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Unmasking the functions of the chromaffin cell α_7 nicotinic receptor by using short pulses of acetylcholine and selective blockers

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ABSTRACT **Methyllycaconitine (MLA), α-conotoxin ImI, and α-bungarotoxin inhibited the release of catecholamines triggered by brief pulses of acetylcholine (ACh) (100 μM, 5 s) applied to fast-superfused bovine adrenal chromaffin cells, with IC_{50}s of 100 nM for MLA and 300 nM for α-conotoxin ImI and α-bungarotoxin. MLA (100 nM), α-conotoxin ImI (1 μM), and α-bungarotoxin (1 μM) halved the entry of $^{45}Ca^{2+}$ stimulated by 5-s pulses of 300 μM ACh applied to incubated cells. These supramaximal concentrations of α_7 nicotinic receptor blockers depressed by 30% (MLA), 25% (α-bungarotoxin), and 50% (α-conotoxin ImI) the inward current generated by 1-s pulses of 100 μM ACh, applied to voltage-clamped chromaffin cells. In *Xenopus* oocytes expressing rat brain α_7 neuronal nicotinic receptor for acetylcholine nAChR, the current generated by 1-s pulses of ACh was blocked by MLA, α-conotoxin ImI, and α-bungarotoxin with IC_{50}s of 0.1 nM, 100 nM, and 1.6 nM, respectively; the current through $\alpha_3\beta_4$ nAChR was unaffected by α-conotoxin ImI and α-bungarotoxin, and weakly blocked by MLA (IC_{50} = 1 μM). The functions of controlling the electrical activity, the entry of Ca^{2+}, and the ensuing exocytotic response of chromaffin cells were until now exclusively attributed to $\alpha_3\beta_4$ nAChR; the present results constitute the first evidence to support a prominent role of α_7 nAChR in controlling such functions, specially under the more physiological conditions used here to stimulate chromaffin cells with brief pulses of ACh.**

In 1977, Wilson and Kirshner (1) first reported the presence of α-bungarotoxin-binding sites in bovine adrenal medullary chromaffin cells. Eighteen years later, a bovine chromaffin cell α_7 subunit of the neuronal nicotinic receptor subtype for acetylcholine (nAChR) was cloned; injection in *Xenopus* oocytes of its cRNA produced α-bungarotoxin-binding sites and functional acetylcholine (ACh) currents that were blocked by α-bungarotoxin (2). α_3 and β_4 subunits of nAChR have been also cloned and characterized in bovine chromaffin cells (3). Thus, the question arises as to why different nAChR are expressed in bovine chromaffin cells and what their functions might be. Early attempts to block with α-bungarotoxin (a selective α_7 receptor blocker), the catecholamine secretory responses generated by various nicotinic receptor agonists produced negative results (1, 4–6) or provided some conflicting data (7). Thus, the actual view is that $\alpha_3\beta_4$ receptors control the catecholamine secretory response in chromaffin cells and that α_7 receptors play no role in the regulation of such function.

By using new protocols (short ACh pulses) and pharmacological tools, i.e., the novel selective α_7 nAChR blockers α-conotoxin ImI (8, 9) and methyllycaconitine (MLA) (10), we obtained evidence that α_7 receptors do have a role in the generation of inward nAChR currents (I_{ACh}), as well as in the triggering of Ca^{2+} entry and catecholamine release in response to ACh stimulation of bovine chromaffin cells. In addition, to strengthen the functional data obtained in chromaffin cells, we present here additional experiments that corroborate the high selectivity of α-conotoxin ImI and methyllycaconitine on the neuronal nAChR of the α_7-subtype and their poor blockade of $\alpha_3\beta_4$-subtype of receptors expressed in *Xenopus* oocytes.

MATERIALS AND METHODS

Isolation of Bovine Chromaffin Cells. Bovine chromaffin cells were isolated as described by Moro *et al.* (11). Cells were suspended in DMEM supplemented with 5% fetal calf serum, 10 μM cytosine arabinoside, 10 μM fluorodeoxyuridine, 50 units ml^{-1} penicillin, and 50 μg ml^{-1} streptomycin. For secretion experiments, 5×10^6 cells were plated in 10-cm diameter Petri dishes, and for ^{45}Ca uptake experiments, 2×10^5 cells per dish were plated in 96-well dishes. For patch-clamp experiments, cells were plated on 1-cm diameter glass coverslips at a density of 50×10^3 cells ml^{-1}. Cells were kept in an incubator at 37°C with 5% CO_2 in a water-saturated atmosphere. Media were renewed after 24 h and every 2–3 days.

