

## RESEARCH PAPER

# Calcium signalling mediated through $\alpha 7$ and non- $\alpha 7$ nAChR stimulation is differentially regulated in bovine chromaffin cells to induce catecholamine release

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## BACKGROUND AND PURPOSE

$\text{Ca}^{2+}$  signalling and exocytosis mediated by nicotinic receptor (nAChR) subtypes, especially the  $\alpha 7$  nAChR, in bovine chromaffin cells are still matters of debate.

## EXPERIMENTAL APPROACH

We have used chromaffin cell cultures loaded with Fluo-4 or transfected with aequorins directed to the cytosol or mitochondria, several nAChR agonists (nicotine, 5-iodo-A-85380, PNU282987 and choline), and the  $\alpha 7$  nAChR allosteric modulator PNU120596.

## KEY RESULTS

Minimal  $[\text{Ca}^{2+}]_c$  transients, induced by low concentrations of selective  $\alpha 7$  nAChR agonists and nicotine, were markedly increased by the  $\alpha 7$  nAChR allosteric modulator PNU120596. These potentiated responses were completely blocked by the  $\alpha 7$  nAChR antagonist  $\alpha$ -bungarotoxin ( $\alpha 7$ -modulated-response). Conversely, high concentrations of the  $\alpha 7$  nAChR agonists, nicotine or 5-iodo-A-85380 induced larger  $[\text{Ca}^{2+}]_c$  transients, that were blocked by mecamylamine but were unaffected by  $\alpha$ -bungarotoxin (non- $\alpha 7$  response).  $[\text{Ca}^{2+}]_c$  increases mediated by  $\alpha 7$  nAChR were related to  $\text{Ca}^{2+}$  entry through non-L-type  $\text{Ca}^{2+}$  channels, whereas non- $\alpha 7$  nAChR-mediated signals were related to L-type  $\text{Ca}^{2+}$  channels;  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$ -release contributed to both responses. Mitochondrial involvement in the control of  $[\text{Ca}^{2+}]_c$  transients, mediated by either receptor, was minimal. Catecholamine release coupled to  $\alpha 7$  nAChRs was more efficient in terms of catecholamine released/ $[\text{Ca}^{2+}]_c$ .

## CONCLUSIONS AND IMPLICATIONS

$[\text{Ca}^{2+}]_c$  and catecholamine release mediated by  $\alpha 7$  nAChRs required an allosteric modulator and low doses of the agonist. At higher agonist concentrations, the  $\alpha 7$  nAChR response was lost and the non- $\alpha 7$  nAChRs were activated. Catecholamine release might therefore be regulated by different nAChR subtypes, depending on agonist concentrations and the presence of allosteric modulators of  $\alpha 7$  nAChRs.

## Abbreviations

$[\text{Ca}^{2+}]_c$ , cytosolic concentration of calcium; BGT,  $\alpha$ -bungarotoxin; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; nAChR, nicotinic acetylcholine receptor; VOCC, voltage operated calcium channel

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## Introduction

Adrenal chromaffin cells derive from the neural crest and are innervated by the preganglionic cholinergic neurons of the splanchnic nerve that release acetylcholine, the ligand that induces catecholamine secretion from chromaffin cells. Binding of the physiological neurotransmitter acetylcholine or nicotine to the nicotinic cholinoreceptors (nAChR; receptor nomenclature follows Alexander *et al.*, 2009) located in the plasma membrane of the chromaffin cell, opens the nicotinic receptor channel allowing the entry of sodium and calcium and the exit of potassium ions by means of an electrochemical gradient. The resultant depolarization of the membrane opens voltage-operated Ca<sup>2+</sup> channels (VOCCs) followed by Ca<sup>2+</sup> entry through these channels to trigger the release of catecholamines into the blood stream.

Neuronal nAChRs are pentameric ligand-gated channels, consisting of the same subunit type (homomeric) or different subunit types (heteromeric). In bovine chromaffin cells,  $\alpha$ 3 (Criado *et al.*, 1992),  $\alpha$ 5 (Campos-Caro *et al.*, 1997)  $\beta$ 4 (Campos-Caro *et al.*, 1997) and  $\alpha$ 7 (Garcia-Guzman *et al.*, 1995) subunits have been cloned. Furthermore,  $\alpha$ 3,  $\alpha$ 5 and  $\beta$ 4 transcripts are co-expressed in chromaffin cells and functional receptors have been obtained upon co-injection of these three subunit cRNAs into *Xenopus* oocytes (Campos-Caro *et al.*, 1997). In primary cultures of bovine chromaffin cells, most studies have shown that the nAChRs are insensitive to  $\alpha$ -bungarotoxin (BGT) and there is wide agreement that they are involved in controlling catecholamine release.

As for the  $\alpha$ 7 nAChR, the presence of BGT-binding sites in the adrenal medullary chromaffin cells was first reported in 1977 (Wilson and Kirshner, 1977). When the  $\alpha$ 7 nAChRs of the bovine chromaffin cell were cloned, injection in *Xenopus* oocytes of its cRNA produced BGT binding sites and functional acetylcholine currents sensitive to BGT (Criado *et al.*, 1992). Later on, BGT binding sites were described mainly in adrenergic but not in noradrenergic cells (Criado *et al.*, 1997). More recently, El-Haij *et al.* (2007) have reported that the  $\alpha$ 7 homomeric population of nAChRs in bovine chromaffin cells was greater than the heteromeric nAChR population.

In terms of the control of catecholamine release, it is well established that Ca<sup>2+</sup> is required for this process, not only after a depolarizing pulse but also after nicotinic stimulation (Douglas and Rubin, 1961a,b). After nAChR activation, the cytosolic concentration of Ca<sup>2+</sup> [Ca<sup>2+</sup>]<sub>c</sub> is raised because of Ca<sup>2+</sup> entry through VOCCs (Rathouz and Berg, 1994) and by Ca<sup>2+</sup>-induced Ca<sup>2+</sup>-release (CICR) from intracellular

stores (Verkhratsky, 2002). The VOCCs present in bovine chromaffin cells include the L, N and P/Q subtypes (Garcia *et al.*, 2006). Each VOCC subtype has been found to contribute to <sup>45</sup>Ca<sup>2+</sup> entry (Ballista *et al.*, 1989; Lopez *et al.*, 1994; Villarroya *et al.*, 1997) and catecholamine release (Lopez *et al.*, 1994; Lara *et al.*, 1998). However, N and P/Q channels are inactivated at depolarizing voltages, in contrast to the L-subtype (Villarroya *et al.*, 1999), and this inactivation blocks Ca<sup>2+</sup> entry and catecholamine release (Artalejo *et al.*, 1986; Michelena *et al.*, 1993).

Although Ca<sup>2+</sup> entry through the nicotinic receptor itself seems to contribute very little to [Ca<sup>2+</sup>]<sub>c</sub> increase after receptor activation (Zhou and Neher, 1993), Ca<sup>2+</sup> permeability associated with a particular nAChR subtype represents an important aspect of its physiological role in cellular processes. The homomeric  $\alpha$ 7 nAChR has the highest Ca<sup>2+</sup>:Na<sup>+</sup> permeability ratio (Seguela *et al.*, 1993; Fucile *et al.*, 2003) but desensitizes very rapidly (Couturier *et al.*, 1990) in comparison with other nAChR subtypes. Another characteristic of the  $\alpha$ 7 nAChR is that nicotinic agonists bind to it with low affinity (Pereira *et al.*, 1993), although these receptors show high affinity for the antagonists BGT and methyllycaconitine (MLA).

As indicated before, the existence of  $\alpha$ 7 nAChRs in bovine chromaffin cells has been well documented by molecular techniques, but its functional relevance in terms of Ca<sup>2+</sup> signalling and catecholamine exocytosis is still a matter of debate. We therefore investigated this issue further, using new pharmacological tools such as the  $\alpha$ 7 nAChR agonist PNU282987 and the  $\alpha$ 7 nAChR allosteric modulator PNU120596.

Our results showed that activation of  $\alpha$ 7 nAChRs, in the presence of an allosteric modulator, can mediate BGT-sensitive [Ca<sup>2+</sup>]<sub>c</sub> signals and catecholamine release. Furthermore, [Ca<sup>2+</sup>]<sub>c</sub> increases mediated by  $\alpha$ 7 nAChR were related to Ca<sup>2+</sup> entry through non-L-VOCCs, whereas non- $\alpha$ 7 nAChR-mediated signals were related to L-type VOCCs. CICR also participated in both responses. Our results indicated that functionality of the  $\alpha$ 7 nAChR in chromaffin cells seems to require the binding of an allosteric modulator together with its agonist and that Ca<sup>2+</sup> signalling mediated through  $\alpha$ 7 and non- $\alpha$ 7 nAChR stimulation is differentially regulated in bovine chromaffin cells.

## Methods

### *Isolation and culture of bovine chromaffin cells*

Bovine adrenal glands were obtained from a local slaughterhouse. Chromaffin cells were isolated by

collagenase digestion as previously described (Livett, 1984) with some modifications (Moro *et al.*, 1990); purification of bovine chromaffin cells was achieved by several consecutive centrifugations. Cells were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum, 50 IU·mL<sup>-1</sup> penicillin and 50 µg·mL<sup>-1</sup> streptomycin. Cells were pre-plated for 30 min and proliferation inhibitors (10 µM cytosine arabinoside, 10 µM fluorodeoxyuridine, and 10 µM leucine methyl ester) were added to the medium to prevent excessive growth of fibroblasts. Cultures were maintained in an incubator at 37°C in a water-saturated atmosphere with 5% CO<sub>2</sub>. Cells were used between 48 and 72 h post-plating.

