BJP British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2011.01596.x
www.brjpharmacol.org

RESEARCH PAPER

Pharmacological characterization of native α7 nicotinic ACh receptors and their contribution to depolarization-elicited exocytosis in human chromaffin cells

Correspondence
Almudena Albillos or Alberto Pérez-Alvarez, Departamento de Farmacología y Terapéutica, Facultad de Medicina, Universidad Autónoma de Madrid, c/ Arzobispo Morcillo 4, 28029 Madrid, Spain. E-mails: almudena.albillos@uam.es, a.perez@uam.es

Keywords
nicotinic receptor; α7; patch clamp; capacitance; human chromaffin cell

Received
28 April 2011
Revised
27 June 2011
Accepted
7 July 2011

Alberto Pérez-Alvarez[1], Alicia Hernández-Vivanco[1], Sergio Alonso y Gregorio[2], Angel Tabernero[2], J Michael McIntosh[3] and Almudena Albillos[1]

[1]*Departamento de Farmacología y Terapéutica, Facultad de Medicina, Universidad Autónoma de Madrid, Madrid, Spain,* [2]*Servicio de Urología, Hospital Universitario La Paz, Madrid, Spain, and* [3]*Department of Biology and Department of Psychiatry, University of Utah, Salt Lake City, Utah, USA*

BACKGROUND AND PURPOSE

Expression of α7 nicotinic acetylcholine receptors (nAChRs) and their role in exocytosis have not yet been examined in human chromaffin cells.

EXPERIMENTAL APPROACH

To characterize these receptors and investigate their function, patch-clamp experiments were performed in human chromaffin cells from organ donors.

KEY RESULTS

The nicotinic current provoked by 300 μM ACh in voltage-clamped cells was blocked by the nicotinic receptor antagonists α-bungarotoxin (α-Bgtx; 1 μM; 6 ± 1.7%) or methyllycaconitine (MLA; 10 nM; 7 ± 1.6%), respectively, in an irreversible and reversible manner, without affecting exocytosis. Choline (10 mM) pulses induced a biphasic current with an initial quickly activated (5.5 ± 0.4 ms rise time) and inactivated component (8.5 ± 0.4 ms time constant) (termed α7), which was blocked by α-Bgtx or MLA, followed by a slower component (non-α7). α7 nAChR currents were dissected by blocking the non-α7 nAChR current component of the ACh and choline response with the α6* nAChR blocker α-conotoxin (α-Ctx) MII[S4A, E11A, L15A]. PNU-282987, an α7 nAChR-specific agonist, elicited rapidly activated and rapidly inactivated currents. α7 nAChR-positive allosteric modulators, such as 5-hydroxyindole (1 mM) and PNU-120596 (10 μM), potentiated responses that were blocked by α-Bgtx or MLA. Exocytosis was evoked by depolarization-elicited α7 nAChR currents, using choline in the presence of α-Ctx MII[MS4A, E11A, L15A] or PNU-282987 as agonists.

CONCLUSIONS AND IMPLICATIONS

Our electrophysiological recordings of pure α7 nAChR currents elicited by rapid application of agonists demonstrated that functional α7 nAChRs are expressed and contribute to depolarization-elicited exocytosis in human chromaffin cells.

Abbreviations

5-HI, 5-hydroxyindole; α-Bgtx, α-bungarotoxin; α-Ctx MII[S4A, E11A, L15A], α-conotoxin MII[S4A, E11A, L15A]; C_m, membrane capacitance; DHβE, dihydro-β-erythroidine; G_m, membrane conductance; G_s, seal conductance; MLA, methyllycaconitine; nAChR, nicotinic acetylcholine receptor; PAM, positive allosteric modulator; VDCC, voltage-dependent Ca^{2+} channels

Introduction

We previously showed that nicotinic acetylcholine receptors (nAChRs; nomenclature follows Alexander *et al.*, 2011) in human chromaffin cells control several stages of the stimulus-secretion coupling process that occurs when these cells are activated with short or long pulses of ACh (Pérez-Alvarez and Albillos, 2007). Also, human chromaffin cells mainly exhibit an 'adrenergic phenotype' (Pérez-Alvarez *et al.*, 2008), i.e., they release more adrenaline than noradrenaline, confirming previous data (Takiyyuddin *et al.*, 1994). Thus, nAChRs play an essential role in the fast and more sustained catecholamine secretion that takes place under stress situations in humans.

In relation to the fast response elicited by short pulses of ACh, nAChRs in human chromaffin cells play a prominent role in exocytosis according to the following evidence from our laboratory (Pérez-Alvarez and Albillos, 2007): (i) 200 ms pulses of ACh elicited a nicotinic current, fully abolished by mecamylamine, that contributed to overall exocytosis at hyperpolarized membrane potentials; this contribution was as large as that of voltage-dependent Ca^{2+} channels (VDCC) at depolarized potentials; and (ii) this ACh-evoked nicotinic current triggered plasma membrane depolarization, recruiting VDCC and eliciting exocytosis.

From a methodological point of view, the ACh secretory response elicited by Ca^{2+} entry through the nicotinic receptor ionophore could be quantified by capacitance measurements at high resolution using the voltage clamp configuration of the 'patch-clamp' technique, which allows the recording of secretory vesicle fusion with the plasma membrane (Neher and Marty, 1982). However, the need to fix the voltage to perform capacitance recordings precludes the recruitment of Na^+ and Ca^{2+} channels by ACh to evoke action potentials, the subsequent depolarization, activation of VDCC and exocytosis. In order to overcome this limitation, we designed a 'triple-step protocol' (Pérez-Alvarez and Albillos, 2007). This method allows the measurement of ACh-elicited exocytosis by recording plasma membrane capacitance (C_m) in the voltage clamp configuration of the patch-clamp technique before (step 1) and after (step 3) the depolarization elicited by the agonist takes place, which must be recorded in the current clamp configuration (step 2).

In this context, we sought to address the subtypes of nAChRs involved in the exocytotic process triggered by short ACh pulses in human chromaffin cells, with special attention paid to the α7 receptor, which is crucial in mediating rapid transmission (Zhang *et al.*, 1996; Ullian *et al.*, 1997; Alkondon *et al.*, 1998; Frazier *et al.*, 1998), especially in periods of high-frequency stimulation (Chang and Berg, 1999; Bibevski *et al.*, 2000). This receptor generates a quickly inactivated current that is readily blocked by α-bungarotoxin (α-Bgtx) or methyllycaconitine (MLA) (Alkondon *et al.*, 1992; Bertrand *et al.*, 1992; Zorumski *et al.*, 1992). α7 receptor currents raise intracellular Ca^{2+} (Vijayaraghavan *et al.*, 1992; Zhang *et al.*, 1994) due to the high Ca^{2+} permeability of α7 nAChRs (Bertrand *et al.*, 1993; Séguéla *et al.*, 1993; Delbono *et al.*, 1997), depolarize the plasma membrane when located postsynaptically (Zhang *et al.*, 1996; Frazier *et al.*, 1998; Chang and Berg, 1999) and presynaptically modulate neurotransmitter release (McGehee *et al.*, 1995; Gray *et al.*, 1996; Guo *et al.*, 1998; Li *et al.*, 1998).

In chromaffin cells from other species where nAChRs have been investigated, there is controversy over the nAChR subunit sensitivity to α-Bgtx, the homomeric or heteromeric distribution of α7 nAChRs and their functionality. In bovine chromaffin cells, expression of α7 nAChRs has been determined by specific α-Bgtx binding (Wilson and Kirshner, 1977; Quik *et al.*, 1987), cloning (García-Guzmán *et al.*, 1995) and antibody detection (El-Hajj *et al.*, 2007). However, in these cells nAChRs have also been found to be insensitive to α-Bgtx (Free *et al.*, 2002) as is the catecholamine release process (Kumakura *et al.*, 1980; Trifaró and Lee, 1980; Kilpatrick *et al.*, 1981; Tachikawa *et al.*, 2001). These findings contrast with those of other researchers who report partial or full α-Bgtx blockade of currents and secretion in bovine chromaffin or PC12 cells (Blumenthal *et al.*, 1997; López *et al.*, 1998; Fuentealba *et al.*, 2004). A recent study by Del Barrio *et al.* (2011) shows that minimal cytosolic Ca^{2+} concentrations transients, induced by low concentrations of the selective α7 nAChR agonist PNU-282987 and their corresponding elicited exocytosis, were markedly increased by PNU-120596, and these responses were blocked by α-Bgtx. Evidence for the presence of α7 nAChRs has been also provided in human (Mousavi *et al.*, 2001) and rat (Rust *et al.*, 1994; Mousavi *et al.*, 2001; Colomer *et al.*, 2010) chromaffin cells through α7 mRNA detection, and in PC12 cells by affinity purification and immunoblotting of α-Bgtx receptors (Drisdel and Green, 2000).

The present study was designed to explore the presence and function of α7 nAChRs in primary cultures of human chromaffin cells. Here, we show pure α7 nAChR currents, recorded for the first time in chromaffin cells. Our data indicate that α7 nAChRs are functionally expressed and contribute to exocytosis of secretory vesicles in human chromaffin cells.

Methods

Cell cultures

The study protocol was approved by the Ethics Committees of the Hospital Ramón y Cajal, Hospital Universitario La Paz and Universidad Autónoma de Madrid. After obtaining informed consent from the donors' relatives, adrenal glands were collected from nine adult organ donors (both sexes; age range: 16–78 years) who had died of cerebral haemorrhage. After confirmation of brain death, adequate organ perfusion was ensured and adrenal glands were obtained during surgical procedures to remove kidneys for transplantation. After removal, glands were maintained at 4°C in sterile Locke's solution and taken to the laboratory within 4 hours. The inherent difficulties in obtaining human adrenal glands limited the number of experiments that could be performed. The method used for isolation and culture of human chromaffin cells has been previously described (Pérez-Alvarez and Albillos, 2007). Experiments were started 48 h after plating to allow recovery of the nicotinic receptor after collagenase treatment (Almazán *et al.*, 1984).

Electrophysiological recordings

Perforated patch recordings were made in the whole-cell configuration of the patch-clamp technique. The external solution used to record nicotinic currents was (in mM): 2 $CaCl_2$, 145 NaCl, 5.5 KCl, 1 $MgCl_2$, 10 HEPES and 10 glucose; the pH was adjusted to 7.4 with NaOH. Intracellular solution composition was (in mM): 145 K-glutamate, 8 NaCl, 1 $MgCl_2$, 10 HEPES and 0.5 amphotericin B (Sigma-Aldrich, Madrid, Spain); the pH was adjusted to 7.2 with KOH.