Measurement of Catecholamine Release from Bovine Chromaffin Cells. Cells were collected by gentle rubbing, centrifuged, resuspended in 200 μl of Krebs-Hepes (composition in mM: NaCl, 144/KCl, 5.9/$MgCl_2$, 1.2/$CaCl_2$, 2/Hepes10/glucose, 11) pH 7.4 at room temperature (22 ± 2°C), and introduced in a microchamber with glass wool. Then, the cells were continuously superfused with Krebs-Hepes at a rate of 0.5 ml min^{-1}; the liquid flowing from the perfusion chamber reached an electrochemical detector model Metrohn 641VA under the amperometric mode, which monitored on-line the amount of catecholamines secreted (12). Catecholamine secretion was stimulated by perfusing for 5 s every 6 min, a Krebs-Hepes solution containing 100 μM ACh. Known concentrations of adrenaline were used as external standards. Secretion was quantified by measuring the peaks (and in some experiments also the areas) of the amperometric signals in nanoamperes.

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Abbreviations: MLA, methyllycaconitine; ACh; acetylcholine; nAChR, neuronal nicotinic receptor for acetylcholine; ImI, α-conotoxin ImI.
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Measurement of $^{45}Ca^{2+}$ Uptake. Cells were washed once with Krebs-Hepes and then the toxins were incubated for 30 min at 37°C. Ca^{2+} uptake was induced by adding $^{45}Ca^{2+}$ (9 μCi ml^{-1}) dissolved in Krebs-Hepes containing 300 μM ACh for 10 s; basal uptake was performed in normal Krebs-Hepes. The reaction was stopped by adding once an ice-cold Krebs-Hepes solution containing 10 mM La^{3+} and OCa^{2+}; excess extracellularly bound $^{45}Ca^{2+}$ was washed five times with a Ca^{2+}-free Krebs-Hepes containing 10 mM $LaCl_3$ and 2 mM EGTA, at 15-s intervals. At the end, 100 μl of 10% trichloroacetic acid was added and the cells were scrapped off with a pipette tip and introduced in a vial; scintillation liquid was added and radioactivity was counted in a Packard scintillation counter Model L1500.

Electrophysiological Recordings of nAChR Currents in Chromaffin Cells. Currents through the nAChR channels were recorded by using the whole-cell configuration of the patch-clamp technique (13). Briefly, nicotinic currents were elicited by fast application of 100 μM ACh through a multibarrel pipette controlled by miniature solenoid valves and placed within ≈100 μm of the cell. The flow rate (0.5–1 ml min^{-1}) was regulated by gravity to achieve a complete replacement of cell surroundings in 50–100 ms. External superfusion medium was (in mM): NaCl, 137; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 2; glucose, 11; and Hepes-NaOH, 10; pH, 7.4. Soft glass patch-clamp electrodes were filled with (in mM): NaCl, 10; CsCl, 100; tetraethylammonium cloride, 20; MgATP, 5; EGTA, 14; GTP.Na, 0.3; and Hepes-KOH, 20; pH 7.2. The holding potential was −80 mV, and electrode resistance ranged from 2 to 5 MΩ.

Expression of $\alpha_3\beta_4$ and α_7 nAChR in Oocytes and Measurements of nAChR Channel Currents. The plasmids pPCA48E, pZPC13, and pHIP306 containing the entire coding regions of rat nAChR α_3, β_4, and α_7 genes were linearized by using the restriction enzymes *Eco*RI, *Xho*I, and *Sma*I, respectively. Capped mRNAs were synthesized *in vitro* from the corresponding plasmids by using SP6 (α_3), T3 (β_4), or T7 (α_7) RNA polymerases and a mCAP RNA-capping Kit (Stratagene). Preparation of oocytes and microinjection of RNAs were done according to Miledi *et al.* (14) with some modifications (15). Oocytes were injected with 50 nl (50 ng) of α_7 cRNA or 50 nl (25:25 ng) of a combination of α_3 and β_4 cRNAs by using a nanoject automatic injector (Drummond Scientific, Broomall, PA). Oocytes were defolliculated with collagenase (type I, 200 units mg^{-1} at a final concentration of 0.5 mg ml^{-1}). Electrophysiological recordings were made 2–5 days after RNA injection.

