinic agonists of α7-GH4C1 cells and the low-affinity mecamylamine component of PC12 cells, revealed differences in potency between the two cell lines while the rank order was the same (cytisine = nicotine > acetylcholine > choline). This rank order is different from that reported for the rat α7 nicotinic acetylcholine receptor expressed in *Xenopus* oocytes (nicotine > cytisine > acetylcholine) (Seguela et al., 1993) and might be due to the different expression systems. Acetylcholine differentiated the recombinant and native α7 receptors. In the functional assay, acetylcholine had a 1.5-fold higher efficacy in PC12 cells and was 3.7-fold more potent on α7-GH4C1 cells. Concentration–response relationships yielded a half-maximal effective concentration (EC_{50}) of 389 μM for acetylcholine in PC12 cells and 105 μM in α7-GH4C1 cells. Zaninetti et al. (2000) found that acetylcholine had an EC_{50} value of 288 μM on neurons of the supraoptic nucleus in hypothalamic slices, a value similar to that obtained in PC12 cells. Furthermore, they found an acetylcholine efficacy higher than nicotine, similar to our results in PC12 cells. In other cells expressing α7 receptors, acetylcholine had a higher potency, similar to that we have obtained on α7-GH4C1 cells (105 μM). For instance, in human neuroblastoma cells expressing rat α7 nicotinic acetylcholine receptors, the EC_{50} value for acetylcholine was 150 μM (Puchacz et al., 1994) and in cultured foetal rat hippocampal neurons which express methyllycaconitine-sensitive nicotinic acetylcholine receptors, the EC_{50} value for acetylcholine was 126 μM (Alkondon et al., 1992). The difference in acetylcholine sensitivity and efficacy between PC12 cells we have studied and the systems examined by others could be due to the association of various nicotinic acetylcholine receptor subunits with an α7 subunit, thus modifying its pharmacological profile as proposed by Zaninetti et al. (2000) for the nicotinic acetylcholine receptors expressed in supraoptic nucleus neurons.

The dihydro-β-erythroidine sensitivity of the acetylcholine-evoked current also differentiated the native and recombinant nicotinic acetylcholine receptors. The antagonist blocked the acetylcholine-evoked current in both receptors, but was 3.4-fold more potent on α7-GH4C1 cells. The sensitivity of the recombinant α7 receptor to dihydro-β-erythroidine (IC_{50} of 2.5 μM at 100 μM acetylcholine; present results) is similar to that of the recombinant chick α7 nicotinic acetylcholine receptor expressed in *Xenopus* oocytes (IC_{50} of 1.6 μM at 100 μM acetylcholine; Bertrand et al., 1992). In PC12 cells, 1 μM dihydro-β-erythroidine blocked only 15% of the current evoked by 300 μM acetylcholine, close to its EC_{50}, whereas in α7-GH4C1 cells, the same concentration of dihydro-β-erythroidine blocked 36% of the current evoked by 100 μM acetylcholine, again close to its EC_{50}. In neurons of the supraoptic nucleus in hypothalamic slices, which are hypothesized to express heteromeric α7 receptors, the current evoked by 300 μM acetylcholine was unaffected by 1 μM dihydro-β-erythroidine (Zaninetti et al., 2000). In our experiments, 10 μM dihydro-β-erythroidine was able to reduce the amplitude of the α7 nicotinic acetylcholine receptor-mediated current evoked by 3 mM choline in either PC12 cells or α7-GH4C1 cells by 76%. For comparison in interneurons of rat hippocampal slices, 10 μM dihydro-β-erythroidine was able to reduce the amplitude of α7 nicotinic acetylcholine receptor-mediated current evoked by 10 mM choline by 40% (Alkondon et al., 1999). Since dihydro-β-erythroidine is a competitive antagonist (Bertrand et al., 1992), the discrepancy in the percentage of block might be due to the different agonist concentrations used (3 mM choline versus 10 mM choline).

The differences between the native α7 nicotinic acetylcholine receptors in PC12 cells and the recombinant α7 receptor in GH4C1 cells revealed by mecamylamine, acetylcholine and dihydro-β-erythroidine, are all consistent with different populations of α7 receptors in the two cell lines. Since PC12 cells express other nicotinic acetylcholine receptor subunits, the α7 subunit may form heteromeric receptors. The existence of native nicotinic acetylcholine receptors forming heteromeric complexes containing α7 subunits has previously been supported by binding experi-

ments with [^{125}I]α-bungarotoxin demonstrating that endogenously expressed α7 nicotinic receptors in chick brain are physiologically and pharmacologically different from heterologously expressed recombinant α7 homomeric channels expressed in *Xenopus* oocytes (Anand et al., 1993). More recently, Yu and Role (1998) presented evidence that the α7 subunit contributes to the function of at least three subtypes of native nicotinic acetylcholine receptors expressed in embryonic chick sympathetic neurons, proposing that these three populations of α7-containing nicotinic acetylcholine receptors are distinct heteromeric complexes that include other α and/or β subunits. Palma et al. (1999) showed that after injection of *Xenopus* oocytes with a mixture of chick α7 and β3 cDNAs, clear evidence was obtained for the plasma membrane assembly of heteromeric nicotinic acetylcholine receptors, although acetylcholine could not activate these receptors, suggesting that the β3 subunit negatively regulated the receptor. Crabtree et al. (1997, 2000, 2001) and Yakel et al. (2001) found that heteromeric combinations of chick α7 with α5 and/or β2 subunits can be expressed in heterologous systems and Ferreira et al. (2001) showed that there is a nicotinic acetylcholine receptor containing the α7 subunit in motoneurons of the dorsal motor nucleus of the vagus that is pharmacologically different from the homomeric α7 subtype. Other members of the nicotinic acetylcholine receptor family form heteromeric receptors. For instance, α4β2 and α4α5β2 receptors are found in the central nervous system, α3α5β4, α3α5β2β4 and α3β4-containing receptors are expressed in the autonomic ganglia and α1β1γδ and α1β1εδ receptors have been described in foetal and adult skeletal muscles (Lukas et al., 1999).

In conclusion, the results presented in this work indicate that in PC12 cells, at least two α7-containing nicotinic acetylcholine receptors are functionally expressed which can be distinguished on the basis of their affinity to mecamylamine. Moreover, the pharmacological properties of the recombinant α7 nicotinic acetylcholine receptor expressed in GH4C1 cells and of the mecamylamine low-affinity α7-containing receptors in PC12 cells were not identical but similar. In fact, different sensitivities to acetylcholine and to dihydro-β-erythroidine block of the acetylcholine-activated current suggest the existence of α7-containing nicotinic receptors in PC12 cells different from the α7 receptor expressed in the heterologous systems. Although it cannot be ruled out that such differences are also influenced by differentially processed α7 subunits (Drisdel and Green, 2000) our results provide further evidence for α7 containing heteromeric nicotinic acetylcholine receptors.

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