An amphotericin B stock solution was prepared daily at a concentration of 50 mg/mL dimethyl sulphoxide (DMSO) and kept protected from light. The final concentration of amphotericin B was prepared by ultrasonicating 10 μL of stock amphotericin B in 1 mL of internal solution in the dark. Pipettes were tip-dipped in amphotericin-free solution for several seconds and back-filled with freshly mixed intracellular amphotericin solution.

The perfusion system for drug application consisted of a multibarrelled polyethylene pipette positioned close to the cell under study. The exchange time of solutions of this system calculated with open-tip experiments (Zhang *et al.*, 1994) was 10 ms. The agonist was always delivered from the same tube. Antagonists or modulators were perfused between pulses (5 min), and this flow was only interrupted during agonist perfusion (200 ms). The level of the bath fluid was continuously controlled by a custom-designed fibre optics system coupled to a pump used to aspirate excess fluid.

Pipettes of 2–3 MΩ resistance were pulled from borosilicate glass capillary tubes, partially coated with wax and fire polished. After seal formation and perforation, only recordings in which the access resistance of the pipette and the leak current were lower than 20 MΩ and 20 pA, respectively, were accepted. The holding potential (V_h) was –80 mV in all cases except in Figure 5 (V_h = –60 mV). Series resistance was 80% compensated. A four-pole Bessel filter set to 2.9 kHz was used. Currents were sampled at 12 kHz.

Simultaneous electrophysiological current and plasma C_m measurements (as an index of exocytosis) were undertaken using an EPC-10 amplifier and PULSE software (HEKA Elektronik, Lambrecht, Germany) running on a PC computer. Increments in C_m accurately measure the fusion of vesicles to the plasma membrane that takes place during the exocytotic event (Neher and Marty, 1982; Albillos *et al.*, 1997; Neher, 1998; 2006) and have been previously employed to record secretory responses induced by nicotinic agonists (Mollard *et al.*, 1995; Pérez-Alvarez and Albillos, 2007).

Capacitance changes were estimated by the Lindau–Neher (Lindau and Neher, 1988; Gillis, 1995) technique implemented in the 'Sine + DC' feature of the 'PULSE' lock-in software. A 1 kHz, 70 mV peak-to-peak amplitude sinewave was applied at the holding potential. Plasma C_m was sampled at 1 kHz. The 'triple-step' protocol employed in this study is a slight modification of the method reported previously (Pérez-Alvarez and Albillos, 2007) to measure agonist-induced exocytosis by means of C_m changes. It combines the voltage clamp and the current clamp configurations of the patch-clamp technique. Initial plasma C_m is recorded in the voltage clamp configuration (step 1), then by switching to the current clamp configuration, the agonist is able to depolarize the plasma membrane (step 2), and finally by returning to the voltage clamp mode, the increase produced in plasma C_m can be recorded, reflecting the overall exocytosis evoked by the agonist (step 3). For analysis of C_m changes, the maximum C_m value in step 3 was taken after at least 100 ms from the start of the step to avoid capacitance artefacts (Horrigan and Bookman, 1994) and compared with the basal value before stimulation.

Analysis of data was conducted on a PC using IGOR Pro software (Wavemetrics, Lake Oswego, OR, USA). Changes in seal conductance (G_s) after the application of agonists or α7 nAChR potentiators, and in membrane conductance (G_m) after the application of ACh were observed. Given that large conductance changes affect capacitance measurements, these were always determined after the G_s and G_m changes had ceased (Pérez-Alvarez and Albillos, 2007). The fast component charge of the choline-evoked current was calculated by determining the integral from the baseline of the recording until 10 ms after the peak current.

Data analysis

Unless otherwise stated, data are given as the mean ± SEM. Paired or unpaired Student's *t*-tests were used to compare data.

Materials

PNU-120596 was purchased from TOCRIS Bioscience (Bristol, UK). ACh, choline, PNU-282987, 5-HI, α-Bgtx and MLA were from Sigma (Madrid, Spain). All drugs were dissolved in water, except PNU-120596, PNU-282987 and 5-hydroxyindole (5-HI) that were dissolved in DMSO. The concentrations of DMSO used to dissolve these drugs were 0.03% for 5-HI, 0.1% for PNU-120596, 0.01% for 3 μM PNU-282987 and 0.1% for 30 μM PNU-282987. Stock aliquots were stored at –20°C. Choline was always used at 10 mM, and ACh at 300 μM.

Results

α7 nAChR antagonists partially block currents elicited by ACh but not exocytosis

Using the voltage clamp mode in the perforated patch configuration of the patch-clamp technique, the presence of functional α7 nAChRs in the plasma membrane was investigated using ACh as stimulus (300 μM, 200 ms pulses applied every 2 min). We chose this concentration of ACh because it was sufficient to evoke substantial currents, without the large rebound produced using 1 mM ACh (Pérez-Alvarez and Albillos, 2007). In addition, 300 μM is the concentration considered to be attained by ACh at the neuromuscular junction (Kuffler and Yoshikami, 1975; Corringer *et al.*, 2000; Changeux and Edelstein, 2005). After obtaining stable responses, α-Bgtx (1 μM) or MLA (1 nM or 10 nM) were perfused to rapidly and selectively block α7 nAChRs. The nicotinic peak currents elicited by ACh (2.6 ± 0.3 nA, n = 10) were blocked by 6.0 ± 1.7% using α-Bgtx (n = 5) and by 7.1 ± 1.6% using 10 nM MLA (n = 5). Blockade was irreversible after wash out of α-Bgtx (Figure 1A and B) but not MLA (Figure 1D and E). Exocytosis, measured as a plasma C_m increase, was unaffected by the antagonists (n = 5) (Figure 1C and F).

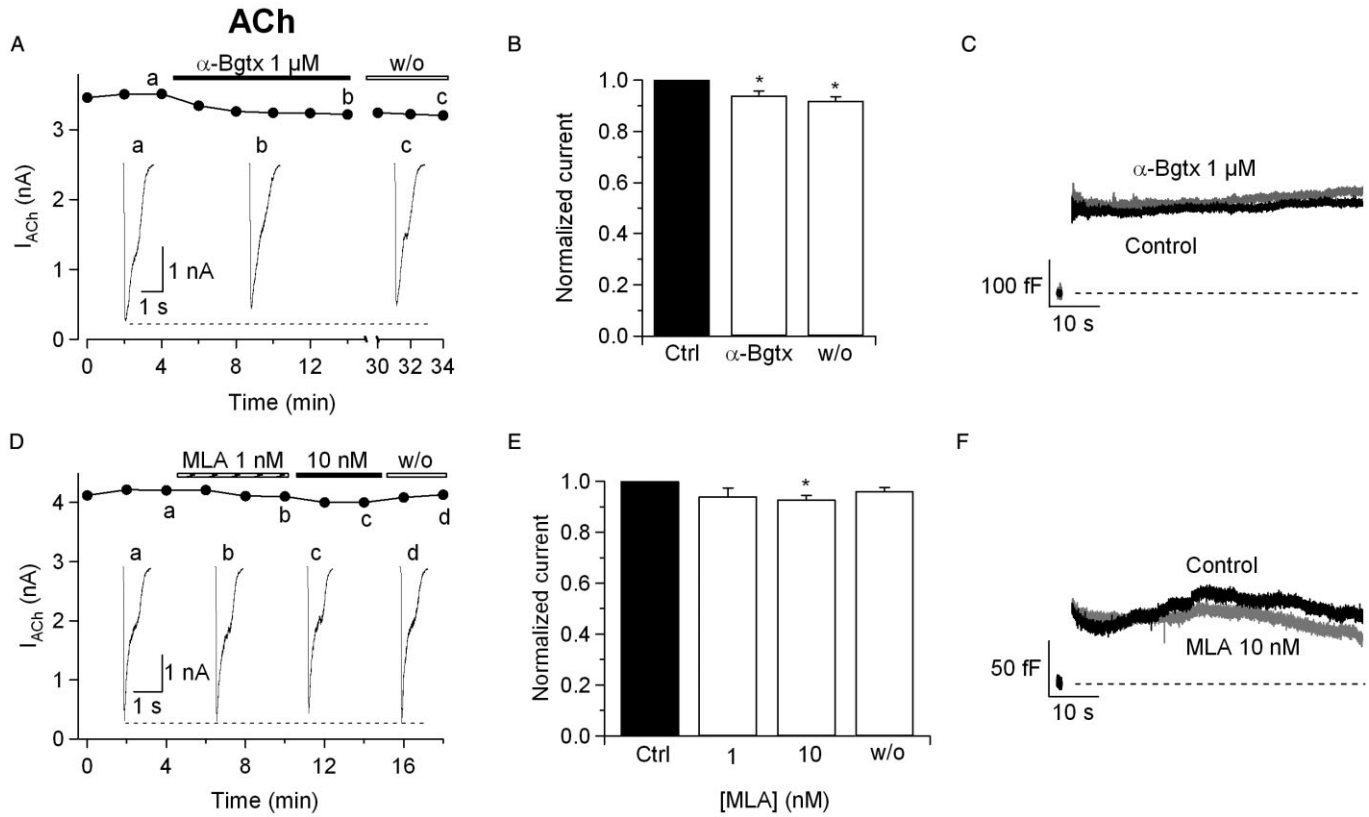

Figure 1

α-Bgtx and MLA blockade of ACh-evoked currents and exocytosis. 200 ms pulses of 300 μM ACh were applied every 2 min. Time course of blockade exerted by α-Bgtx 1 μM (A) or MLA 1–10 nM (D) on the ACh-elicited currents; perfusion of blocker was followed by washout (w/o\) to test reversibility. Bar diagrams represent means ± SEM and show the extent and reversibility of blockade by α-Bgtx (n = 5) (B) or MLA (n = 5) (E). Exocytotic responses as measured by C_m were unaffected by α-Bgtx (C) or MLA (F). Normalized data were obtained as the ratio of the agonist response in the presence of the antagonist, with respect to control conditions. *P < 0.05, significantly different from control; paired Student's t-test.