Inward currents through nAChR were recorded at room temperature (22 ± 2°C) in Ringer's solution containing (in mM): NaCl, 115; KCl, 2; $CaCl_2$, 1.8; and Hepes, 5; buffered to pH 7.4 with NaOH. To block endogenous muscarinic receptors, atropine (1 μM) was used in all the solutions employed for the experiments expressing the combination $\alpha_3\beta_4$ subunits. Membrane currents were recorded with a two-electrode voltage clamp amplifier (OC-725-B Warner Instrument Corporation, Hamden, CT) by using microelectrodes with resistance of 0.5–5 MΩ made from borosilicate glass (GC100TF-15, Clark Electromedical Instruments, Pangbourne, UK) and filled with KCl (3 M). The holding potential in all experiments was −60 mV. Single oocytes were held in a chamber with a volume of 0.3 ml and constantly superfused with Ringer's solution by gravity (4 ml min^{-1}). Toxins were superfused during variable periods of time (ranging from 3 to 20 min) until a maximal and stable effect was observed in some cases, or the absence of any effect was confirmed in others. Voltage protocols, ACh pulses and data acquisition were controlled by using a Digidata 1200 interface and CLAMPEX software (Axon Instruments, Foster City, CA). The IC_{50} of the toxins on ACh-induced currents were estimated through nonlinear regression analysis, by GRAPHPAD Software (San Diego, CA) for a PC computer.

Materials and Solutions. Acetylcholine was purchased from Sigma and $^{45}Ca^{2+}$ with a specific activity 10–40 mCi mg^{-1}, from Amersham. α-Conotoxin ImI was obtained as described by Myers *et al.* (16) and McIntosh *et al.* (8). α-Bungarotoxin was purchased from Research Biochemicals (Natick, MA) and Sigma; methyllycaconitine was also from Research Biochemicals. Toxins were dissolved in distilled water at the concentration of 10^{-3} M, aliquoted, and maintained at −20°C until use. Before each experiment, they were dissolved in the saline solutions used in each experiment and diluted to the desired final concentration.

Statistics. Differences between means of group data fitting a normal distribution were assessed by using the Student's *t* test. A *P* value equal or <0.05 was taken as the limit of significance.

RESULTS

Blockade of ACh-Induced Secretory Responses by Selective Antagonists of α_7 nAChR. To study the effects of toxins on catecholamine release responses, chromaffin cells were continuously superfused with Krebs-Hepes solution, and after an initial equilibration period (≈10 min), they were stimulated at 6-min intervals with short ACh pulses (100 μM, 5 s). The initial secretory response (345 ± 39 nA in 19 different cell batches) declined by 30% after eight ACh pulses (control curve in Fig. 1*B*). A typical experimental protocol to study the effects of a given toxin on secretion appears in Fig. 1*A*, where original secretory spikes are shown; the toxin (one single concentration in each cell batch) was applied during 12 min (6 min before an ACh pulse and 6 min before the subsequent pulse). Fig. 1*A* shows an example of the blockade of secretion induced by 1 μM α-conotoxin ImI as well as the recovery of the ACh responses after the toxin washout. Fig. 1*B* shows averaged results for three selective α_7 nAChR blockers. α-Bungarotoxin blocked the response by 44% after 6 min and by 63% after 12 min; α-conotoxin ImI (1 μM) caused 70% inhibition at 6 and 12 min. Methyllycaconitine (0.1 μM) caused 70% inhibition of secretion in 6 min. The effects of α-conotoxin ImI and methyllycaconitine reversed quickly upon washout, whereas those of α-bungarotoxin did not. Concentration-response curves for three of the toxins are shown in Fig. 1*C*; approximate IC_{50}s were 300 nM for α-conotoxin ImI and α-bungarotoxin, and 100 nM for MLA.

Inhibition by α_7 Blockers of ACh-Induced $^{45}Ca^{2+}$ Uptake. The physiological catecholamine release response triggered by ACh in the intact adrenal gland implies the previous entry into the cytosol of extracellular Ca^{2+} (17) through voltage-dependent Ca^{2+} channels (18). So, it was expected that the blockade of secretion by α-toxins would parallel the inhibition of Ca^{2+} entry. Chromaffin cells incubated in Krebs-Hepes solution for 10 s took up 70 ± 5.7 cpm as $^{45}Ca^{2+}$ (n = 18 wells). A pulse of 300 μM ACh for 10 s was necessary to get a near 4-fold increase of $^{45}Ca^{2+}$ uptake (277 ± 14 cpm in 18 wells from different cell batches).