### Measurement of calcium signals with fluorescent dyes

**Fluo-4 measurements in populations of bovine chromaffin cells.** Bovine chromaffin cells were plated in black 96 well plates at a density of 200 000 cells per well. Cells were loaded with 4 µM Fluo-4/AM for 1 h at 37°C in DMEM. Then, cells were washed twice with Krebs-HEPES solution (composition (in mM): 140 NaCl, 5.6 KCl, 1.2 MgCl<sub>2</sub>, 2 CaCl<sub>2</sub>, 10 HEPES, 11 D-glucose, pH 7.4) and kept at room temperature for 30 min before the beginning of the experiment. When BGT (100 nM), mecamylamine (30 µM) nimodipine (3 µM) or  $\omega$ -conotoxin MVIIC (2 µM) were employed, these were incubated with the cells for 15 min before injecting the nicotinic agonists.

Fluorescence measurements were carried out for 10 s, as the agonists were injected in a microplate reader (FLUOstar Optima, BMG, Offenburg, Germany). Wavelengths of excitation and emission were 485 and 520 nm respectively.

At the end of the experiment, 75 µL of Triton (5%) was added to each well to calculate maximum fluorescence ( $F_{\max}$ ) and then 50 µL of MnCl<sub>2</sub> (1 M) to obtain the minimum fluorescence ( $F_{\min}$ ). Drug-evoked responses were expressed as percentage of the fluorescence values at each time point ( $F$ ) minus minimum fluorescence values ( $F_0$ ) divided by  $F_{\max} - F_{\min}$  as follows:

$$F_{520} = (F - F_0) / (F_{\max} - F_{\min}) \%$$

The maximum value of  $F_{520}$ , obtained for each experiment, was considered as the Peak  $F_{520}$  value.

**Aequorin measurements.** The construction of the aequorin chimeras targeted to either the cytosol or the mitochondria has been described previously (Rizzuto *et al.*, 1992; Brini *et al.*, 1995). Chromaffin cells were plated on coverslips and infected with adenoviruses for expression of both constructs of

targeted aequorin. Adenoviruses were prepared as described previously (Santodomingo *et al.*, 2008). Infection was carried out the day after cell isolation and Ca<sup>2+</sup> measurements were performed 48–72 h after infection. For aequorin reconstitution, cells expressing either cytosolic or mitochondrial aequorin were incubated for 1–2 h at room temperature with 1 µM of native coelenterazine in standard medium (145 mM NaCl, 5 mM KCl, 1 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 10 mM glucose, and 10 mM HEPES, pH 7.4). Cells were then placed in the perfusion chamber of a purpose-built luminometer maintained at 37°C. Calibration of the luminescence data into [Ca<sup>2+</sup>] was made using an algorithm as previously described (Barrero *et al.*, 1997).

### Catecholamine measurements

Bovine chromaffin cells were seeded in 12 well plates at a density of 500 000 cells per well. Catecholamines released from the cells were analyzed by high-performance liquid chromatography (HPLC; Agilent Technologies 1200 series) using electrochemical detection (BioRad, model 1640, Bio-Rad Laboratories, Irvine, CA, USA). A C<sub>18</sub> reverse phase column (ZORBAX Eclipse XDB, Agilent Technologies, Santa Clara, CA, USA), eluted with a mobile phase (pH 4; 0.1 M sodium acetate, 0.1 M citric acid, 0.7 mM sodium octyl sulphate and 0.57 mM EDTA containing 10% methanol, v/v) was employed. The system was run continuously with the mobile phase being recycled back into the reservoir and used for up to one week. Flow rate was 1 mL·min<sup>-1</sup>, at a pressure of 2200 psi. Fixed potentials against H<sub>2</sub>/H<sup>+</sup> reference electrodes were: conditioning electrode 0.4V; pre-oxidation electrode +0.10V; working electrode +0.35V. Adrenaline and noradrenaline concentrations were calculated from the chromatographic peak areas by using external standards. The chromatograms were collected, stored and processed with Clinical Data Management Software (BioRad CDM 1.10).

### Data and statistical analysis

Data are given as means  $\pm$  SEM. Differences between groups were determined by applying a one-way ANOVA followed by Newman-Keuls test. In the aequorin experiments, Student's *t*-test was used to determine statistical differences between groups. Differences were considered to be statistically significant when  $P \leq 0.05$ .

### Materials

Nicotine, choline,  $\alpha$ -bungarotoxin (BGT), mecamylamine, cadmium, N-methyl glucamine, dimethyl sulfoxide (DMSO), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) and nimodipine were from Sigma (Madrid, Spain).

PNU282987, PNU120596, 5-iodo-A-85380 dihydrochloride and  $\omega$ -conotoxin MVIIIC from Tocris (Biogen Científica, Madrid, Spain). DMEM, fetal bovine serum and penicillin/streptomycin were purchased from Invitrogen (Barcelona, Spain). Fluo-4 AM and coelenterazine were from Molecular Probes (Invitrogen, Madrid, Spain).

## Results

### Nicotine and 5-iodo-A-85380 induce intracellular calcium signals that are not sensitive to BGT

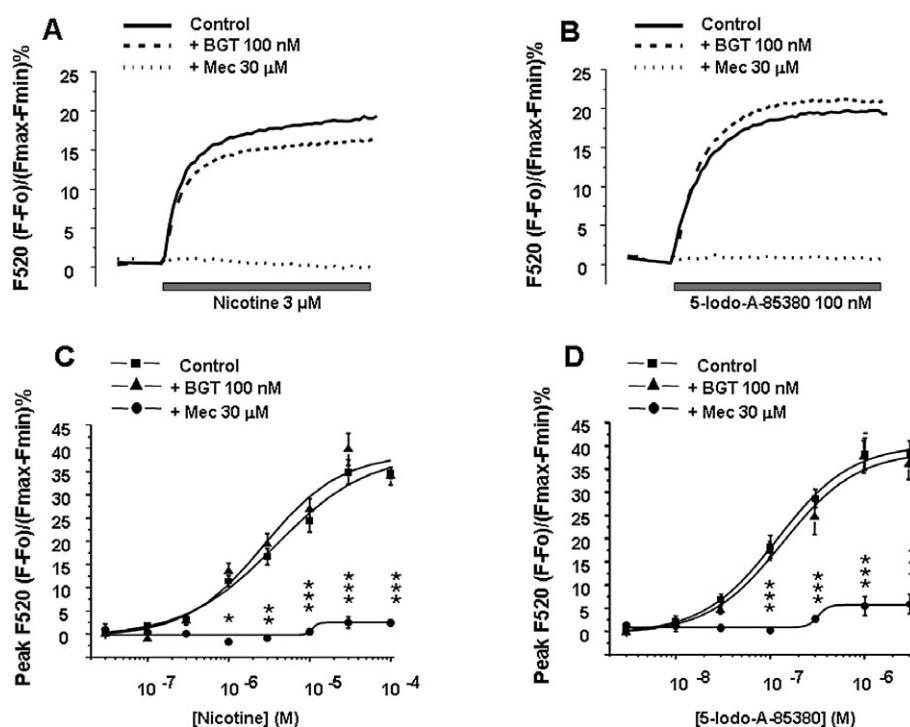
In Fluo-4 loaded chromaffin cells, addition of increasing concentrations of nicotine (0.03 to 100  $\mu$ M) showed that this nicotinic agonist caused  $[Ca^{2+}]_c$  increases in a concentration-dependent manner with an EC<sub>50</sub> of  $3.73 \pm 0.71 \mu$ M (Figure 1C). The  $[Ca^{2+}]_c$  increases induced by nicotine were inhibited by mecamylamine but were unaffected by BGT (see Figure 1A for representative fluorescence tracings performed with 3  $\mu$ M nicotine).

For comparative purposes we used another nAChR agonist, 5-iodo-A-85380, which has been described to have a fivefold higher affinity for  $\alpha$ 3 $\beta$ 4 than for  $\alpha$ 7 nAChRs (Mukhin *et al.*, 2000). 5-Iodo-A-85380 (0.003 to 3  $\mu$ M) produced concentration-dependent  $[Ca^{2+}]_c$  signals with an EC<sub>50</sub> of  $0.11 \pm 0.01 \mu$ M (Figure 1D). As in the case of nicotine, the  $[Ca^{2+}]_c$  increases following 5-iodo-A-85380 were fully blocked by mecamylamine, but were unaffected by BGT (see Figure 1B for representative fluorescence tracings performed with 100 nM 5-iodo-A-85380).

These results suggest that these two agonists, under these experimental conditions, stimulate non- $\alpha$ 7 nAChRs and from now on we will refer to these responses as to non- $\alpha$ 7.

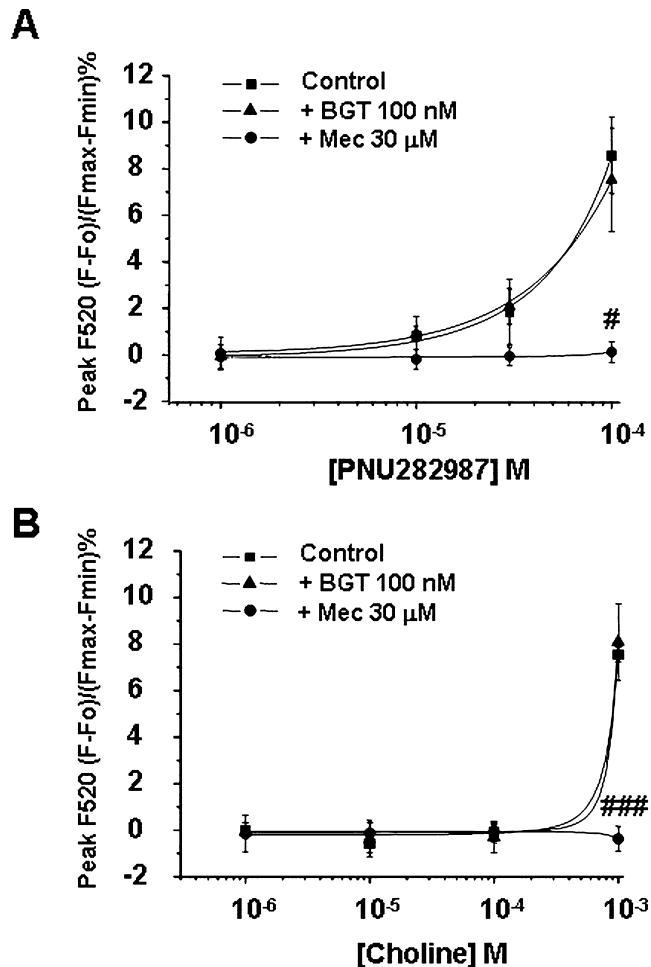
### Cytosolic calcium signals mediated by the $\alpha$ 7 nAChR agonists PNU282987 and choline