α7 nAChR-positive allosteric modulators (PAMs) potentiate ACh-elicited current and exocytosis

As the magnitude of the α7 current was small, an additional strategy was used to confirm the presence of α7 nAChRs. PAMs such as PNU-120596 (Hurst *et al.*, 2005; Gronlien *et al.*, 2007) or 5-hydroxyindole (5-HI) (Zwart *et al.*, 2002) can increase the current through homomeric α7 nAChRs. We tested these compounds in human chromaffin cells to assess their effects on the nicotinic currents elicited by ACh.

Perfusion with PNU-120596 (10 μM) potentiated the nicotinic current charge (1.7 ± 0.2 nC to 3.4 ± 0.9 nC) and the corresponding ACh-elicited exocytosis (116.2 ± 69 fF to 414 ± 233 fF) (n = 4) but did not increase the peak current (Fig 2). This effect was reversible after wash out (Figure 2A and B), and fully and irreversibly abolished after α-Bgtx perfusion in the presence of PNU-120596 (Supporting Information Figures S1A and B).

5-HI, at a concentration of 1 mM, blocked the peak current and the current charge elicited by ACh by 37 ± 6% and 64 ± 3% (Figure 2C) (n = 5), without significantly affecting exocytosis. This could be explained by the blockade exerted on non-α7 nAChRs by 5-HI, documented by other authors (Zwart *et al.*, 2002). A summary of the effects of these compounds on the peak current, charge and exocytosis is provided in Figure 2D.

The isolated α7 current component of the ACh response does not evoke exocytosis

To dissect the α7 current component of the ACh response, we assessed the effect of α-conotoxin (α-Ctx) MII[S4A,E11A,L15A], which has been shown to primarily target α6* nAChRs (Azam *et al.*, 2008), the main nAChR subtype expressed in human chromaffin cells (Albillos *et al.*, 2011). Perfusion with the toxin at 500 nM concentration showed a clear blockade of the peak current and exocytotic responses evoked by ACh (Figure 3A). The percentage of peak current remaining (~5%, n = 5) was consistent with the α7 contribution to the total current. Nevertheless, the slow kinetics of current inactivation and the exocytotic response observed in the presence of the toxin argued against a total blockade of non-α7 nAChRs. Application of higher concentrations (1–2 μM) unveiled a fast nicotinic current that was irreversibly blocked upon perfusion with α-Bgtx 1 μM (n = 5) (Figure 3B). The overall non-α7-current blockade was also reflected in the total abolition of the secretory response.

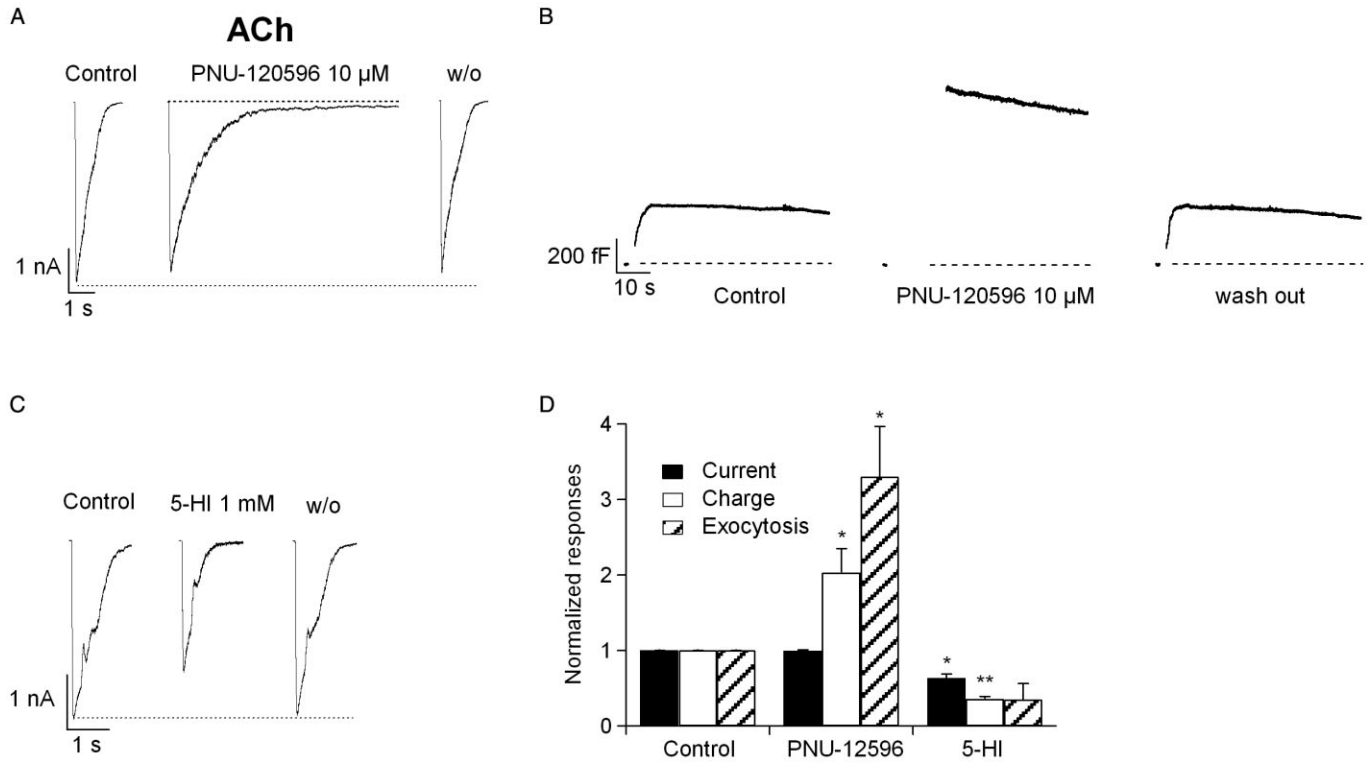

Figure 2

Effect of α7 nAChR PAMs on ACh-evoked currents and exocytosis. 200 ms pulses of 300 μM ACh were applied every 5 min. Original recordings of currents (A) and C_m (B) evoked by ACh in the presence or absence of PNU-120596 10 μM. Perfusion and washout (w/o) of 5-HI (1 mM) (C). Bar diagram comparing the effects of PNU-120596 ($n = 4$) and 5-HI ($n = 5$) on the ACh current, charge and exocytosis (D). Normalized data were obtained as the ratio of the agonist response in the presence of the modulator, with respect to control conditions. *$P < 0.05$, **$P < 0.01$, significantly different from control; paired Student's *t*-test.

Choline and PNU-282987 evoke α7 nicotinic currents that do not trigger exocytosis in human chromaffin cells

We used choline (10 mM), an α7 nAChR agonist (Papke *et al.*, 1996; Alkondon *et al.*, 1997; 1999; Frazier *et al.*, 1998), to characterize the α7 current and to assess the effects of selective α7 nAChR antagonists or PAMs. Choline binds with millimolar affinity to α7 nAChRs (Papke *et al.*, 1996; Alkondon *et al.*, 1997). It was used at 10 mM (EC_{100} on α7 nAChRs, Alkondon *et al.*, 1997; Fuentealba *et al.*, 2004) to ensure activation of α7 nAChRs.

Cell stimulation with choline 10 mM (200 ms) evoked a biphasic current with two clearly defined components. The first component was rapidly activated and inactivated. The second current component exhibited a slow activated stage with no inactivation (Figure 4A). The peak current amounted to 130 ± 16 pA ($n = 31$). However, the Ca^{2+} entry elicited by choline could not trigger exocytosis of secretory vesicles (Figure 4A, inset). In response to perfusion with the α7 receptor antagonist α-Bgtx, only the quickly activated and inactivated component was blocked (98 ± 1%, $n = 6$), and this component did not recover after toxin washout (Figure 4B). When tested in different cells, the α7 receptor antagonist MLA also blocked the initial current component elicited by choline by 97 ± 2% ($n = 8$). MLA washout resulted in recovery of the fast component of the nicotinic current (Figure 4C). A summary of the effects of α7 receptor antagonist blockade and washout is provided in Figure 4D.

Choline is a partial non-α7 agonist (Papke *et al.*, 1996; Fuentealba *et al.*, 2004). The α7 component of the choline response could be isolated by applying α-Ctx MII[S4A, E11A, L15A] at a concentration of 500 nM and this component was fully and reversibly blocked with 10 nM MLA ($n = 6$) (Figure 4E). These data show that the initial component of the biphasic current elicited by choline corresponds to activation of α7 nAChRs.

In addition, we evaluated the effect of PNU-282987, a synthetic selective α7 nAChR agonist. This compound evoked a peak current of 42 ± 9.8 pA at 3 μM and of 57.7 ± 9.2 pA at 30 μM ($n = 6$). Choline was applied to the same cells as PNU-282987 to compare their responses (Figure 5A and B). The PNU-282987 peak current amounted to 37 ± 5% (at 3 μM) and 42 ± 6% (at 30 μM) of the peak current response shown by the choline α7 component (Figure 5C). MLA (10 nM) fully abolished the nicotinic current elicited by PNU-282987 as well as the initial peak current evoked by choline in the same cells, confirming the α7 nature of the PNU-282987 response (Figure 5A).