Toxins (0.1 μM) were kept in contact with the cells for 30 min before applying the ACh pulse. α-Bungarotoxin blocked the $^{45}Ca^{2+}$ uptake by 44 ± 3.7%, α-conotoxin ImI by 45.6 ± 3.1%, and MLA by 42 ± 4.2% (Fig. 2). Blockade of $^{45}Ca^{2+}$ entry could be due to a direct action of the toxins on Ca^{2+} channels, which are activated secondarily to ACh stimulation of nAChR, causing cell depolarization. However, none of the toxins inhibited the $^{45}Ca^{2+}$ uptake induced by high K^+ (70 mM, 10 s), thus precluding a direct effect on Ca^{2+} channels (data not shown). $^{45}Ca^{2+}$ influx into chromaffin cells stimulated with ACh may occur via nicotinic receptors directly and/or via voltage-dependent Ca^{2+} channels. The present experiments do not distinguish between these possibilities.

Effects of α-Toxins on Inward Currents Through Native Nicotinic Receptors in Chromaffin Cells. If the α_7 blockers

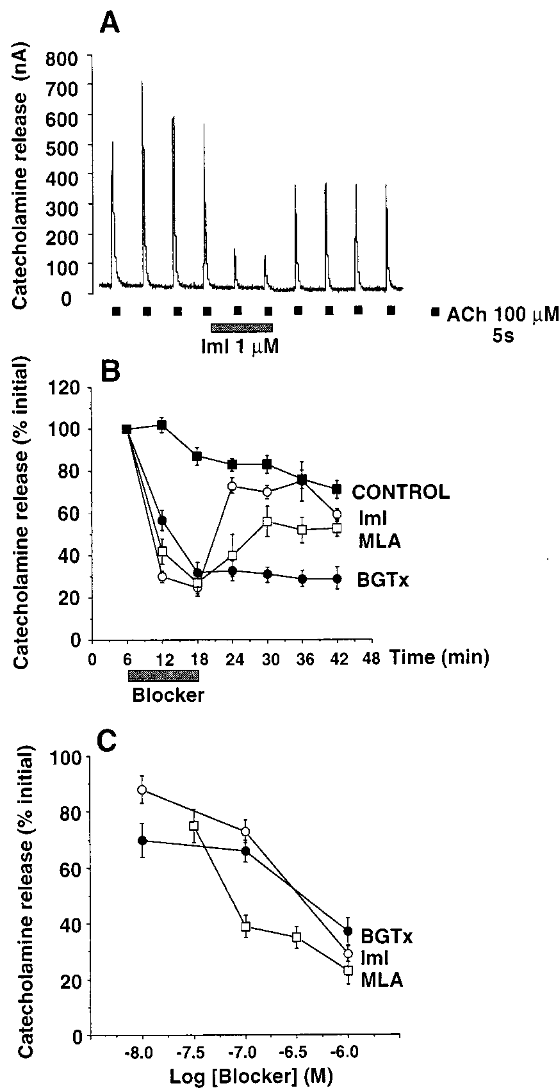

FIG. 1. Blockade and recovery of ACh-induced secretory responses after superfusion of chromaffin cells with MLA (0.1 μM), α-bungarotoxin (BGTx; 1 μM), and α-conotoxin ImI (ImI; 1 μM). The blockers were given during the time period indicated on the bottom horizontal bar in *A* and *B* (12 min). In each individual experiment, cells were stimulated 10 times at 6 min intervals with ACh pulses (100 μM, 5 s) (see example of *A* using α-conotoxin ImI as a blocker). The first secretory pulse was discarded and the secretory peak of the second pulse was normalized to 100% (initial, *B*). The rest of the peaks were expressed as % of the initial secretion peak. Data are means ± SE of 6–11 experiments (*B*). (*C*) Concentration-response curves for BGTx, α-conotoxin ImI, and MLA for blockade of ACh-evoked secretion (protocols as in *A*); the value of secretion after 12 min of toxin superfusion was taken to calculate the % blockade of secretion. A separate batch of cells was used for each blocker concentration. Data are means ± SE of 6–8 experiments performed with different batches of cell cultures.

inhibited Ca^{2+} entry and secretion stimulated by nicotinic receptors, then they also should affect the ACh-induced currents through native nAChR of chromaffin cells. The application of 1-s pulses of ACh (100 μM) to a voltage-clamped (holding potential −80 mV) chromaffin cell, at 30 s intervals, generated inward currents (I_{ACh}); the current amplitude was reproducible during the application of at least 18 ACh pulses (Fig. 3*A*). In 98 cells, the average peak current amounted to 1,736 ± 80 pA.