We first used the selective  $\alpha$ 7 nAChR agonist PNU282987 (Hajos *et al.*, 2005). At concentrations ranging from 1 to 30  $\mu$ M, PNU282987 failed to produce any significant  $[Ca^{2+}]_c$  increase; however, at



**Figure 1**

Intracellular calcium signals induced by nicotine and 5-iodo-A-85380 in Fluo-4 loaded bovine chromaffin cells. The upper part of the figure shows representative Fluo-4 fluorescence traces induced by nicotine at 3  $\mu$ M (A) and 5-iodo-A-85380 at 100 nM (B) measured over 10 s of continuous agonist stimulation alone or in cells pre-treated for 15 min with  $\alpha$ -bungarotoxin (BGT; 100 nM) or mecamylamine (Mec; 30  $\mu$ M). (C) The mean maximum peak fluorescence after increasing concentrations of nicotine (0.03–100  $\mu$ M) alone or in cells pre-incubated for 15 min with BGT or mecamylamine. (D) Data using the same protocol as used in Figure C, when cells were stimulated by the nicotinic agonist 5-iodo-A-85380 (0.003–3  $\mu$ M). Bars show the mean  $\pm$  SEM of five independent experiments; each experiment was performed in triplicate. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; significantly different from agonist alone; one-way ANOVA followed by a post hoc Newman–Keuls test.



**Figure 2**

Intracellular  $\text{Ca}^{2+}$  responses induced by the  $\alpha 7$  nicotinic acetylcholine receptor (nAChR) agonists PNU282987 and choline. Fluo-4 loaded bovine chromaffin cells were stimulated with increasing concentrations of the  $\alpha 7$  nAChR agonists (A) PNU282987 (1–100  $\mu\text{M}$ ) or (B) choline (1–1000  $\mu\text{M}$ ). The antagonists mecamylamine (Mec; 30  $\mu\text{M}$ ) or  $\alpha$ -bungarotoxin (BGT; 100 nM) were pre-incubated 15 min before injecting the agonists. Data represent the mean  $\pm$  SEM of four independent experiments; each experiment was performed in triplicate. # $P < 0.05$ , ### $P < 0.001$ , significantly different from agonist in the absence of antagonist. One-way ANOVA followed by a post hoc Newman–Keuls test.

100  $\mu\text{M}$  it caused a  $[\text{Ca}^{2+}]_c$  increase that was completely blocked by mecamylamine but unaffected by BGT, suggesting that this calcium increase was due to stimulation of non- $\alpha 7$  nAChRs (Figure 2A).

We also used the endogenous  $\alpha 7$  nAChR agonist choline (Alkondon *et al.*, 1997). A similar pattern to that observed for PNU282987 was observed for this agonist. Choline did not induce a measurable  $[\text{Ca}^{2+}]_c$  increase at concentrations ranging from 1 to 100  $\mu\text{M}$ ; at 1000  $\mu\text{M}$ , choline gave a  $[\text{Ca}^{2+}]_c$  signal that was blocked by mecamylamine but not by BGT (Figure 2B).

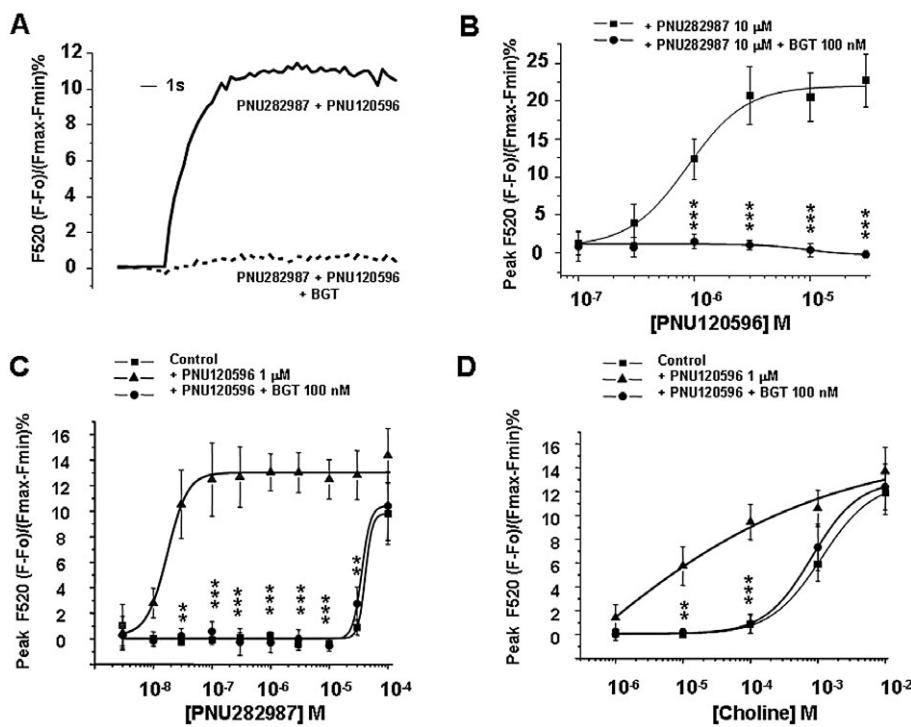
### The $\alpha 7$ nAChR allosteric modulator PNU120596 potentiates the $[\text{Ca}^{2+}]_c$ increases induced by low concentrations of PNU282987, choline and nicotine: the modulated $\alpha 7$ nAChR response

We measured the effects of the  $\alpha 7$  nAChR positive allosteric modulator PNU120596 (Hurst *et al.*, 2005) on the  $[\text{Ca}^{2+}]_c$  increases mediated by the  $\alpha 7$  nAChR agonists PNU282987 and choline. Figure 3A illustrates a typical Fluo-4 recording showing that PNU282987 at 10  $\mu\text{M}$ , a concentration that did not modify basal  $[\text{Ca}^{2+}]_c$  (see Figure 2A), increased by 12-fold the  $[\text{Ca}^{2+}]_c$  signal in cells pre-treated with the  $\alpha 7$  nAChR allosteric modulator PNU120596. This potentiated response was completely blocked by BGT. The  $\alpha 7$  allosteric modulator potentiated responses to the  $\alpha 7$  nAChR agonist PNU282987 (10  $\mu\text{M}$ ) in a concentration-dependent manner, with an  $\text{EC}_{50}$  of  $0.89 \pm 0.14 \mu\text{M}$  and this modulated response was sensitive to BGT (Figure 3B).

We then performed a concentration response-curve with PNU282987 in bovine chromaffin cells pre-treated with a fixed concentration of the  $\alpha 7$  allosteric modulator PNU120596 (1  $\mu\text{M}$ ). Under these experimental conditions, we observed a 12-fold potentiation of the PNU282987 response, especially at those concentrations of PNU282987 that *per se* did not induce detectable  $[\text{Ca}^{2+}]_c$  signals (Figure 3C). The potentiated responses were completely blocked by BGT, suggesting that they were mediated by  $\alpha 7$  nAChRs. At higher concentrations of PNU282987 (10–100  $\mu\text{M}$ ), the proportion of the response that was sensitive to BGT was reduced (Figure 3C).

We also used the endogenous  $\alpha 7$  nAChR agonist choline. In chromaffin cells pre-treated with the  $\alpha 7$  nAChR allosteric modulator. The  $[\text{Ca}^{2+}]_c$  signals induced by choline were significantly potentiated, most clearly at low concentrations and this potentiated response was fully blocked by BGT (Figure 3D). These results suggest that  $\alpha 7$  nAChRs in chromaffin cells require the binding of an allosteric modulator together with lower concentrations of agonists to produce a measurable  $[\text{Ca}^{2+}]_c$  signal, mediated by  $\alpha 7$  nAChRs.

Lastly, we measured the  $[\text{Ca}^{2+}]_c$  increases mediated by the non-specific nAChR agonist nicotine, in cells pre-incubated with PNU120596. The allosteric modulator was able to potentiate the  $[\text{Ca}^{2+}]_c$  signal induced by low concentrations of nicotine (0.3 and 1  $\mu\text{M}$ ) (Figure 4A,C); the potentiated response was again fully blocked by BGT. However, concentrations of nicotine above 1  $\mu\text{M}$  were not significantly potentiated by PNU120596 (Figure 5B,C). These results suggest that low concentrations of nicotine, in the presence of an  $\alpha 7$  nAChR allosteric

**Figure 3**

Intracellular Ca<sup>2+</sup> responses induced by the  $\alpha$ 7 nicotinic acetylcholine receptor (nAChR) agonists PNU282987 and choline in the presence of the  $\alpha$ 7 allosteric modulator PNU120596. (A) Representative Fluo-4 fluorescence traces showing the calcium increase induced by 10  $\mu$ M PNU282987 plus the allosteric modulator at 1  $\mu$ M alone or in cells pre-treated with  $\alpha$ -bungarotoxin (BGT) 100 nM. (B) The Ca<sup>2+</sup> signals induced by 10  $\mu$ M PNU282987 were potentiated in a concentration-dependent manner by the allosteric modulator (from 0.1 to 30  $\mu$ M) PNU120596; such potentiation was completely blocked in cells pre-treated with BGT 100 nM. (C,D) Concentration-response curves of PNU282987 (0.003–100  $\mu$ M) and choline (1  $\mu$ M–10 mM) in the presence of the  $\alpha$ 7 allosteric modulator PNU120596 (1  $\mu$ M). Control cells were pre-incubated for 15 min in the presence of the same volume of DMSO (0.1% (v/v)) which was used to assess the maximum concentration of PNU120596. Data represent the mean  $\pm$  SEM of four independent experiments; each experiment was performed in triplicate. \*\* $P$  < 0.01; \*\*\* $P$  < 0.001; significantly different from agonists plus PNU120596: one-way ANOVA followed by a post hoc Newman–Keuls test.

modulator, can induce [Ca<sup>2+</sup>]<sub>c</sub> increases via  $\alpha$ 7 nAChRs whereas, at concentrations above 1  $\mu$ M, the allosteric potentiation is reduced and nicotine seems to induce [Ca<sup>2+</sup>]<sub>c</sub> increases, predominantly, via non- $\alpha$ 7 nAChRs.