The α7 current kinetics elicited by choline and PNU-282987 were analysed in order to be compared with previous

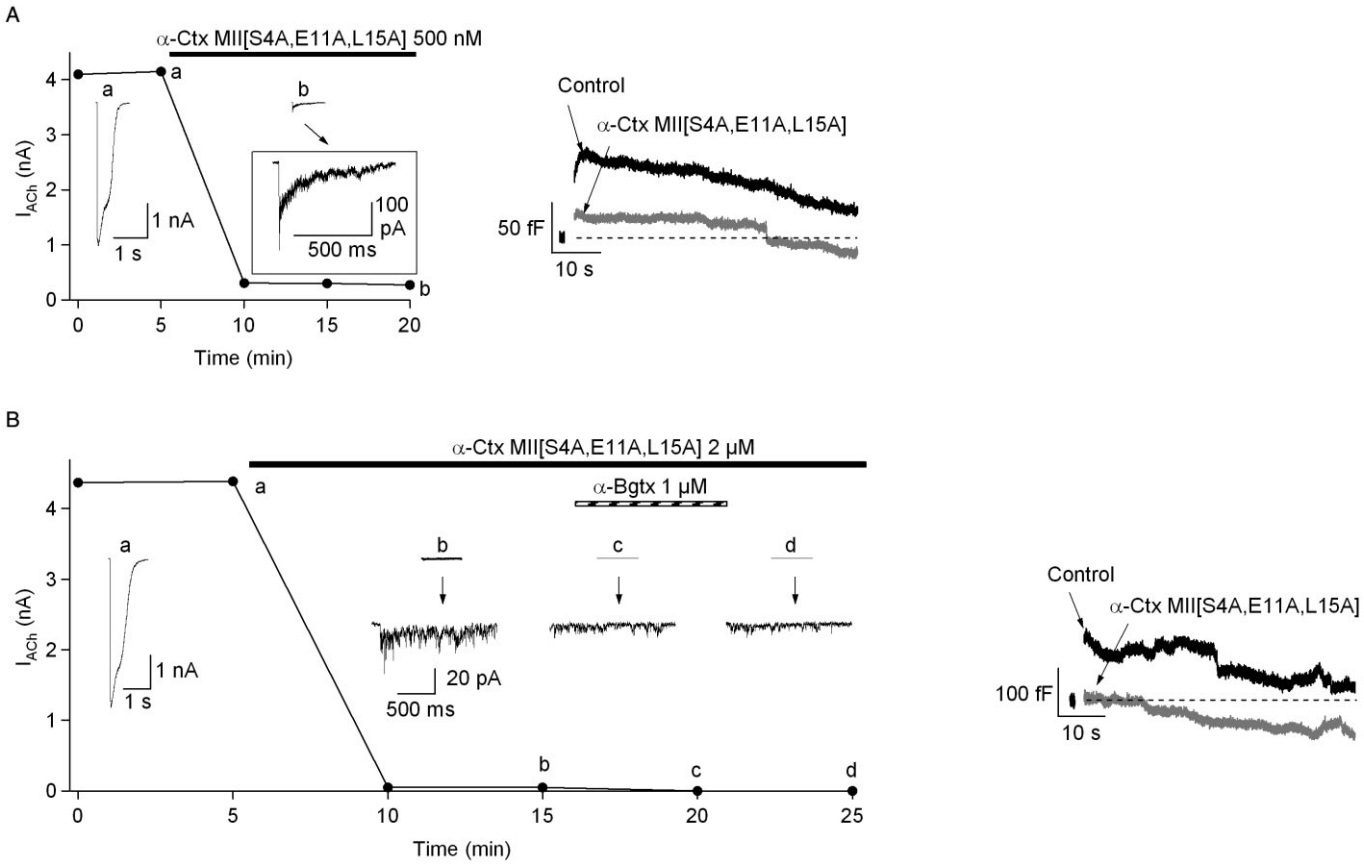

Figure 3

Characterization of the α7 current component and the corresponding exocytosis elicited by ACh. Panel A (left) shows the time course of blockade exerted by α-Ctx MII[S4A,E11A,L15A] (500 nM) on ACh-induced currents. The insets show original recordings of ACh before and after perfusion with the toxin. On the right, the original traces of the exocytotic responses elicited by ACh before and after perfusion with α-Ctx MII[S4A,E11A,L15A] are displayed. Panel B shows the time course of blockade exerted by α-Ctx MII[S4A,E11A,L15A] at a higher concentration (2 μM) on ACh-induced currents. α-Bgtx perfusion exerted an additional and irreversible blockade on the α-Ctx MII[S4A,E11A,L15A]-resistant current (n = 5). The insets show original recordings of ACh before and after perfusion with the toxins. On the right, the original traces of the exocytotic responses elicited by ACh before and after perfusion with α-Ctx MII[S4A,E11A,L15A] are shown.

data reported in other cell systems (Supporting Information Figure S2). In the case of choline, the time measured for 10–90% activation (t_{10-90}) was 5.5 ± 0.4 ms. The inactivation phase could be fitted to a single exponential curve, giving a time constant of inactivation ($\tau_{inactiv}$) of 8.5 ± 0.4 ms (n = 31). The kinetic study of the currents yielded by PNU-282987 indicated quickly activated and inactivated stages for both concentrations, with t_{10-90} = 7.1 ± 0.4 ms and $\tau_{inactiv}$ = 10.0 ± 0.9 ms for 3 μM, and t_{10-90} = 5.5 ± 0.4 ms and $\tau_{inactiv}$ = 9.8 ± 1.8 ms for 30 μM (n = 6).

α7 nAChR PAMs potentiate choline- and PNU-282987- elicited responses

When the agonist used was choline, 5-HI (1 mM) evoked a large increase in the initial peak current (α7 component as previously described). The potentiation achieved by 5-HI was 3.5-fold in the cell shown in Figure 6A. On average, the peak current elicited by choline was augmented (132 ± 43 pA to 426 ± 139 pA) (n = 5) (Figure 6B). This increase was abolished, together with the initial peak current elicited by choline, after perfusion with MLA (10 nM) and was fully restored after washout. Potentiation of α7 peak current by 5-HI, however, did not cause overall current charge (α7 and non-α7) potentiation. This may be explained by the blocking effects of 5-HI on the non-α7 component of the choline-elicited current (Figure 6A and B).

PNU-120596 caused a marked potentiation of the choline response, which was reversible after washout. The choline-evoked peak current was increased to 1.5 ± 0.4 nA and the current charge to 5.7 ± 2.2 nC (n = 6) (Figure 6C–E). Exocytosis was also largely potentiated although a numerical value for the potentiation from a null value could not be calculated. Current, charge and exocytosis increments were irreversibly abolished after application of α-Bgtx (1 μM) (Supporting Information Figure S3).

When the agonist used was PNU-282987 (3 μM), currents and exocytosis were also increased by PNU-120596. Peak currents were increased to 2.4 ± 0.2 nA and the current charge to 12.5 ± 2.9 nC (n = 5) (Figure 6F–H).

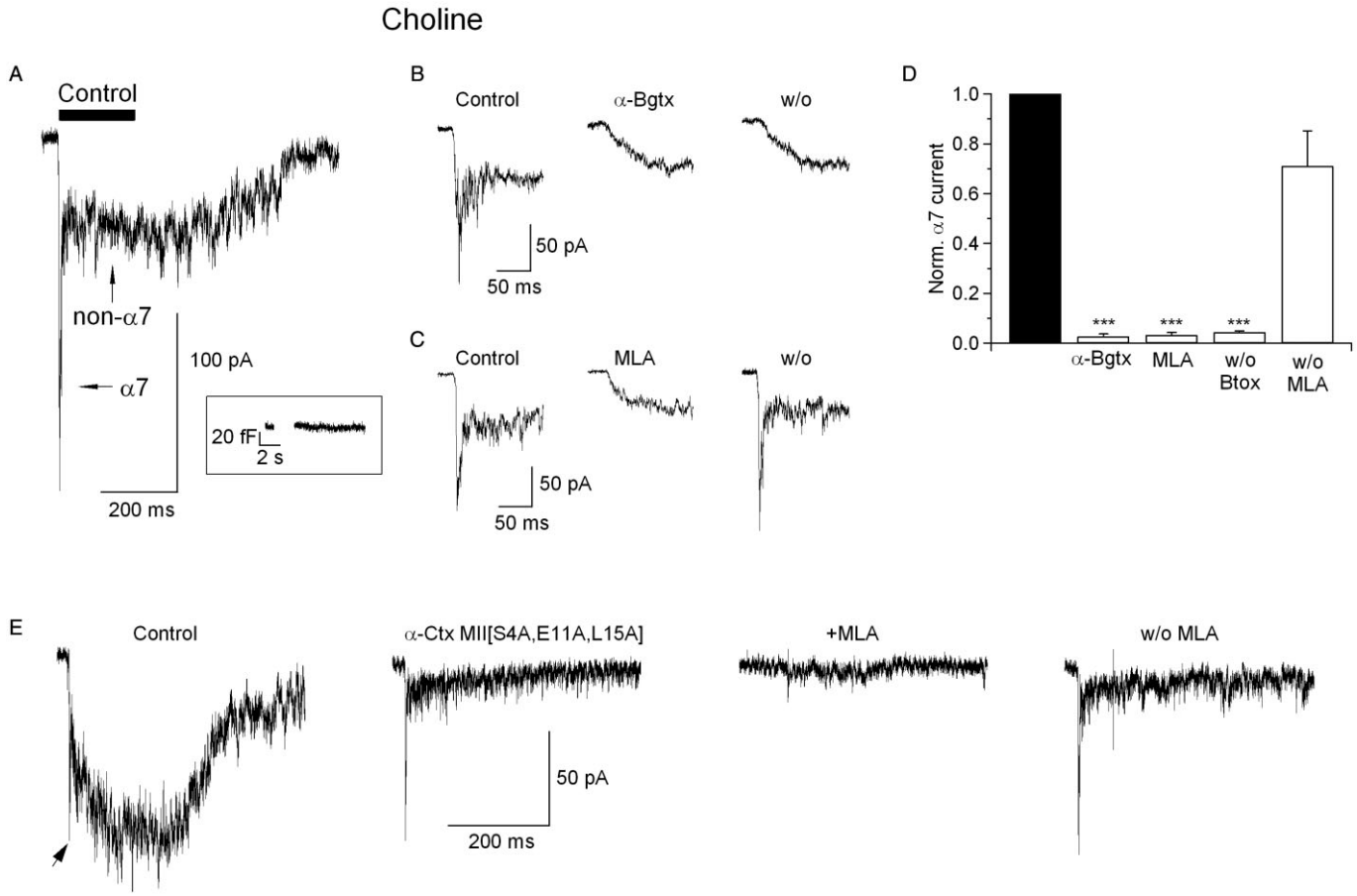

Figure 4

Characterization of α7 currents and the corresponding exocytosis evoked by the α7 nAChR agonist choline. Choline pulses (10 mM, bar represents 200 ms of drug application) elicit a biphasic current response (A) with a fast (α7) and slow component (non-α7). There is no sizeable exocytotic response to this stimulus (see inset for the C_m recording). Original recordings showing selective blockade of the α7 component by α-Bgtx 1 μM (B) or MLA 10 nM (C). In (D), summary results of the block and washout(w/o) of the α7 component, exerted by α-Bgtx (n = 6) and MLA (n = 8). Normalized data were obtained as the ratio of the agonist response in the presence of the antagonist, with respect to control conditions. ***P < 0.001, significantly different from control; paired Student's t-test. In (E), original recordings of the 10 nM MLA blockade and wash out (w/o) of the α7 currents elicited by choline after perfusion of 500 nM α-Ctx MII[S4A,E11A,L15A].