The time courses of the effects of α_7 blockers on I_{ACh} are shown in Fig. 3 *B* and *C*. After breaking into the cell with the patch pipette, pulses of ACh (1 s, 100 μM) were applied at 30-s intervals. Once the I_{ACh} peaks stabilized, 1 μM α-conotoxin ImI was applied as shown on the top horizontal bars (Fig. 3*B*). The inhibition with each concentration was achieved in a single step and was maximum after 30 s of superfusion with the toxin. Note also that the blockade was reversible and that the current recovered its initial size after 3 min of toxin washout. The addition of the toxin for a second time produced similar effects. The *Inset* shows original I_{ACh} traces in the absence (control) and in the presence of 1 μM toxin; the kinetics of I_{ACh} was not apparently modified by α-conotoxin ImI. Fig. 3*C* shows similar time course experiments for I_{ACh} blockade induced by MLA (0.1 μM) and by α-bungarotoxin (1 μM). MLA caused a fast blockade of I_{ACh} (≈65%) that quickly reversed after washout of the compound. The subsequent addition of α-bungarotoxin caused ≈40% blockade of I_{ACh} that reversed slowly and partially upon washout.

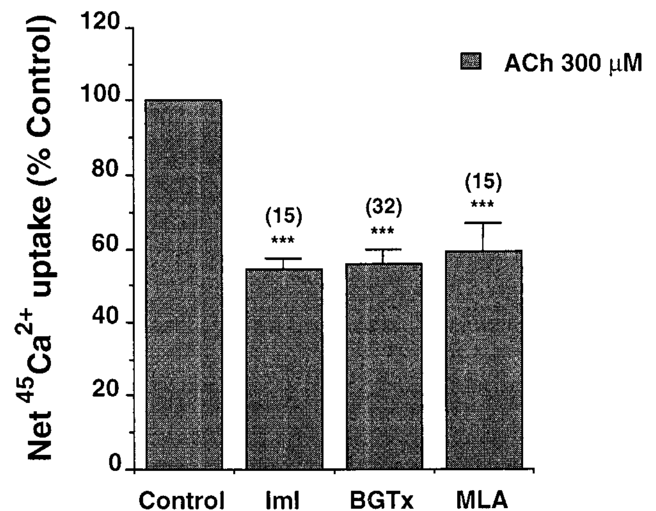

FIG. 2. Effects of α-conotoxins ImI (ImI), α-bungarotoxin (BGTx), and MLA on $^{45}Ca^{2+}$ entry into chromaffin cells stimulated with ACh. Cells were incubated for 30 min with the compounds at the concentration of 100 nM. Data are means ± SE of the number of wells shown in parentheses on top of each bar, from at least four different batches of cells. $^{***}P < 0.001$, compared with controls.

Effects of α_7 Blockers on ACh Currents in *Xenopus* Oocytes Expressing the $\alpha_3\beta_4$ and α_7 Subtypes of nAChR. The selectivity of the three α_7 blockers used above was studied in *Xenopus* oocytes injected with RNAs coding for α_7 and $\alpha_3\beta_4$ receptors. Fig. 4*A* shows the kinetics of I_{ACh} in oocytes expressing α_7 and $\alpha_3\beta_4$ receptors. The application of long pulses of ACh (100 μM, 20 s) generated inward currents that in the case of α_7 receptors inactivated promptly with a τ of 1.5 s. In contrast, the current generated by $\alpha_3\beta_4$ receptors suffered a very tiny inactivation (only ≈5–10% of current loss was observed at the end of the 20 s ACh pulse). The size of peak currents also differed between α_7-injected oocytes (80 ± 9 nA, n = 25 oocytes) and $\alpha_3\beta4$ oocytes (3.4 ± 0.73 μA; n = 20 oocytes), 3–4 days after injecting the mRNAs.

The effects of α-toxins on I_{ACh} were tested in oocytes stimulated with 1-s pulses of 100 μM ACh, applied at 1-min intervals. With this protocol, I_{ACh} was quite reproducible during several minutes; so, it was possible to test the blocking effects of a given compound as well as the recovery of I_{ACh} after washing out such compound. Fig. 4*B* shows that 1 μM α-conotoxin ImI completely suppressed the α_7 I_{ACh}, which recovered quickly after 5-min washout. α-Bungarotoxin (0.1 μM) suppressed I_{ACh} through α_7 receptors, and the current did not recover 10 min after the toxin washout (not shown).