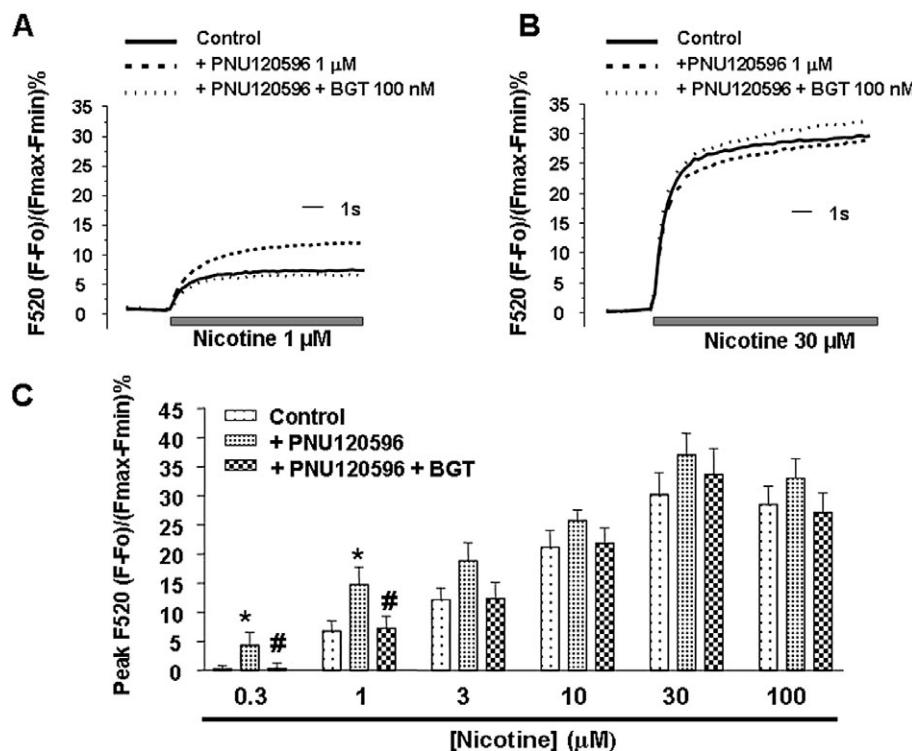
#### *Inactivation of the $\alpha$ 7-nAChR-mediated [Ca<sup>2+</sup>]<sub>c</sub> signals*

To further understand the behavior of the  $\alpha$ 7 nAChRs in bovine chromaffin cells, we analyzed the kinetics of  $\alpha$ 7 [Ca<sup>2+</sup>]<sub>c</sub> signals, mediated solely by  $\alpha$ 7 nAChRs, using PNU282987 as the agonist. Cells pre-incubated with a fixed concentration of PNU120596 (1  $\mu$ M) alone or in the presence of BGT (100 nM), were stimulated with increasing concentrations of PNU282987 (1–100  $\mu$ M) for 5 min to achieve a plateau in the  $\alpha$ 7 desensitization state. To estimate the response mediated solely by  $\alpha$ 7 nAChRs, for a given concentration of the agonist, we subtracted the response that was not blocked by 100 nM BGT (non- $\alpha$ 7 response) from the total response (PNU

282987 + PNU 120596) (see Figure S1 for example). After this correction, the [Ca<sup>2+</sup>]<sub>c</sub> signals changed as the concentration of the agonist was increased. The maximum peak of the [Ca<sup>2+</sup>]<sub>c</sub> signal became lower as the concentration of the  $\alpha$ 7 nAChR agonist increased (Figure 5). These results correlated with the reduction of the  $\tau_{on}$  (activation time constant) and  $\tau_{off}$  (inactivation time constant) (see Table 1). Most probably, the lower maximum peak of the [Ca<sup>2+</sup>]<sub>c</sub> signals obtained with higher concentrations of the agonist could be related to greater inactivation of the  $\alpha$ 7 nAChR.

#### *Participation of VOCCs in the [Ca<sup>2+</sup>]<sub>c</sub> increases mediated by $\alpha$ 7 and non- $\alpha$ 7 nAChRs*

To explore the contribution of the different VOCCs on the  $\alpha$ 7 and non- $\alpha$ 7 nAChR responses, we pre-incubated the cells with a blocker of L-type (nimodipine, 3  $\mu$ M) or a blocker of non-L-type ( $\omega$ -conotoxin MVIIIC, 2  $\mu$ M) VOCCs. As illustrated

**Figure 4**

Dual effect of nicotine on the  $\alpha 7$  and non- $\alpha 7$  nicotinic acetylcholine receptors (nAChRs). (A) Representative traces of intracellular calcium measured as Fluo-4 fluorescence in cells stimulated with a low concentration of nicotine (1  $\mu$ M); this response was potentiated by PNU120596 (1  $\mu$ M) and such potentiation was fully blocked by  $\alpha$ -bungarotoxin (BGT) 100 nM, an  $\alpha 7$  nAChR response. (B) A similar experiment as earlier but at a higher concentration of nicotine (30  $\mu$ M). In this case, the nicotinic response was unaffected by PNU120596 or BGT: a non- $\alpha 7$  nAChR response. (C) Concentration-response curve of nicotine alone (control) or in cells pre-incubated with PNU120596 (1  $\mu$ M) alone or PNU120596 (1  $\mu$ M) plus BGT (100 nM). The  $\text{Ca}^{2+}$  signals induced by low concentrations of nicotine (0.3 and 1  $\mu$ M) were potentiated by the allosteric modulator; this response was blocked by BGT.  $\text{Ca}^{2+}$  increases induced by higher concentrations of nicotine (above 1  $\mu$ M) were not significantly potentiated by PNU120596 and were not affected by BGT. Data represent the mean  $\pm$  SEM of five independent experiments; each experiment was performed in triplicate. \* $P < 0.05$ , significantly different from control. # $P < 0.05$ , significantly different from nicotine plus PNU120596. One-way ANOVA followed by a post hoc Newman–Keuls test.

**Table 1**

Activation constant ( $\tau_{on}$ ), inactivation constant ( $\tau_{off}$ ) and mean maximum peak Fluo-4 fluorescent values of the net  $\alpha 7$ -nicotinic acetylcholine receptor (nAChR)-mediated  $\text{Ca}^{2+}$  response

PNU 282987 concentration	$\tau_{on}$	$\tau_{off}$	Peak max
1 $\mu$ M	7.51 $\pm$ 1.18	67.5 $\pm$ 16.2	16.5 $\pm$ 2.8
10 $\mu$ M	4.34 $\pm$ 0.24	64.3 $\pm$ 14.3	15.6 $\pm$ 2.7
30 $\mu$ M	4.00 $\pm$ 0.70	51.5 $\pm$ 13.5	12.5 $\pm$ 1.8
100 $\mu$ M	2.90 $\pm$ 0.68	22.2 $\pm$ 1.8	8.4 $\pm$ 2.68

To obtain the net  $\alpha 7$  nAChR-mediated response, the non- $\alpha 7$  nAChR response, that is, the fluorescence increases that were not blocked by  $\alpha$ -bungarotoxin, were subtracted from the PNU282987 + PNU120596 fluorescence value. Once these curves were obtained (see Figure 4),  $\tau_{on}$ ,  $\tau_{off}$  (seconds) and maximum peak were calculated.

in Figure 6A, the  $\alpha 7$  nAChR-mediated  $[\text{Ca}^{2+}]_c$  response (PNU282987 at 10  $\mu$ M plus PNU120596 at 1  $\mu$ M) (see Figure 3B,C) was not affected by nimodipine. However, the non-L-type channel blocker,  $\omega$ -conotoxin MVIIIC, decreased the  $\text{Ca}^{2+}$  signal by 19.7  $\pm$  7% with respect to control. In contrast, the non- $\alpha 7$  nAChR response to nicotine at 10  $\mu$ M (see Figure 1C) was decreased by the L-type blocker nimodipine by 16.9  $\pm$  7.7% and was unaffected by  $\omega$ -conotoxin MVIIIC (Figure 6B).

To further test the involvement of VOCCs in the  $[\text{Ca}^{2+}]_c$  increases mediated by stimulation of these different nAChR subtypes, we used two other experimental tools: (i) the non-specific  $\text{Ca}^{2+}$  channel blocker cadmium; and (ii) substitution of the extracellular  $\text{Na}^+$  for N-methyl glucamine to prevent  $\text{Na}^+$ -mediated depolarization. Both cadmium and N-methyl glucamine diminished the responses induced by  $\alpha 7$  and non- $\alpha 7$  nAChRs. Blockade obtained by cadmium or  $\text{Na}^+$  substitution by

N-methyl glucamine was not different to that obtained with  $\omega$ -conotoxin MVIIIC in the case of  $\alpha$ 7 nAChR stimulation (Figure 6A) or nimodipine in the case of non- $\alpha$ 7 nAChR stimulation (Figure 6B).