The kinetics of the fast current component were preserved in the 5-HI-potentiated choline-elicited currents (t_{10-90} = 7.2 ± 0.7 ms, $\tau_{inactiv}$ = 10.1 ± 0.6 ms), in good agreement with the kinetic properties of type I PAMs, which do not influence inactivation (Gronlien *et al.*, 2007). However, in the presence of PNU-120596, the current elicited by choline showed significantly slower kinetics (t_{10-90} = 776.2 ± 33.3 ms, $\tau_{inactiv}$ = 2.32 ± 0.2 s) consistent with the actions of a type II PAM (Gronlien *et al.*, 2007). When the agonist was PNU-282987, PNU-120596 potentiated current kinetics were also significantly slower as compared with controls (t_{10-90} = 890.7 ± 113.8 ms, $\tau_{inactiv}$ = 4.8 ± 0.6 s) (n = 5) (Supporting Information Figure S4).

α7 activation triggers exocytosis by depolarizing the cell membrane

Although Ca^{2+} entry through the ionophore associated with the α7 nAChR yielded no secretory response, we next tried to determine whether this receptor could contribute to the exocytosis by means of plasma membrane depolarization and subsequent recruitment of Ca^{2+} channels, as reported for nAChRs in human chromaffin cells (Pérez-Alvarez and Albillos, 2007). This goal was achieved by investigating the exocytotic response elicited by the selective stimulation of the α7 nAChRs, and consequent evoked depolarization, by choline in the presence of 500 nM α-Ctx MII[S4A, E11A, L15A] or PNU-282987. Exocytosis elicited by agonist depolarization was measured using the 'triple-step' protocol. The exocytotic response elicited by 10 mM choline after perfusion with 500 nM α-Ctx MII[S4A, E11A, L15A] or 30 μM PNU-282987 was 26 ± 4 fF (n = 5) and 50 ± 13 fF (n = 7), respectively (Figure 7A and B), calculated as the difference between the C_m values of steps 3 and 1.

Finally, PAMs are being developed to improve cognitive disorders (Hajós and Rogers, 2010). In this sense, it would be relevant to analyse the effect of these drugs on the exocytotic

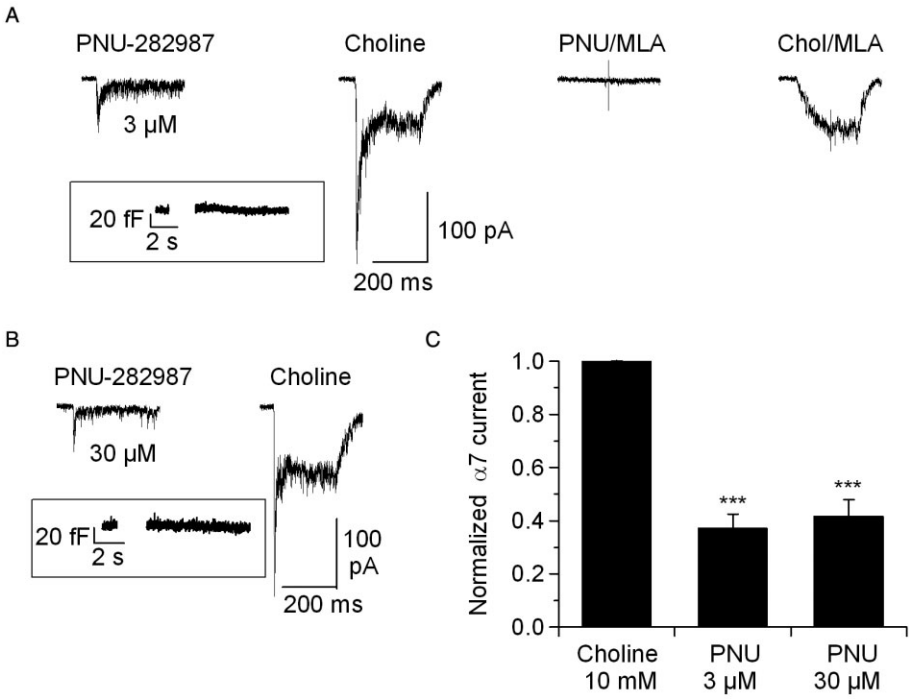

Figure 5

Characterization of α7 currents and the corresponding exocytosis evoked by the α7 nAChR agonist PNU-282987. Original recordings of currents and membrane capacitances elicited by the selective α7 agonist PNU-282987 (3 μM) and choline in the same cell (A). MLA abolished the α7 component activated by both agonists. PNU-282987 at 30 μM elicited an α7 current of similar magnitude to 3 μM and no exocytosis (B). In (C), summary results of the current and charge elicited by pulses of PNU-282987 (3 μM and 30 μM), normalized to the choline response in the same cell. ***$P < 0.001$, significantly different from choline alone; paired Student's *t*-test ($n = 6$).

process that would lead to catecholamine release in human chromaffin cells. PNU-120596 (1 μM) was tested on the agonist depolarization (ACh or choline)-elicited exocytosis, which was measured using the 'triple-step' protocol. The depolarization elicited by 300 μM ACh generated a secretory response of 61.7 ± 7 fF ($n = 4$), which was potentiated by 1 μM PNU-120596. The depolarization duration also largely increased in these cells (Supporting Information Figure S5A). When 10 mM choline was used as agonist, secretion was 40 ± 13 fF ($n = 5$). In the cell shown in Supporting Information Figure S5B, the choline secretory response was potentiated by PNU-120596, from 15 fF to 914 fF, and the duration of the depolarization from 0.9 s to 28.8 s.

Discussion

Although the presence of mRNA for α7 nAChRs in human chromaffin cells has been previously reported (Mousavi *et al.*, 2001), our results constitute the first electrophysiological recordings and characterization of functional α7 nAChRs in primary cultures of human chromaffin cells and provide evidence for their contribution to depolarization-elicited exocytosis in these cells. ACh-evoked currents attributable to α7 nAChRs have also been described in the peripheral nervous system (Zhang *et al.*, 1994; Cuevas and Berg, 1998) and CNS (Zorumski *et al.*, 1992; Alkondon and Albuquerque, 1993) of other mammals.

Choline behaves as an agonist of α7 nAChRs (Papke *et al.*, 1996; Alkondon *et al.*, 1997; 1999; Frazier *et al.*, 1998), and its dual effect has been reported of potentiating or inhibiting α4β4 nAChRs (Zwart and Vijverberg, 2000), as well as activating α3β4 nAChRs expressed in *Xenopus* oocytes (Fuentealba *et al.*, 2004). This could explain the biphasic current, showing an initial peak and a second plateau stage, elicited by this agonist in the present study. This initial peak current was triggered through activation of rapidly activated and inactivated α7 nAChRs, as it was blocked by the α7 nAChR antagonists MLA or α-Bgtx and was potentiated by the α7 nAChR PAMs 5-HI or PNU-120596. The α7 nAChR current could be isolated using the selective α6* receptor blocker α-Ctx MII[S4A, E11A,L15A] (Azam *et al.*, 2008), and further abolished by MLA. Rapid inactivation of this initial α7 current peak in the presence of choline (8.5 ± 0.4 ms) was of the same order of magnitude as the ACh-elicited current reported in native systems, such as cultured postnatal rat hippocampal neurons (8 ± 2 ms) (Zorumski *et al.*, 1992), or choline-elicited responses in tuberomammillary histamine neurons of the posterior hypothalamus (9.65 ± 1.1 ms) (Uteshev *et al.*, 2002). The second stage of the choline-evoked current was produced by activation of non-α7 nAChRs, as indicated by the blockade of these nAChRs by α-Ctx MII[S4A, E11A,L15A].

α-Bgtx and MLA have also been shown to block α9* nAChRs (Elgoyhen *et al.*, 1994; 2001; Verbitsky *et al.*, 2000). However, the irreversible nature of the α-Bgtx blockade