Quantitative data on toxin blockade of I_{ACh} in α_7- and $\alpha_3\beta_4$-injected oocytes are presented in Figs. 4 *C* and *D*, respectively. α-Conotoxin ImI blocked α_7 currents with an IC_{50}

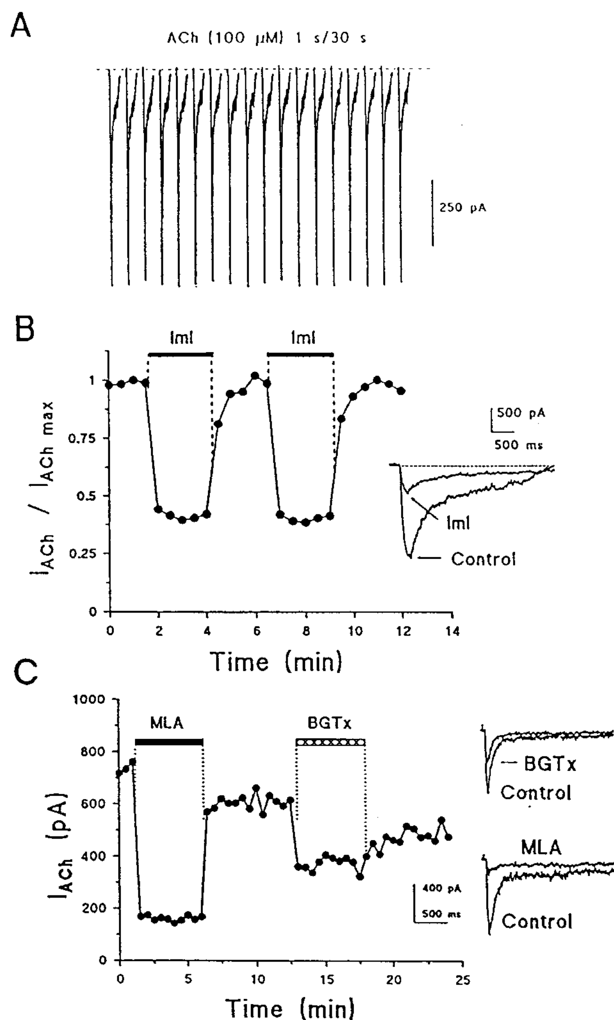

FIG. 3. Effects of α-conotoxin ImI (ImI; *B*), α-bungarotoxin (BGTx; *C*), and MLA (*C*) on inward currents induced by ACh pulses (100 μM, 1 s, given at 30-s intervals), in voltage-clamped chromaffin cells (holding potential -80 mV). (*A*) Reproducibility of inward currents upon repeated application of ACh pulses. (*B*) Time course of I_{ACh} before, during, and after adding ImI (1 μM; top horizontal bars). (*Insets*) Two original inward current traces generated by ACh pulses before or in the presence of the blocker. (*C*) Time course of the blocking effects of both MLA (0.1 μM) and BGTx (1 μM). These results were reproduced in 4–9 cells for each blocker.

of 100 nM; $\alpha_3\beta_4$ currents were unaffected even by 3 μM of this toxin. α-Bungarotoxin blocked α_7 currents with an IC_{50} of 1.6 nM and had no effects on $\alpha_3\beta_4$ currents at concentrations up to 3 μM. Finally, methyllycaconitine blocked α_7 currents with an IC_{50} of 0.1 nM and $\alpha_3\beta_4$ currents with an IC_{50} of 1 μM.

DISCUSSION

In this study, we used three blockers with high selectivity for α_7 nAChR to explore their effects on native nicotinic receptor currents as well as on Ca^{2+} uptake and catecholamine release triggered by stimulation of chromaffin cells with short pulses of ACh. Their claimed selectivity (8, 10) was corroborated in our hands, since the brain homomeric α_7 nicotinic receptor currents in oocytes were potently blocked by α-bungarotoxin, α-conotoxin ImI, and methyllycaconitine; α-bungarotoxin and α-conotoxin ImI did not affect the I_{ACh} through heteromeric $\alpha_3\beta_4$ receptors whereas MLA blocked it with a potency four orders of magnitude lower than that exhibited for α_7 receptors (Fig. 4). The IC_{50} values for MLA and α-bungarotoxin blockade of α_7 receptors expressed in oocytes were slightly higher than those obtained by other authors (9, 19); this could be explained on the basis of the shorter exposure periods used in our experiments or the lower superfusion rates used. Because the bovine adrenal chromaffin cells express α_7 receptors with high homology to brain receptors (2), we used these specific blockers as tools to investigate whether such receptors had a functional role in chromaffin cells.