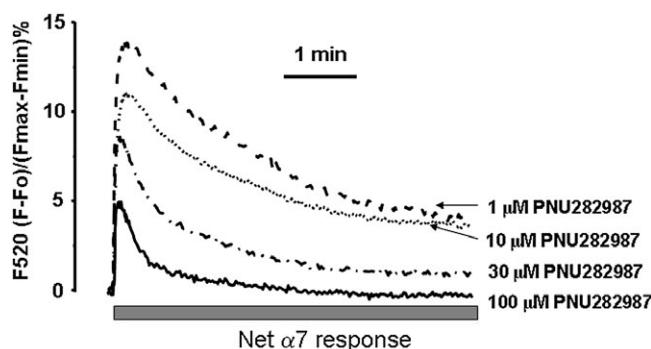
### Magnitude and inactivation of $\alpha$ 7 and non- $\alpha$ 7 nAChRs-mediated [Ca<sup>2+</sup>]<sub>c</sub> transients measured with cytosolic targeted aequorins

As the experimental conditions used above did not allow consecutive stimulation of the cells or quan-

tification of the intracellular calcium concentration, we used another experimental approach that allowed superfusion of the cells, consecutive stimulation-washout cycles and estimation of the magnitude of the [Ca<sup>2+</sup>]<sub>c</sub> transients. For this purpose we used chromaffin cells transfected with aequorins targeted to the cytosol (Montero *et al.*, 2000).

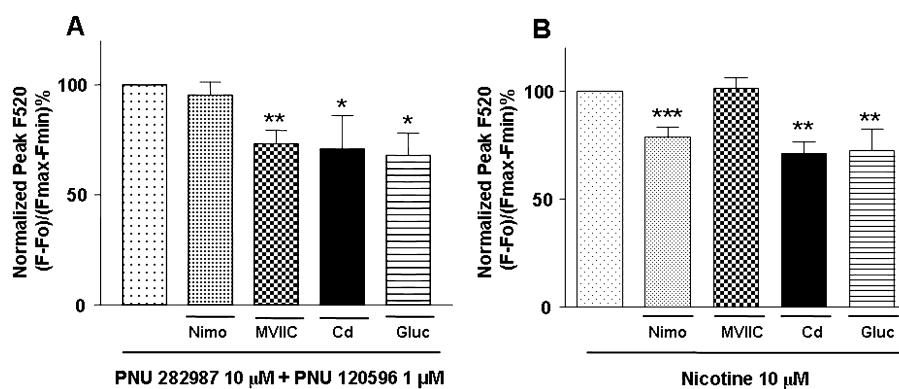
Figure 7A shows a typical experiment. Addition of PNU282987 plus PNU120596 (at concentrations that gave an  $\alpha$ 7 nAChR-mediated response) produced a mean [Ca<sup>2+</sup>]<sub>c</sub> peak around 200 nM, whereas perfusion of nicotine (at a concentration that gave a non- $\alpha$ 7 nAChR response) produced larger peaks of around 600 nM (see statistics in Figure 7C). The nicotine-induced [Ca<sup>2+</sup>]<sub>c</sub> peak was also wider than that induced by PNU120596 plus PNU282987. Thus, when the total [Ca<sup>2+</sup>]<sub>c</sub> response was measured in terms of area under the curve, the [Ca<sup>2+</sup>]<sub>c</sub> response induced by the allosterically modulated  $\alpha$ 7 nAChRs was only 14 ± 3% (mean ± SEM = 6) of that induced by nicotine. When the cells were pre-treated with 100 nM BGT, an irreversible  $\alpha$ 7 nAChR antagonist, the [Ca<sup>2+</sup>]<sub>c</sub> increase induced by PNU120596 plus PNU282987 was abolished whereas the nicotinic response remained unaltered (Figure 7B,C). These results indicated that with this new experimental approach, we could also distinguish between an  $\alpha$ 7 and non- $\alpha$ 7-nAChR-mediated response.

When the cells were stimulated twice with nicotine, this agonist gave two [Ca<sup>2+</sup>]<sub>c</sub> peaks of similar magnitude; however, a second stimulation with PNU120596 plus PNU282987 produced a much smaller response, indicating a slower recovery from



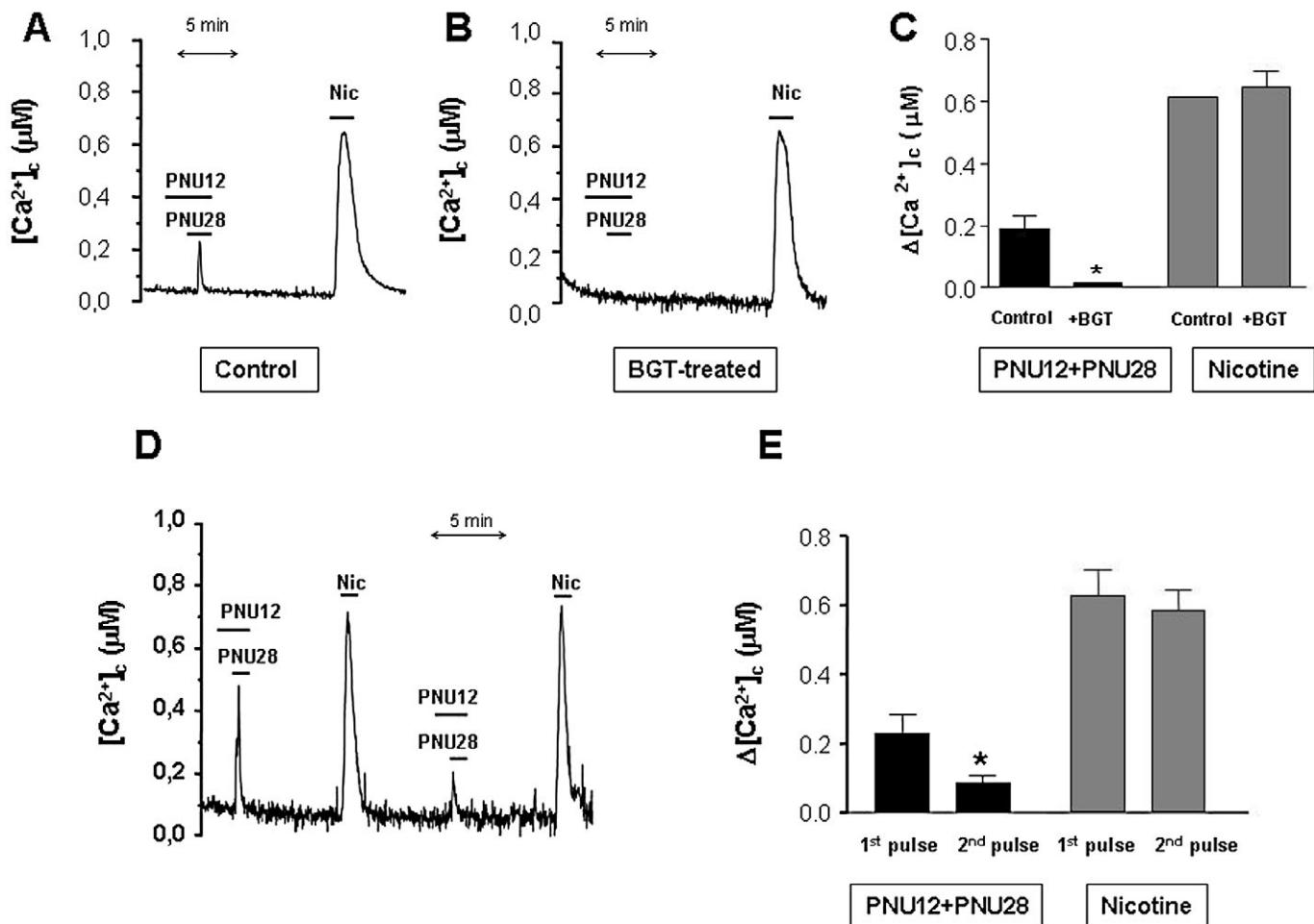
**Figure 5**

Intracellular Ca<sup>2+</sup> traces of pure  $\alpha$ 7 responses mediated by increasing concentrations of PNU282987 plus PNU120596 (1  $\mu$ M). Mean traces of the net  $\alpha$ 7-nicotinic acetylcholine receptor (nAChR)-mediated Ca<sup>2+</sup> responses that correspond to increasing concentrations of the  $\alpha$ 7 nAChR agonist PNU282987 (1–100  $\mu$ M) plus the  $\alpha$ 7 nAChR allosteric modulator PNU120596 at 1  $\mu$ M. To obtain the net  $\alpha$ 7 nAChR response, we subtracted the non- $\alpha$ 7 nAChR response, that is, the fluorescence increases that were not blocked by  $\alpha$ -bungarotoxin from the PNU282987 + PNU120596 values. Data correspond to the mean of five independent experiments.



**Figure 6**

Effect of voltage operated calcium channels (VOCC) blockers on increases in intracellular Ca<sup>2+</sup> mediated by  $\alpha$ 7 and non- $\alpha$ 7 nicotinic acetylcholine receptors (nAChRs). Cells were stimulated with PNU282987 10  $\mu$ M + PNU 120596 1  $\mu$ M (A), 10  $\mu$ M nicotine (B), in the presence of 50  $\mu$ M Cd<sup>2+</sup> (Cd), in Krebs-HEPES with N-methyl glucamine in substitution for sodium (gluc) or in the presence of the L-type VOCC blocker nimodipine (nimo 3  $\mu$ M);, or the non-L VOCC blocker  $\omega$ -conotoxin MVIIIC (MVIIIC 2  $\mu$ M) during 10 s. Control cells, to compare with nimodipine, were supplemented with the same percentage of DMSO (0.1%) that was used to dissolve nimodipine. Data represent the mean and SEM of at least four independent experiments performed in triplicate. \*P < 0.05, \*\*P < 0.01; \*\*\*P < 0.001, significantly different from control: one-way ANOVA followed by a post hoc Newman-Keuls test.