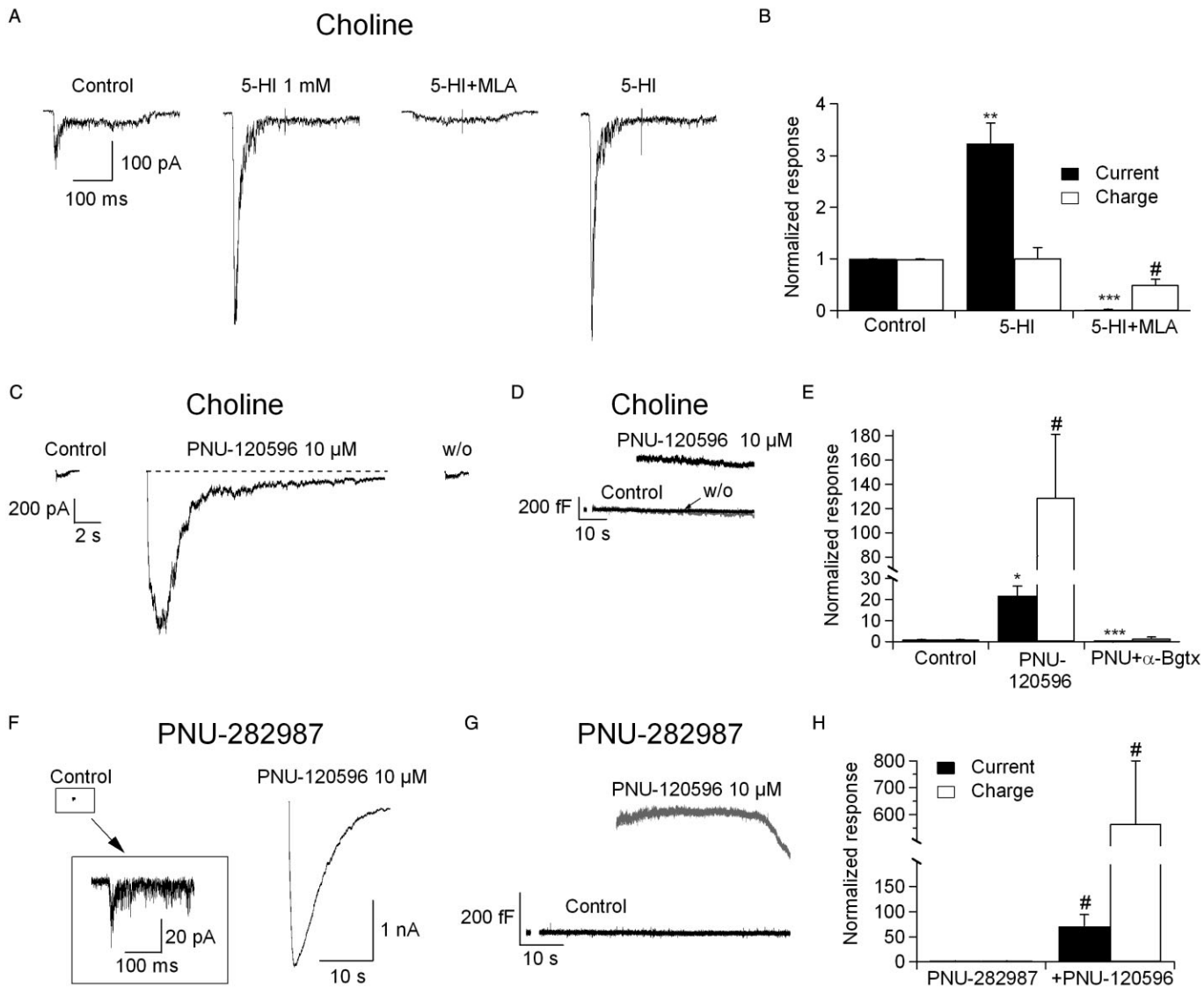

Figure 6

Potentiation of the α7 nAChR current and exocytosis by PAMs. Original recordings of potentiation and blockade of choline currents by 5-HI 1 mM and 5-HI+MLA (A). In (B), summary results of the effects on choline responses exerted by 5-HI ($n = 5$) or 5-HI+MLA. Normalized data were obtained as the ratio of the agonist response in the presence of the modulator, with respect to control conditions. $^{\#}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$, significantly different from control; paired Student's *t*-test.The potentiation by 10 μM PNU-120596 of choline-elicited currents is shown in panel C along with washout (w/o). The corresponding exocytotic response was also selectively potentiated (D). In (E), summary results of the effect on choline responses exerted by PNU-120596 ($n = 6$) or PNU-120596 plus α–Bgtx. Normalized data were obtained as the ratio of the agonist response in the presence of the modulator, with respect to control conditions. $^{\#}P < 0.05$, $^{*}P < 0.01$, $^{***}P < 0.001$, significantly different from control; paired Student's *t*-test. Currents (F) and exocytosis (G) elicited by 3 μM PNU-282987 were also strongly potentiated by PNU-120596. In (H), summary results of the effect of PNU-120596 on the 3 μM PNU-282987 responses. Normalized data were obtained as the ratio of the agonist response in the presence of the antagonist or modulator respect to control conditions. $^{\#}P < 0.05$, significantly different from control (PNU-282987 alone); paired Student's *t*-test. ($n = 5$).

observed here, the fast activation and desensitization kinetics of the currents involved, and the selective action of the modulators on α7 nAChRs (Zwart *et al.*, 2002; Hurst *et al.*, 2005) make it unlikely that the nAChR subtypes sensitive to α-Bgtx and MLA reported here are of the α9* subtype.

In human adult chromaffin cells, mRNAs for α7 and β2 receptor subunits have been detected (Mousavi *et al.*, 2001). Given the confirmation by Liu *et al.* (2009) of earlier data (Khiroug *et al.*, 2002) supporting the presence of a functional α7β2 receptor in rat brain, it could be that such a receptor is expressed in human chromaffin cells. However, two lines of evidence refute this possibility: first, the α7 current observed in our study exhibits fast activation and inactivation kinetics, comparable to those shown by homomeric receptors (Zorumski *et al.*, 1992) yet contrary to the slow current kinetics reported for α7β2 heteromeric nAChRs (Khiroug *et al.*, 2002;

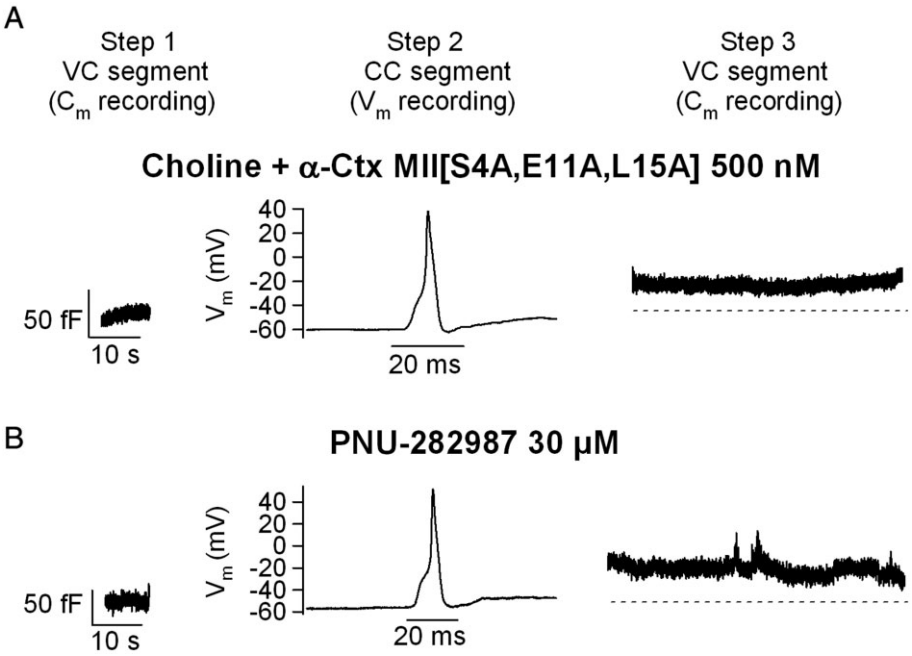

Figure 7

Contribution of α7 nAChRs to depolarization-elicited exocytosis revealed by their selective potentiation and activation. A modification of the 'triple-step' protocol described by Pérez-Alvarez and Albillos, (2007) was used to measure the exocytotic responses evoked by the selective activation of α7 nAChRs by choline plus α-Ctx-MII[S4A, E11A, L15A] 500 nM (A) or PNU-282987 30 μM (B), applied in the current clamp (CC) configuration to induce depolarization. In the first step, the basal C_m was recorded by applying a sine wave in the voltage clamp (VC) mode. Step 2 was performed under the current clamp configuration to allow the agonist to depolarize the plasma membrane and evoke exocytosis, which was measured in step 3 by recording C_m in the voltage clamp configuration. V_h was –60 mV, as a potential close to the resting membrane potential of human chromaffin cells.

Liu *et al.*, 2009); and second, the lack of an effect of 3 μM dihydro-β-erythroidine (DHβE; data not shown) on human α7 nAChRs here reported, a concentration well above the IC_{50} of 0.17 μM described for the highly sensitive α7β2 receptor (Liu *et al.*, 2009).

Evidence for functional α7 nAChRs has been reported in bovine chromaffin cells as the blockade by α7 nAChR antagonists of the current or secretory response elicited by long pulses of agonists (1–5 s) (López *et al.*, 1998), or as the cytosolic Ca^{2+} transient or secretion induced by low concentrations of selective α7 nAChR agonists perfused for 10–30 s in the presence of PNU-120596 (Del Barrio *et al.*, 2011). However, in this latter study, direct recording of the α7 nAChR Ca^{2+} signal using α7 nAChR agonists was not possible, probably because of the long stimuli and static system of drug application used. Indeed, it has been reported that rapid drug application is crucial to record fast activated and inactivated α7 nAChR currents (Vijayaraghavan *et al.*, 1992; Zhang *et al.*, 1994). Neither have functional α7 nAChRs been found in rat chromaffin cells (Di Angelantonio *et al.*, 2003), most likely because 100 μM nicotine mainly activates non-α7 nAChRs as shown in bovine chromaffin cells (Del Barrio *et al.*, 2011). In effect, the present results are the first description of the recording of isolated native α7 nAChR currents in chromaffin cells using short α7 nAChR agonist stimuli and a rapid agonist perfusion system. Thus, it seems that the fast activation and inactivation kinetics of α7 nAChR currents dictate a need for high resolution techniques and fast agonist perfusion systems to record these currents.

Interestingly, autoradiography of bovine adrenal gland slices with α-Bgtx indicates that α7 nAChRs are restricted to medullary areas adjacent to the adrenal cortex and co-localize with the enzyme PNMT, which methylates noradrenaline to adrenaline and thus confers the ability to release adrenaline on chromaffin cells (the 'adrenergic phenotype') (Criado *et al.*, 1997). In addition, glucocorticoid-activated PNMT expression increases with stress (Wurtman and Axelrod, 1965; Kvetnansky *et al.*, 2006; Tai *et al.*, 2007). Accordingly, because human chromaffin cells mainly display an adrenergic phenotype (99% of cells) (Pérez-Alvarez *et al.*, 2008), it is plausible that α7 nAChR expression also increases with stress to produce the faster neurotransmitter response necessary. However, in a recent study by Colomer *et al.*, (2010) in rat chromaffin cells, no changes in the expression levels of α7 nAChR transcripts were observed.

Here we also show that α7 nAChRs contribute to exocytosis when plasma membrane depolarization is evoked by specific α7 nAChR agonists (i.e. choline after perfusion with α-Ctx MII[S4A, E11A, L15A] or PNU-282987). In bovine chromaffin cells, α7 nAChRs were shown to contribute to exocytosis using α7 nAChR antagonists to block ACh-evoked catecholamine release from a population of cells (López *et al.*, 1998). Also, the α7 agonist PNU-282987, in the presence of the allosteric potentiator PNU-120596, stimulated catechola-

mine release in a population of bovine cells (Del Barrio *et al.*, 2011). In the present study, through the use of a high-resolution patch-clamp technique and brief exposure to agonists, we were able to record the exocytotic responses produced to α7 nAChR agonists. In other cell systems, α7 nAChRs have been found to presynaptically modulate (McGehee *et al.*, 1995; Gray *et al.*, 1996; Guo *et al.*, 1998; Li *et al.*, 1998) or influence (Coggan *et al.*, 1997) neurotransmitter release. Postsynaptically, they are known to generate depolarizing currents (Frazier *et al.*, 1998) but no evidence of a role in exocytosis has been reported.

There is also mounting evidence for a role of choline as a modulator of synaptic cholinergic neurotransmission at the splachnic nerve chromaffin cell synapse (Holz and Senter, 1981) acting through α7 nAChRs (Fuentealba *et al.*, 2004). However, the incomplete blockade exerted by 100 nM of α-Bgtx and MLA on the choline-elicited secretory response has cast doubts on the nature of the nAChR subtype involved in such choline effects (Fuentealba *et al.*, 2004). Here we show that choline activated α7 nAChRs and, thus, could have a functional role in regulating cholinergic synaptic activity by acting on these receptors.