Our chromaffin cell cultures are enriched in epinephrine-containing cells (11), where α_7 receptors seem to be preferentially expressed (20). We cannot exclude the expression of native heteromeric receptors formed by the combination of α_7 and $\alpha_3\beta_4$ subunits, as the case is in oocytes injected with $\alpha_3\beta_4$ and β_3 subunits (21). However, it is likely that the whole-cell native currents through nAChR, generated by 1-s pulses of ACh, are mainly the result of the simultaneous activation of a mixed population of α_7 and $\alpha_3\beta_4$ receptors. This is supported by the fact that the three α_7 receptor blockers used in this study inhibited only partially the ACh-stimulated inward currents (Fig. 3), the entry of $^{45}Ca^{2+}$ (Fig. 2), and the release of catecholamines (Fig. 1), in chromaffin cells, at concentrations that inhibited fully the ACh-induced current in oocytes expressing α_7 nAChR (Fig. 4).

The reasons for the failure to show a functional role for chromaffin cell α_7 nAChR in earlier studies (1, 4–6, 22, 25), might rest in the use of long periods of stimulation (minutes) of chromaffin cells. As shown for brain receptors (26; and Fig. 4*A*), the bovine chromaffin cell α_7 receptor desensitizes in <1 s when expressed in oocytes (2). If the native α_7 receptor had similar kinetics in bovine chromaffin cells, exposure of these cells to nAChR agonists during several minutes in those earlier studies, would necessarily mask any participation of α_7 receptors in the fast component of the secretory response. As seen in Fig. 4*A*, $\alpha_3\beta_4$ receptors expressed in oocytes did not desensitize even after 20 s of ACh application; it seems therefore that under the experimental conditions used in earlier reports (minutes of ACh stimulation) the observed secretory responses will be due to $\alpha_3\beta_4$ receptor stimulation. But this by no means excludes that under more physiological conditions as those used here (1–5 s stimulation with ACh), α_7 receptors do certainly participate in the triggering and regulation of fast Ca^{2+} entry and catecholamine release.

Finding a function for α_7 nAChR in brain tissues have proven to be also elusive. Earlier experiments showing specific and saturable binding sites for α-bungarotoxin could not find that the toxin affected some functions in several synapses (27–32). However, more recent studies have provided data implicating α_7 receptors in some neural functions. For instance, α_7 receptors have been suggested to enhance fast excitatory transmission via a presynaptic mechanism (33), the increase of cytosolic Ca^{2+} (34, 35), the guidance of nerve fibers (36), the inhibition of neurite extension (37), the expression of neurotrophic growth factor in rat hippocampus (38), the death of motoneurons (39), the modulation of cell proliferation in lung tumor cells (40, 41), and the generation of postsynaptic neuronal responses (42, 43).

With the same blockers used in the present study, Codignola *et al.* (40) also found that α-conotoxin ImI inhibits nicotine-evoked hormone secretion in human small cell lung carcinoma, and Vijayaraghavan *et al.* (34) found that α-bungarotoxin and methyllycaconitine inhibit the increase of $[Ca^{2+}]_i$ induced by 2- to 4-s pulses of nicotine in chicken ciliary ganglion neurons. Interestingly, the latter authors found that the inhibition was higher, the lower the concentration of nicotine used. Thus, it seems that depending on the conditions of stimulation (duration of the stimulus, concentration of agonist used), the functions associated to the activation of α_7 receptors can be unmasked or not. This might explain the extensive controversy that has surrounded the numerous attempts to find a function for α_7 receptors. In this context, we believe that our present results clearly establish a functional role in the control of Ca^{2+}