**Figure 7**

Magnitude and inactivation of [Ca<sup>2+</sup>]<sub>c</sub> transients mediated by α7 and non-α7 nicotinic acetylcholine receptors (nAChRs) agonists measured with aequorin. Bovine chromaffin cells expressing cytosolic aequorin were reconstituted with coelenterazine and then luminescence was monitored under continuous perfusion with 2 mM Ca<sup>2+</sup>-containing external medium. (A) Shows a representative recording illustrating [Ca<sup>2+</sup>]<sub>c</sub> transients obtained with submaximal concentrations of PNU282987 10 μM + PNU120596 10 μM and nicotine 30 μM given in the same population of cells. (B) Data from an experiment as in A, but in cells pre-treated with 100 nM α-bungarotoxin (BGT), 15 min prior the experiment, to block the α7 nAChRs. (C) Summary data from several experiments as in A and B, bars correspond to the mean ± SEM of seven individual experiments. \*P < 0.01, significantly different from control. (D) Tracing representing the effects of several additions of either PNU282987 10 μM + PNU120596 10 μM or 30 μM nicotine, as indicated. (E) Summary data from seven experiments (mean ± SEM). Note the differences between the first and second stimulation with α7 or non-α7 nAChR stimulation. \*P < 0.05, significantly different from the first pulse.

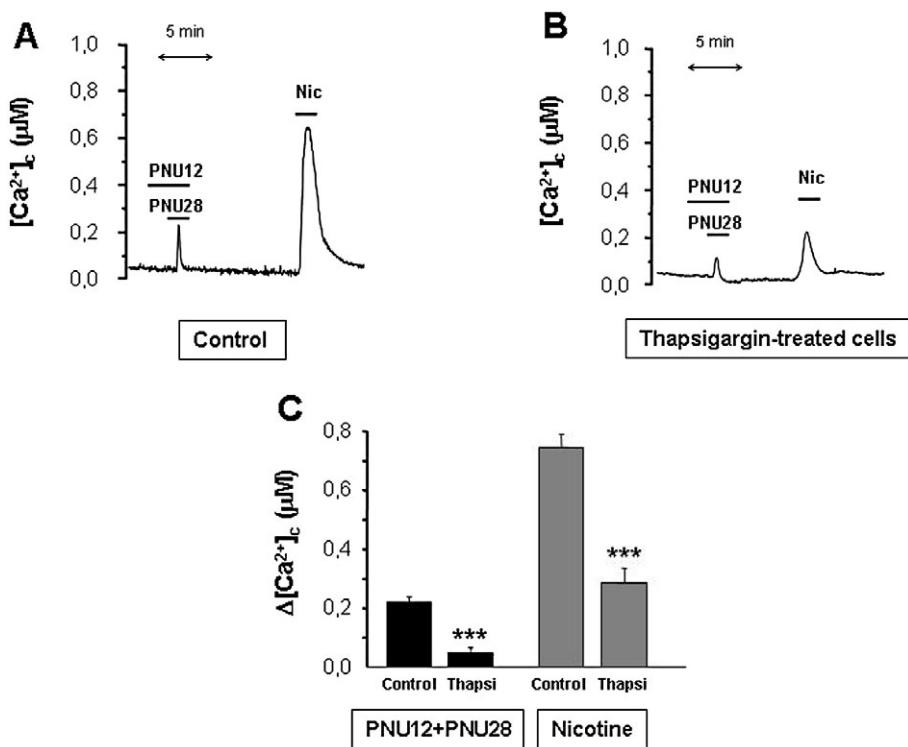
inactivation (Figure 7D,E). Similar results were given when the order of the agonists was changed.

#### Contribution of the endoplasmic reticulum (ER) and mitochondria to the [Ca<sup>2+</sup>]<sub>c</sub> and [Ca<sup>2+</sup>]<sub>m</sub> transients induced by stimulation of α7 and non-α7 nAChR

Figure 8A shows a representative trace of cells consecutively stimulated with PNU120596 plus PNU282987 (α7 nAChR response) and nicotine (non-α7 nAChR response) in control cells expressing cytosolic aequorin. To determine the contribution of the ER to the α7 and non-α7 nAChR-mediated [Ca<sup>2+</sup>]<sub>c</sub> increase, we pre-treated

the cells with thapsigargin, an irreversible blocker of the ER Ca<sup>2+</sup> pump (SERCA), 15 min before nicotinic agonist exposure. Emptying the ER Ca<sup>2+</sup> store with thapsigargin reduced, by over 80%, the [Ca<sup>2+</sup>]<sub>c</sub> peaks induced by PNU120596 plus PNU282987 and by 60% the [Ca<sup>2+</sup>]<sub>c</sub> peaks induced by nicotine (Figure 8B,C). These results were confirmed in chromaffin cells loaded with fluo-4 AM (Figure S2). Contribution of the ER was significantly higher (*P* < 0.001; *n* = 3) for the α7 nAChR response, compared with the non-α7 nAChR response.

The other intracellular organelle that participates in the regulation of intracellular calcium

**Figure 8**

Contribution of the endoplasmic reticulum to the  $\alpha$ 7 and non- $\alpha$ 7 cytosolic Ca<sup>2+</sup> transients. Representative tracings of [Ca<sup>2+</sup>]<sub>c</sub> induced by  $\alpha$ 7 (PNU282987 10  $\mu$ M + PNU120596 10  $\mu$ M) and non- $\alpha$ 7 (nicotine 30  $\mu$ M) nicotinic acetylcholine receptors (nAChRs) in control (A) and in cells treated with 1  $\mu$ M thapsigargin for 15 min prior to the experiment (B). (C) Summary data from several similar experiments as those shown in A and B. Bars represent the mean and SEM of at least six independent experiments from different cultures. \*\*\* $P < 0.001$ , significantly different from control.

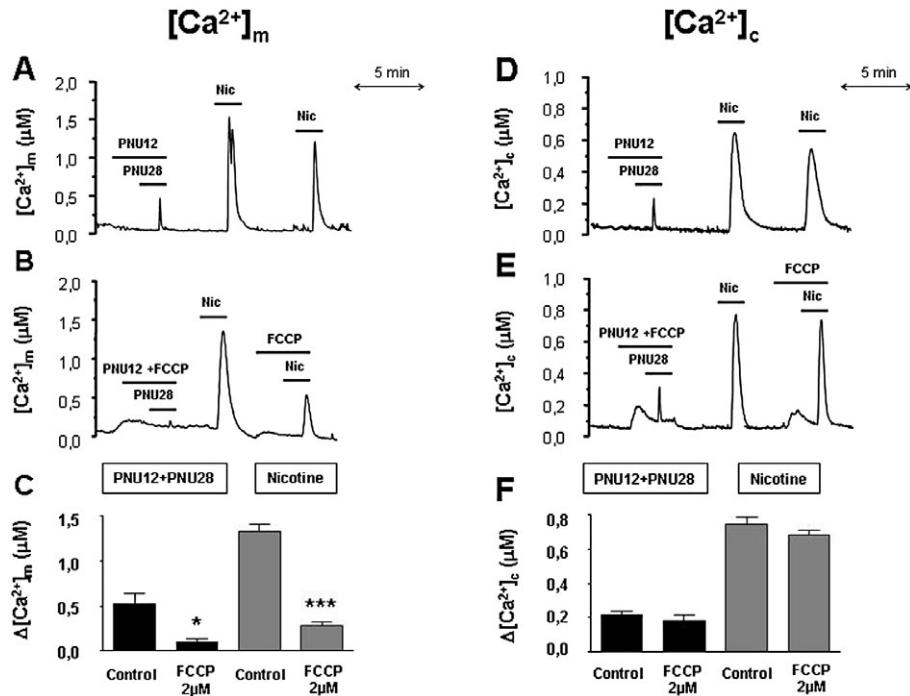
homeostasis is the mitochondria. We therefore measured mitochondrial [Ca<sup>2+</sup>] ([Ca<sup>2+</sup>]<sub>m</sub>) with aequorins targeted to this organelle. PNU120596 plus PNU282987 and nicotine elicited larger responses in the mitochondria (Figure 9A,C) than those measured in the cytosol (Figure 9D,F). Stimulation with the  $\alpha$ 7 agonist and the allosteric modulator, induced [Ca<sup>2+</sup>]<sub>m</sub> peaks of  $516 \pm 119$  nM and [Ca<sup>2+</sup>]<sub>c</sub> peaks of  $218 \pm 20$  nM (the ratio of [Ca<sup>2+</sup>]<sub>m</sub>/[Ca<sup>2+</sup>]<sub>c</sub> was  $2.4 \pm 0.8$ ). Stimulation with nicotine induced [Ca<sup>2+</sup>]<sub>m</sub> peaks of  $1324 \pm 83$  nM and [Ca<sup>2+</sup>]<sub>c</sub> peaks of  $745 \pm 44$  nM ([Ca<sup>2+</sup>]<sub>m</sub>/[Ca<sup>2+</sup>]<sub>c</sub> was  $1.8 \pm 0.2$ ). These ratio values, [Ca<sup>2+</sup>]<sub>m</sub>/[Ca<sup>2+</sup>]<sub>c</sub>, were not different from each other, indicating that mitochondria captured Ca<sup>2+</sup> in the same way, regardless of which nAChR subtype is stimulated. The higher [Ca<sup>2+</sup>]<sub>m</sub> measurements are probably a consequence of the ability of mitochondria to amplify the [Ca<sup>2+</sup>]<sub>c</sub> variations.