Finally, our data could also have therapeutic implications. α7 nAChRs are considered pharmacological targets to treat several diseases, and α7 nAChR PAMs are being investigated as candidates for improving cognitive deficits and neurodegeneration (Hurst *et al.*, 2005; Dunlop *et al.*, 2009; Dinklo *et al.*, 2011). Our data show that exocytosis is greatly enhanced in the presence of PNU-120596, suggesting that treatment with PAMs might have some effect on peripheral catecholamine secretion via actions on chromaffin cells. This effect would need to be considered as these medications are developed. In addition, our study validates the use of human chromaffin cells as a model to investigate the functional roles of α7 nAChRs.

Acknowledgements

The authors thank the donors of the human adrenal glands and their relatives. We also thank Dr Agustín Albillos and the transplant teams of the Hospital Ramón y Cajal and Hospital Universitario La Paz for their excellent coordination in supplying the human adrenal glands. AHV holds a fellowship award from the Universidad Autónoma de Madrid. This work was supported by a grant from the Ministerio de Ciencia y Tecnología No. BFU2008-01382/BFI awarded to AA, and NIH Grants MH53631 and GM48677 to JMM.

Conflicts of interest

None declared.

References

Albillos A, Dernick G, Horstmann H, Almers W, Alvarez de Toledo G, Lindau M (1997). The exocytotic event in chromaffin cells revealed by patch amperometry. Nature 389: 509–512.

Albillos A, Pérez-Alvarez A, McIntosh JM, Alonso y Gregorio S, Tabernero A (2011). nAChRs in human chromaffin cells are mainly composed by α6β4* subunits. Nicotinic Acetylcholine Receptors 2011. Wellcome Trust Conferences, Hinxton, UK.

Alexander SPH, Mathie A, Peters JA (2011). Guide to receptors and channels (GRAC), 5th edition. Br J Pharmacol 164 (Suppl. 1): S1–S324.

Alkondon M, Albuquerque EX (1993). Diversity of nicotinic acetylcholine receptors in rat hippocampal neurons. I. Pharmacological and functional evidence for distinct structural subtypes. J Pharmacol Exp Ther 265: 1455–1473.

Alkondon M, Pereira EF, Wonnacott S, Albuquerque EX (1992). Blockade of nicotinic currents in hippocampal neurons defines methyllycaconitine as a potent and specific receptor antagonist. Mol Pharmacol 41: 802–808.

Alkondon M, Pereira EF, Cortes WS, Maelicke A, Albuquerque EX (1997). Choline is a selective agonist of alpha7 nicotinic acetylcholine receptors in the rat brain neurons. Eur J Neurosci 9: 2734–2742.

Alkondon M, Pereira EF, Albuquerque EX (1998). Alpha-bungarotoxin- and methyllycaconitine-sensitive nicotinic receptors mediate fast synaptic transmission in interneurons of rat hippocampal slices. Brain Res 810: 257–263.

Alkondon M, Pereira EF, Eisenberg HM, Albuquerque EX (1999). Choline and selective antagonists identify two subtypes of nicotinic acetylcholine receptors that modulate GABA release from CA1 interneurons in rat hippocampal slices. J Neurosci 19: 2693–2705.

Almazán G, Aunis D, García AG, Montiel C, Nicolas GP, Sanchez-García P (1984). Effects of collagenase on the release of [3H]-noradrenaline from bovine cultured adrenal chromaffin cells. Br J Pharmacol 81: 599–610.

Azam L, Yoshikami D, McIntosh JM (2008). Amino acid residues that confer high selectivity of the alpha6 nicotinic acetylcholine receptor subunit to alpha-conotoxin MII[S4A,E11A,L15A]. J Biol Chem 283: 11625–11632.

Bertrand D, Bertrand S, Ballivet M (1992). Pharmacological properties of the homomeric alpha 7 receptor. Neurosci Lett 146: 87–90.

Bertrand D, Galzi JL, Devillers-Thiery A, Bertrand S, Changeux JP (1993). Mutations at two distinct sites within the channel domain M2 alter calcium permeability of neuronal alpha 7 nicotinic receptor. Proc Natl Acad Sci USA 90: 6971–6975.

Bibevski S, Zhou Y, McIntosh JM, Zigmond RE, Dunlap ME (2000). Functional nicotinic acetylcholine receptors that mediate ganglionic transmission in cardiac parasympathetic neurons. J Neurosci 20: 5076–5082.

Blumenthal EM, Conroy WG, Romano SJ, Kassner PD, Berg DK (1997). Detection of functional nicotinic receptors blocked by alpha-bungarotoxin on PC12 cells and dependence of their expression on post-translational events. J Neurosci 17: 6094–6104.

Chang KT, Berg DK (1999). Nicotinic acetylcholine receptors containing alpha7 subunits are required for reliable synaptic transmission in situ. J Neurosci 19: 3701–3710.

Changeux JP, Edelstein SJ (2005). Nicotinic Acetylcholine Receptors: From Molecular Biology to Cognition. Odile Jacob: New York.

Coggan JS, Paysan J, Conroy WG, Berg DK (1997). Direct recording of nicotinic responses in presynaptic nerve terminals. J Neurosci 17: 5798–5806.

Colomer C, Olivos-Ore LA, Vincent A, McIntosh JM, Artalejo AR, Guerineau NC (2010). Functional characterization of alpha9-containing cholinergic nicotinic receptors in the rat adrenal medulla: implication in stress-induced functional plasticity. J Neurosci 30: 6732–6742.

Corringer PJ, Le Novere N, Changeux JP (2000). Nicotinic receptors at the amino acid level. Annu Rev Pharmacol Toxicol 40: 431–458.

Criado M, Dominguez del Toro E, Carrasco-Serrano C, Smillie FI, Juiz JM, Viniegra S *et al*. (1997). Differential expression of alpha-bungarotoxin-sensitive neuronal nicotinic receptors in adrenergic chromaffin cells: a role for transcription factor Egr-1. J Neurosci 17: 6554–6564.

Cuevas J, Berg DK (1998). Mammalian nicotinic receptors with alpha7 subunits that slowly desensitize and rapidly recover from alpha-bungarotoxin blockade. J Neurosci 18: 10335–10344.

Del Barrio L, Egea J, Leon R, Romero A, Ruiz A, Montero M *et al*. (2011). Calcium signalling mediated through alpha7 and non-alpha7 nAChR stimulation is differentially regulated in bovine chromaffin cells to induce catecholamine release. Br J Pharmacol 162: 94–110.

Delbono O, Gopalakrishnan M, Renganathan M, Monteggia LM, Messi ML, Sullivan JP (1997). Activation of the recombinant human alpha 7 nicotinic acetylcholine receptor significantly raises intracellular free calcium. J Pharmacol Exp Ther 280: 428–438.

Di Angelantonio S, Matteoni C, Fabbretti E, Nistri A (2003). Molecular biology and electrophysiology of neuronal nicotinic receptors of rat chromaffin cells. Eur J Neurosci 17: 2313–2322.

Dinklo T, Shaban H, Thuring JW, Lavreysen H, Stevens KE, Zheng L *et al*. (2011). Characterization of 2-[[4-fluoro-3-(trifluoromethyl) phenyl]amino]-4-(4-pyridinyl)-5-thiazoleme thanol (JNJ-1930942), a novel positive allosteric modulator of the {alpha}7 nicotinic acetylcholine receptor. J Pharmacol Exp Ther 336: 560–574.

Drisdel RC, Green WN (2000). Neuronal alpha-bungarotoxin receptors are alpha7 subunit homomers. J Neurosci 20: 133–139.

Dunlop J, Lock T, Jow B, Sitzia F, Grauer S, Jow F *et al*. (2009). Old and new pharmacology: positive allosteric modulation of the alpha7 nicotinic acetylcholine receptor by the 5-hydroxytryptamine(2B/C) receptor antagonist SB-206553 (3,5-dihydro-5-methyl-N-3-pyridinylbenzo[1,2-b:4,5-b′]di pyrrole-1(2H)-carboxamide). J Pharmacol Exp Ther 328: 766–776.

Elgoyhen AB, Johnson DS, Boulter J, Vetter DE, Heinemann S (1994). Alpha 9: an acetylcholine receptor with novel pharmacological properties expressed in rat cochlear hair cells. Cell 79: 705–715.

Elgoyhen AB, Vetter DE, Katz E, Rothlin CV, Heinemann SF, Boulter J (2001). Alpha10: a determinant of nicotinic cholinergic receptor function in mammalian vestibular and cochlear mechanosensory hair cells. Proc Natl Acad Sci USA 98: 3501–3506.

El-Hajj RA, McKay SB, McKay DB (2007). Pharmacological and immunological identification of native alpha7 nicotinic receptors: evidence for homomeric and heteromeric alpha7 receptors. Life Sci 81: 1317–1322.

Frazier CJ, Buhler AV, Weiner JL, Dunwiddie TV (1998). Synaptic potentials mediated via alpha-bungarotoxin-sensitive nicotinic acetylcholine receptors in rat hippocampal interneurons. J Neurosci 18: 8228–8235.

Free RB, McKay SB, Boyd RT, McKay DB (2002). Evidence for constitutive expression of bovine adrenal a3beta4* nicotinic acetylcholine receptors. Ann N Y Acad Sci 971: 145–147.

Fuentealba J, Olivares R, Ales E, Tapia L, Rojo J, Arroyo G *et al*. (2004). A choline-evoked [Ca2+]c signal causes catecholamine release and hyperpolarization of chromaffin cells. FASEB J 18: 1468–1470.

García-Guzmán M, Sala F, Sala S, Campos-Caro A, Stuhmer W, Gutierrez LM *et al*. (1995). alpha-Bungarotoxin-sensitive nicotinic receptors on bovine chromaffin cells: molecular cloning, functional expression and alternative splicing of the alpha 7 subunit. Eur J Neurosci 7: 647–655.

Gillis KD (1995). Techniques for membrane capacitance measurements. In: Sackmann B, Neher E (eds). Single Channel Recording, 2nd edn. Plenum: New York, pp. 155–198.

Gray R, Rajan AS, Radcliffe KA, Yakehiro M, Dani JA (1996). Hippocampal synaptic transmission enhanced by low concentrations of nicotine. Nature 383: 713–716.