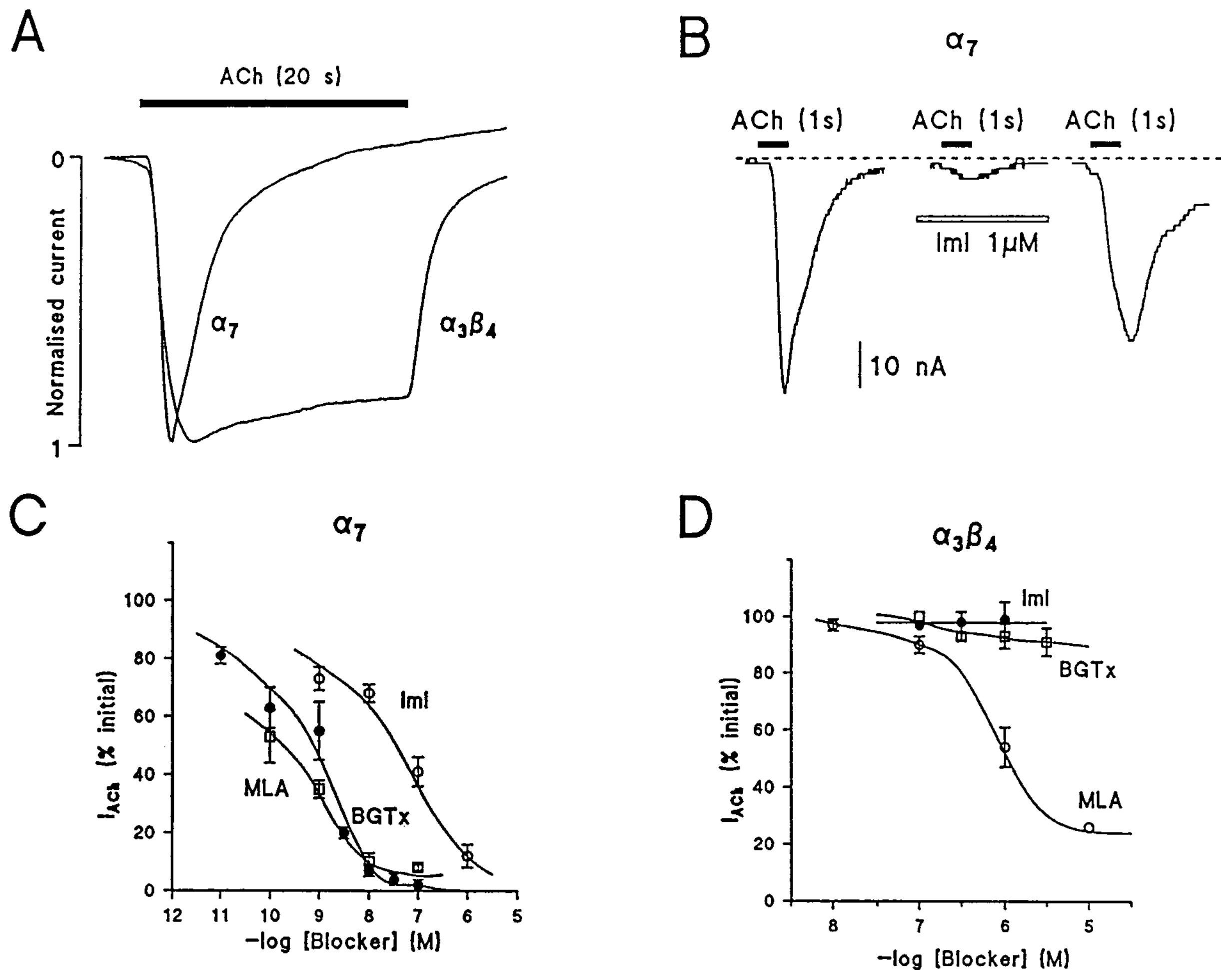

FIG. 4. Kinetics and blockade by α-toxins of I_{ACh}, in *Xenopus* oocytes injected with cRNAs for α_7 or $\alpha_3\beta_4$ nAChR. Oocytes maintained at a holding potential of −60 mV, were stimulated with pulses of ACh (100 μM) as shown by the top horizontal bars. (*A*) Two original I_{ACh} traces (normalized currents) obtained by stimulation with ACh (20 s) in one oocyte expressing α_7 receptors and in another expressing $\alpha_3\beta_4$ receptors. (*B*) Effect of α-conotoxin ImI on I_{ACh} generated by 1 s pulses of ACh (100 μM) applied to oocytes expressing α_7 receptors. (*C* and *D*) Concentration-response curves of α-toxins to block I_{ACh} in oocytes expressing α_7 nAChR (*C*) or $\alpha_3\beta_4$ receptors (*D*). Protocols to estimate the blockade exerted by each toxin on I_{ACh} were as those described in *B*. Repeated pulses of ACh (1s, 100 μM) generated inward currents that were normalized to 100% (before adding each toxin concentration). The blockade exerted by each toxin concentration was expressed as percentage of the initial current. Data are means ± SE of 5–10 oocytes for each blocker.

entry and secretion triggered by the physiological cholinergic neurotransmitter in the adrenal medullary chromaffin cells. Why two (or more) nAChR subtypes (α_7, $\alpha_3\beta_4$) are required to control the secretory process is still a mystery. It may be related to the need to secrete differentially norepinephrine or epinephrine under particular stressful conditions. Or it may be related to the generation of a fast but transient secretory response (α_7 are fast desensitizing receptors) or a more sustained catecholamine release response ($\alpha_3\beta_4$ are slow desensitizing receptors).

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