In addition, as could be expected, the [Ca<sup>2+</sup>]<sub>m</sub> peaks induced by PNU120596 plus PNU282987 and nicotine were markedly reduced by the protonophore FCCP (Figure 9B,C). However, mitochondrial depolarization with FCCP hardly modified [Ca<sup>2+</sup>]<sub>c</sub> caused by  $\alpha$ 7 or non- $\alpha$ 7 nAChR activation

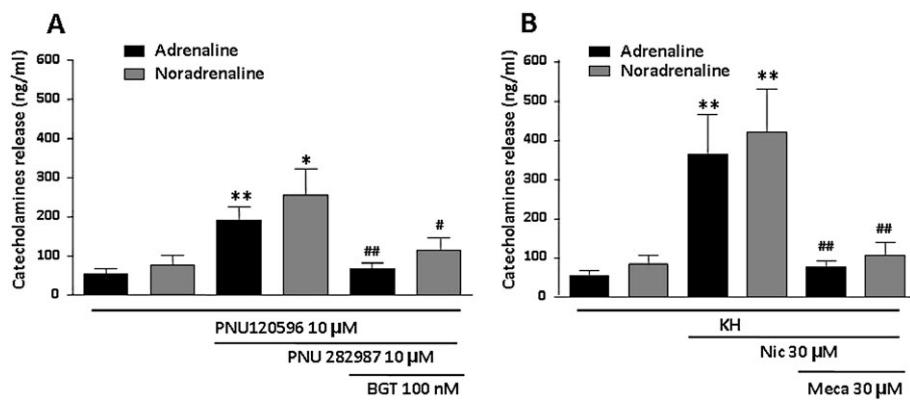
(Figure 9E,F). Therefore these results indicate that the ER but not the mitochondria participate in the [Ca<sup>2+</sup>]<sub>c</sub> transients caused by stimulation of  $\alpha$ 7 and non- $\alpha$ 7 nAChRs.

#### Catecholamine release induced by $\alpha$ 7 and non- $\alpha$ 7 nAChR stimulation

To determine if the Ca<sup>2+</sup> signals were functional in terms of inducing exocytosis, we measured, by HPLC, secretion of adrenaline and noradrenaline induced by  $\alpha$ 7 and non- $\alpha$ 7 nAChR stimulation. Basal adrenaline release from  $10^6$  bovine chromaffin cells was  $54 \pm 15$  ng·mL<sup>-1</sup> whereas that of noradrenaline was  $84 \pm 25$  ng·mL<sup>-1</sup>. The  $\alpha$ 7 nAChR allosteric modulator PNU120596 at 10  $\mu$ M did not modify basal levels of adrenaline or noradrenaline. However, in the presence of 10  $\mu$ M PNU282987, adrenaline release increased to  $191 \pm 38$  ng·mL<sup>-1</sup> (354% increase) and noradrenaline to  $255 \pm 72$  ng·mL<sup>-1</sup> (305% increase); this catecholamine release was completely blocked by BGT (Figure 10A). Nicotine at 30  $\mu$ M increased adrenaline release to  $364 \pm 111$  ng·mL<sup>-1</sup> (674%) and noradrenaline to

**Figure 9**

Contribution of the mitochondria to the mitochondrial and cytosolic Ca<sup>2+</sup> transients induced by stimulation of  $\alpha 7$  or non- $\alpha 7$  nicotinic acetylcholine receptors (nAChRs). Bovine chromaffin cells expressing mitochondrially-or- cytosolic targeted aequorin were reconstituted with coelenterazine and then luminescence was monitored under continuous perfusion with 2 mM Ca<sup>2+</sup>-containing external medium. Left panels show representative traces of [Ca<sup>2+</sup>]<sub>m</sub> transients induced by  $\alpha 7$  (PNU28987 10  $\mu$ M + PNU120596 10  $\mu$ M) and non- $\alpha 7$  (nicotine 30  $\mu$ M) nAChRs agonists, in control cells (A) and in cells treated with FCCP (2  $\mu$ M), 2 min prior to the stimulus (B). (C) Summary data from several similar experiments as those shown in A and B. Right panels show representative traces of [Ca<sup>2+</sup>]<sub>c</sub> induced by  $\alpha 7$  (PNU28987 10  $\mu$ M + PNU120596 10  $\mu$ M) and non- $\alpha 7$  (nicotine 30  $\mu$ M) nAChR agonists in control cells (D) and in cells treated with FCCP(2  $\mu$ M), 2 min prior to and during the stimulus (E). (F) Summary data from several similar experiments as those shown in D and E. Data in panels C and F correspond to the mean and SEM of three to five independent experiments. \*P < 0.05 and \*\*\*P < 0.001, significantly different from control.

**Figure 10**

Differential release of adrenaline and noradrenaline induced by  $\alpha 7$  (A) and non- $\alpha 7$  (B) nicotinic acetylcholine receptor (nAChR) stimulation. Cells were stimulated for 30 s with both PNUs or nicotine and medium were collected to measure catecholamine release by high-performance liquid chromatography. Antagonists were pre-incubated for 5 min and were present during agonist stimulation. Bars show the mean  $\pm$  SEM from seven independent experiments. \*P < 0.05, \*\*P < 0.01, significantly different from PNU120596 alone for panel A or Krebs-HEPES for panel B. #P < 0.05, ##P < 0.01, significantly different from both PNUs for A or nicotine for B. The statistical analysis used was a one-way ANOVA followed by a post hoc Newman-Keuls test.

$422 \pm 116 \text{ ng} \cdot \text{mL}^{-1}$  (502%); such secretion was fully blocked by mecamylamine (Figure 10B).

When normalized catecholamine release (total amount of catecholamines/basal catecholamines secreted) was divided by the normalized peak [Ca<sup>2+</sup>]<sub>i</sub> (total peak [Ca<sup>2+</sup>]<sub>i</sub>/basal [Ca<sup>2+</sup>]<sub>i</sub>) the proportion obtained for the  $\alpha$ 7 nAChR response was 1.5 and for the non- $\alpha$ 7 nAChR response was 0.6. These results suggest that for a given [Ca<sup>2+</sup>]<sub>i</sub> peak, the modulated  $\alpha$ 7 nAChR stimulus secretes more catecholamines, by a factor of 2.5, than a non- $\alpha$ 7 nAChR stimulus.

## Discussion

Bovine chromaffin cells are a model extensively used to study calcium signalling and exocytosis, but the contribution of the different subtypes of nAChRs to the control of these parameters are still in debate, especially for the  $\alpha$ 7 nAChR subtype. By using new pharmacological and selective tools, we have systematically studied how activation of  $\alpha$ 7 and non- $\alpha$ 7 nAChRs of bovine chromaffin cells mediated intracellular Ca<sup>2+</sup> signals and catecholamine secretion.

We first characterized the non- $\alpha$ 7 nAChR-mediated response. The intracellular Ca<sup>2+</sup> transients induced by nicotine and 5-iodo-A-85380 were related to non- $\alpha$ 7 nAChRs, as they were fully blocked by mecamylamine but were unaffected by the  $\alpha$ 7 nAChR antagonist BGT. This non- $\alpha$ 7 nAChR response was similar to those previously reported in bovine chromaffin cells and PC12 cells (Afar *et al.*, 1994; Innocent *et al.*, 2008).

Because nicotine has been reported to act on different nAChR subtypes in PC12 cells (Nakayama *et al.*, 2001) and in chick ciliary ganglion neurons (Vijayaraghavan *et al.*, 1992), we used PNU120596, an  $\alpha$ 7 nAChR allosteric modulator that is known to potentiate the  $\alpha$ 7 nAChR agonist response in PC12 and SHSY5Y cells (Dickinson *et al.*, 2007; Innocent *et al.*, 2008). When we used PNU120596, which does not display any effect on  $\alpha$ 3 $\beta$ 4 nAChRs expressed in *Xenopus* oocytes (Gronlien *et al.*, 2007), we observed that the minimal [Ca<sup>2+</sup>]<sub>c</sub> transients induced by low concentrations of nicotine (1  $\mu\text{M}$  or below) were markedly increased and that this potentiated response was fully blocked by the selective  $\alpha$ 7 nAChR antagonist BGT. However, nicotine at concentrations above 3  $\mu\text{M}$ , provided concentration-dependent [Ca<sup>2+</sup>]<sub>c</sub> transients in the absence of the allosteric modulator and this response was blocked by mecamylamine but not by BGT. Therefore, low concentrations of nicotine, in the presence of an  $\alpha$ 7 nAChR allosteric modulator, gave an  $\alpha$ 7 nAChR-

modulated response, whereas high concentrations of nicotine alone give a non- $\alpha$ 7 nAChR-mediated signal.

To further investigate how activation of  $\alpha$ 7 nAChRs could raise intracellular Ca<sup>2+</sup> and catecholamine secretion we used the selective  $\alpha$ 7 nAChR agonists like PNU282987 and choline. In spite of PNU282987 being highly selective for  $\alpha$ 7 nAChRs (Bodnar *et al.*, 2005; Hajos *et al.*, 2005) and showing almost negligible blockade by  $\alpha$ 3 $\beta$ 4 nAChR antagonists (Wishka *et al.*, 2006), we found that this agonist, at concentrations ranging from 1 to 10  $\mu\text{M}$ , did not induce measurable [Ca<sup>2+</sup>]<sub>c</sub> increases. However at concentrations above 30  $\mu\text{M}$ , it did induce [Ca<sup>2+</sup>]<sub>c</sub> increases that were fully blocked by mecamylamine but were unaffected by BGT. We also found a similar pattern with the endogenous  $\alpha$ 7 nAChR agonist choline. In rat superior cervical ganglion neurones, such BGT-insensitive responses to choline have also been reported (Alkondon *et al.*, 1997; Seddik *et al.*, 2003). However, in the presence of the  $\alpha$ 7 nAChR allosteric modulator PNU120596, the  $\alpha$ 7 nAChR agonist and choline, at concentrations that did not elicit measurable [Ca<sup>2+</sup>]<sub>c</sub> increases by themselves, were markedly increased. These potentiated responses were completely blocked by BGT, indicating that it was an  $\alpha$ 7-nAChR-mediated response.

The activation and inactivation constants as well as the maximum peak of the  $\alpha$ 7 nAChR-mediated [Ca<sup>2+</sup>]<sub>c</sub> responses decreased, as the concentration of the  $\alpha$ 7 nAChR agonist increased (Fatt, 1950; Katz and Thesleff, 1957), suggesting a rapid desensitization of the receptor (Couturier *et al.*, 1990). The rapid inactivation of this receptor could also explain the loss of the  $\alpha$ 7 nAChR response at higher concentrations of the agonists.