Gronlien JH, Hakerud M, Ween H, Thorin-Hagene K, Briggs CA, Gopalakrishnan M *et al*. (2007). Distinct profiles of alpha7 nAChR positive allosteric modulation revealed by structurally diverse chemotypes. Mol Pharmacol 72: 715–724.

Guo JZ, Tredway TL, Chiappinelli VA (1998). Glutamate and GABA release are enhanced by different subtypes of presynaptic nicotinic receptors in the lateral geniculate nucleus. J Neurosci 18: 1963–1969.

Hajós M, Rogers BN (2010). Targeting alpha7 nicotinic acetylcholine receptors in the treatment of schizophrenia. Curr Pharm Des 16: 538–554.

Holz RW, Senter RA (1981). Choline stimulates nicotinic receptors on adrenal medullary chromaffin cells to induce catecholamine secretion. Science 214: 466–468.

Horrigan FT, Bookman RJ (1994). Releasable pools and the kinetics of exocytosis in adrenal chromaffin cells. Neuron 13: 1119–1129.

Hurst RS, Hajós M, Raggenbass M, Wall TM, Higdon NR, Lawson JA *et al*. (2005). A novel positive allosteric modulator of the alpha7 neuronal nicotinic acetylcholine receptor: *in vitro* and *in vivo* characterization. J Neurosci 25: 4396–4405.

Khiroug SS, Harkness PC, Lamb PW, Sudweeks SN, Khiroug L, Millar NS *et al*. (2002). Rat nicotinic ACh receptor alpha7 and beta2 subunits co-assemble to form functional heteromeric nicotinic receptor channels. J Physiol 540: 425–434.

Kilpatrick DL, Slepetis R, Kirshner N (1981). Inhibition of catecholamine secretion from adrenal medulla cells by neurotoxins and cholinergic antagonists. J Neurochem 37: 125–131.

Kuffler SW, Yoshikami D (1975). The number of transmitter molecules in a quantum: an estimate from iontophoretic application of acetylcholine at the neuromuscular synapse. J Physiol 251: 465–482.

Kumakura K, Karoum F, Guidotti A, Costa E (1980). Modulation of nicotinic receptors by opiate receptor agonists in cultured adrenal chromaffin cells. Nature 283: 489–492.

Kvetnansky R, Kubovcakova L, Tillinger A, Micutkova L, Krizanova O, Sabban EL (2006). Gene expression of phenylethanolamine N-methyltransferase in corticotropin-releasing hormone knockout mice during stress exposure. Cell Mol Neurobiol 26: 735–754.

Li X, Rainnie DG, McCarley RW, Greene RW (1998). Presynaptic nicotinic receptors facilitate monoaminergic transmission. J Neurosci 18: 1904–1912.

Lindau M, Neher E (1988). Patch-clamp techniques for time-resolved capacitance measurements in single cells. Pflugers Arch 411: 137–146.

Liu Q, Huang Y, Xue F, Simard A, DeChon J, Li G *et al.* (2009). A novel nicotinic acetylcholine receptor subtype in basal forebrain cholinergic neurons with high sensitivity to amyloid peptides. J Neurosci 29: 918–929.

López MG, Montiel C, Herrero CJ, García-Palomero E, Mayorgas I, Hernandez-Guijo JM *et al.* (1998). Unmasking the functions of the chromaffin cell alpha7 nicotinic receptor by using short pulses of acetylcholine and selective blockers. Proc Natl Acad Sci USA 95: 14184–14189.

McGehee DS, Heath MJ, Gelber S, Devay P, Role LW (1995). Nicotine enhancement of fast excitatory synaptic transmission in CNS by presynaptic receptors. Science 269: 1692–1696.

Mollard P, Seward EP, Nowycky MC (1995). Activation of nicotinic receptors triggers exocytosis from bovine chromaffin cells in the absence of membrane depolarization. Proc Natl Acad Sci USA 92: 3065–3069.

Mousavi M, Hellstrom-Lindahl E, Guan ZZ, Bednar I, Nordberg A (2001). Expression of nicotinic acetylcholine receptors in human and rat adrenal medulla. Life Sci 70: 577–590.

Neher E (1998). Vesicle pools and Ca2+ microdomains: new tools for understanding their roles in neurotransmitter release. Neuron 20: 389–399.

Neher E (2006). A comparison between exocytic control mechanisms in adrenal chromaffin cells and a glutamatergic synapse. Pflugers Arch 453: 261–268.

Neher E, Marty A (1982). Discrete changes of cell membrane capacitance observed under conditions of enhanced secretion in bovine adrenal chromaffin cells. Proc Natl Acad Sci USA 79: 6712–6716.

Papke RL, Bencherif M, Lippiello P (1996). An evaluation of neuronal nicotinic acetylcholine receptor activation by quaternary nitrogen compounds indicates that choline is selective for the alpha 7 subtype. Neurosci Lett 213: 201–204.

Pérez-Alvarez A, Albillos A (2007). Key role of the nicotinic receptor in neurotransmitter exocytosis in human chromaffin cells. J Neurochem 103: 2281–2290.

Pérez-Alvarez A, Hernandez-Vivanco A, Cano-Abad M, Albillos A (2008). Pharmacological and biophysical properties of Ca2+ channels and subtype distributions in human adrenal chromaffin cells. Pflugers Arch 456: 1149–1162.

Quik M, Geertsen S, Trifaró JM (1987). Marked up-regulation of the beta-bungarotoxin site in adrenal chromaffin cells by specific nicotinic antagonists. Mol Pharmacol 31: 385–391.

Rust G, Burgunder JM, Lauterburg TE, Cachelin AB (1994). Expression of neuronal nicotinic acetylcholine receptor subunit genes in the rat autonomic nervous system. Eur J Neurosci 6: 478–485.

Séguéla P, Wadiche J, Dineley-Miller K, Dani JA, Patrick JW (1993). Molecular cloning, functional properties, and distribution of rat brain alpha 7: a nicotinic cation channel highly permeable to calcium. J Neurosci 13: 596–604.

Tachikawa E, Mizuma K, Kudo K, Kashimoto T, Yamato S, Ohta S (2001). Characterization of the functional subunit combination of nicotinic acetylcholine receptors in bovine adrenal chromaffin cells. Neurosci Lett 312: 161–164.

Tai TC, Claycomb R, Siddall BJ, Bell RA, Kvetnansky R, Wong DL (2007). Stress-induced changes in epinephrine expression in the adrenal medulla in vivo. J Neurochem 101: 1108–1118.

Takiyyuddin MA, Brown MR, Dinh TQ, Cervenka JH, Braun SD, Parmer RJ *et al.* (1994). Sympatho-adrenal secretion in humans: factors governing catecholamine and storage vesicle peptide co-release. J Auton Pharmacol 14: 187–200.

Trifaró JM, Lee RW (1980). Morphological characteristics and stimulus-secretion coupling in bovine adrenal chromaffin cell cultures. Neuroscience 5: 1533–1546.

Ullian EM, McIntosh JM, Sargent PB (1997). Rapid synaptic transmission in the avian ciliary ganglion is mediated by two distinct classes of nicotinic receptors. J Neurosci 17: 7210–7219.

Uteshev VV, Meyer EM, Papke RL (2002). Activation and inhibition of native neuronal alpha-bungarotoxin-sensitive nicotinic ACh receptors. Brain Res 948: 33–46.

Verbitsky M, Rothlin CV, Katz E, Elgoyhen AB (2000). Mixed nicotinic-muscarinic properties of the alpha9 nicotinic cholinergic receptor. Neuropharmacology 39: 2515–2524.

Vijayaraghavan S, Pugh PC, Zhang ZW, Rathouz MM, Berg DK (1992). Nicotinic receptors that bind alpha-bungarotoxin on neurons raise intracellular free Ca2+. Neuron 8: 353–362.

Wilson SP, Kirshner N (1977). The acetylcholine receptor of the adrenal medulla. J Neurochem 28: 687–695.

Wurtman RJ, Axelrod J (1965). Adrenaline synthesis: control by the pituitary gland and adrenal glucocorticoids. Science 150: 1464–1465.

Zhang ZW, Vijayaraghavan S, Berg DK (1994). Neuronal acetylcholine receptors that bind alpha-bungarotoxin with high affinity function as ligand-gated ion channels. Neuron 12: 167–177.

Zhang ZW, Coggan JS, Berg DK (1996). Synaptic currents generated by neuronal acetylcholine receptors sensitive to alpha-bungarotoxin. Neuron 17: 1231–1240.

Zorumski CF, Thio LL, Isenberg KE, Clifford DB (1992). Nicotinic acetylcholine currents in cultured postnatal rat hippocampal neurons. Mol Pharmacol 41: 931–936.

Zwart R, Vijverberg HP (2000). Potentiation and inhibition of neuronal alpha4beta4 nicotinic acetylcholine receptors by choline. Eur J Pharmacol 393: 209–214.

Zwart R, De Filippi G, Broad LM, McPhie GI, Pearson KH, Baldwinson T *et al.* (2002). 5-Hydroxyindole potentiates human alpha 7 nicotinic receptor-mediated responses and enhances acetylcholine-induced glutamate release in cerebellar slices. Neuropharmacology 43: 374–384.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Blockade of PNU-120596 potentiation of ACh-elicited currents and exocytosis by α-Bgtx 1 μM are shown in panels A and B, respectively.

Figure S2 Kinetics of α7 currents. Current activation and inactivation kinetics elicited by choline or PNU-282987 3 and 30 μM were analysed and compared. Activation was determined as the time between 10 and 90% activation (t10–90), while inactivation was fitted to a single exponential curve (dashed line is the fitted curve).

Figure S3 Blockade of PNU-120596 potentiation of choline-elicited currents and exocitosis by α-Bgtx 1 μM.

Figure S4 Kinetics of potentiated α7 currents. The current activation and inactivation kinetics elicited by choline 10 mM in the presence of 5-HI 1 mM or PNU-120596 10 μM, or PNU-282987 plus PNU-120596, were analysed and compared. Activation was determined as the time between 10 and 90% activation (t10–90), while inactivation was fitted to a single exponential curve (dashed line is the fitted curve).

Figure S5 Contribution of α7 nAChRs to depolarization-elicited secretion revealed by their selective potentiation. Perfusion of PNU-120596 1 μM increases the depolarizing and secretory responses following nAChR activation by ACh (a) or choline (b).

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.