The faster inactivating profile of the  $\alpha$ 7 nAChR-mediated response was corroborated in the aequorin experiments, where a second stimulation with PNU282987 plus PNU120596 gave practically no response. However, two consecutive nicotine pulses, a non- $\alpha$ 7 nAChR-mediated response, gave [Ca<sup>2+</sup>]<sub>c</sub> transients of similar amplitude, i.e. there was hardly any inactivation. Although it has been reported that PNU120596 increases the mean opening time of the  $\alpha$ 7 nAChR channel even in the desensitized state (Bodnar *et al.*, 2005; Gronlien *et al.*, 2007), our results (Figure 4) suggest a time-dependent decrease of the Ca<sup>2+</sup> signal after prolonged exposure to the agonist.

Cell depolarization and participation of VOCCs in the [Ca<sup>2+</sup>]<sub>c</sub> transients observed with PNU282987 plus PNU120596 ( $\alpha$ 7 response) and nicotine (non- $\alpha$ 7 response) were corroborated with the N-methyl glucamine and cadmium experiments.

Substitution of  $\text{Na}^+$  by N-methyl glucamine, which prevents membrane depolarization mediating  $\text{Na}^+$  entry through its channels, reduced the  $\alpha 7$  and non- $\alpha 7$  nAChR-mediated increases in  $[\text{Ca}^{2+}]_c$  to levels that were not statistically different to those obtained with  $\omega$ -conotoxin MVIIIC in the case of the  $\alpha 7$  response or nimodipine in the case of the non- $\alpha 7$  nAChR response. Moreover, the observation that the non-specific  $\text{Ca}^{2+}$  channel blocker cadmium did not further increase the blockade of the  $[\text{Ca}^{2+}]_c$  responses induced by nimodipine or  $\omega$ -conotoxin MVIIIC in the  $\alpha 7$  or non- $\alpha 7$ -mediated responses, respectively, further supports the idea that non-L-type  $\text{Ca}^{2+}$  channels were coupled to the  $\alpha 7$  nAChRs, whereas L-type  $\text{Ca}^{2+}$  channels were coupled to non- $\alpha 7$  nAChRs.

Differential coupling of L and non-L-type VOCCs to a specific nAChR subtype can also contribute to the interpretation of the kinetics of the  $\text{Ca}^{2+}$  signals mediated by these receptor subtypes, and the dual activation pattern of a given agonist. For example,  $\text{Ca}^{2+}$  entry through L-type VOCCs favoured the inhibition of N and P/Q channels in voltage-clamped bovine adrenal chromaffin cells (Rosa *et al.*, 2009). Therefore, when a nicotinic agonist acts on non- $\alpha 7$  nAChRs, coupled to L-type  $\text{Ca}^{2+}$  channels, the  $\alpha 7$  nAChRs are inactivated because they are coupled to non-L  $\text{Ca}^{2+}$  channels that inactivate more rapidly (Villarroya *et al.*, 1999). Also, depolarization itself can inactivate N-and-P/Q-type- $\text{Ca}^{2+}$  channels (Villarroya *et al.*, 1999) that are more prone to inactivation than L-type  $\text{Ca}^{2+}$  channels; this action translates into a reduction of  $[\text{Ca}^{2+}]_c$  and catecholamine release (Artalejo *et al.*, 1986; Michelena *et al.*, 1993). Therefore, rapid inactivation of  $\alpha 7$  nAChRs could be related to the fact that they seem to be coupled to non-L-type  $\text{Ca}^{2+}$  channels that inactivate faster, whereas non- $\alpha 7$  nAChRs, which are coupled to the slower inactivating L-type  $\text{Ca}^{2+}$  channels, show less inactivation. However, in other systems like GH3 rat pituitary cells expressing homomeric  $\alpha 7$  nAChR,  $[\text{Ca}^{2+}]_c$  transients induced by ACh were blocked by isradipine, an L-type  $\text{Ca}^{2+}$  channel antagonist (Feuerbach *et al.*, 2005).

Besides  $\text{Ca}^{2+}$  entry through VOCCs, CICR from the ER can also contribute to the nAChR-mediated  $[\text{Ca}^{2+}]_c$  transients. Our results in bovine chromaffin cells indicated that CICR made a major contribution to  $[\text{Ca}^{2+}]_c$  transients upon nAChR stimulation, in comparison with  $\text{Ca}^{2+}$  entry through VOCC. We also found, as previously reported in PC12 cells (Dickinson *et al.*, 2007), that CICR was a major contributor during  $\alpha 7$  nAChR stimulation, when compared with non- $\alpha 7$  nAChR stimulation.

As to the participation of the mitochondria, the  $[\text{Ca}^{2+}]_c$  transients induced by both  $\alpha 7$  and non- $\alpha 7$

nAChRs were slightly and similarly amplified in this organelle, resulting in  $[\text{Ca}^{2+}]_m$  transients of about twice the magnitude compared with the corresponding cytosolic transients. In spite of this observation, we did not observe significant differences between mitochondrial  $\text{Ca}^{2+}$  uptake, relative to cytosolic  $\text{Ca}^{2+}$  levels, which could be related to the nAChR subtype producing  $\text{Ca}^{2+}$  entry. Moreover, blockade of mitochondrial  $\text{Ca}^{2+}$  uptake with the protonophore FCCP produced no changes in the  $[\text{Ca}^{2+}]_c$  peaks induced by either  $\alpha 7$  or non- $\alpha 7$  nAChR responses, indicating that under these experimental conditions, mitochondrial  $\text{Ca}^{2+}$  sequestration was relatively small and could not significantly modify the overall  $[\text{Ca}^{2+}]_c$  transient. As shown before, massive  $\text{Ca}^{2+}$  accumulation into mitochondria and its implication in catecholamine modulation requires the application of much more potent stimuli (Herrington *et al.*, 1996; Montero *et al.*, 2000; 2001). Therefore in this case, the physiological significance of the observed increases in the  $[\text{Ca}^{2+}]_m$  would be more related with the activation of energy production than with the buffering of cytosolic  $[\text{Ca}^{2+}]$ .

Activation of postsynaptic nAChRs located in chromaffin cells, by acetylcholine, is responsible of catecholamine secretion under stressful conditions. The results of this study show that calcium entry through the modulated  $\alpha 7$  nAChR seems to be more effective in inducing exocytosis, in comparison with the  $\alpha 3\beta 4/\alpha 3\beta 4\alpha 5$  receptor (Tachikawa *et al.*, 2001), in terms of secretion/ $[\text{Ca}^{2+}]_c$  ratios. However, the magnitude of catecholamine release induced by nicotine, at concentrations related to non- $\alpha 7$  nAChRs was much higher compared with the  $\alpha 7$  nAChR-modulated response. The fact that  $\alpha 7$  nAChRs seem to be coupled to non-L type VOCC, which have been described to be located closer to the exocytotic sites (Lopez *et al.*, 1994; Lara *et al.*, 1998), could explain why  $\text{Ca}^{2+}$  entry through this receptor seems to be more efficient in inducing exocytosis. Although it had been previously reported (Criado *et al.*, 1997) that BGT-labelled cells were mainly adrenergic in adrenal slices, we have not found statistical differences between adrenaline and noradrenaline release when secretion was stimulated via  $\alpha 7$  or non- $\alpha 7$  nAChRs. This finding could be probably attributed to differences in the preparation used; in this study we have employed isolated bovine chromaffin cells in culture instead of adrenal slices.

In conclusion, although the functional role of the  $\alpha 7$  nAChR in the CNS has been well characterized (Barrantes *et al.*, 1995; Meyer *et al.*, 1998; Martin *et al.*, 2004; Ulloa, 2005), its functional properties in the peripheral nervous system have been

less well defined (Fucile *et al.*, 2005). The expression density of this subtype of nAChR in plasma membranes from bovine chromaffin cells is similar ( $[^{125}\text{I}] \alpha\text{BGT } 49.9 \pm 7.6 \text{ fmol}\cdot\text{mg}^{-1}$  protein) (El-Hajj *et al.*, 2007) to those reported for rat hippocampal membranes ( $[^{125}\text{I}] \alpha\text{BGT } 51.1 \text{ fmol}\cdot\text{mg}^{-1}$  protein) (Garcia-Guzman *et al.*, 1995). So, the question is why if bovine chromaffin cells express  $\alpha 7$  nAChRs, most of the attempts to establish their functional role have produced negative results? (Afar *et al.*, 1994; Tachikawa *et al.*, 2001). Nevertheless, we (Lopez *et al.*, 1998) were able to partially block ACh-induced calcium signals and catecholamine secretion with a selective  $\alpha 7$  nAChR antagonist, which was an indirect indication to its participation in these processes. However, detection of calcium signals or exocytosis by direct activation of  $\alpha 7$  nAChRs in these cells have yet not been clearly demonstrated. Our data offer at least three reasons why a functional role for the chromaffin  $\alpha 7$  nAChRs, in terms of increasing cytosolic Ca<sup>2+</sup> and evoking catecholamine release, has been difficult to find: (i) the  $\alpha 7$  nAChRs of bovine chromaffin cells need the presence of an allosteric modulator and low concentrations of agonists to achieve a measurable  $[\text{Ca}^{2+}]_c$  transient; (ii) the magnitude of the  $[\text{Ca}^{2+}]_c$  signal, compared with the non- $\alpha 7$ nAChR-mediated response, is about 25%, in terms of maximum peak response, and around 15%, in terms of total calcium entry; and (iii) the  $\alpha 7$  nAChR inactivates very rapidly.

In summary, the results of this study indicate that  $\alpha 7$  nAChRs, in bovine chromaffin cells, are able to mediate measurable calcium signals and catecholamine exocytosis under circumstances that require an allosteric modulator and low concentrations of agonists. Notably, these requirements are not necessary for the effects mediated by the non- $\alpha 7$  nAChRs.

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## Conflicts of interest

None to declare.

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## Supporting information

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

**Figure S1** To determine the pure  $\alpha$ 7 response (Trace C), we subtracted the response that was not blocked by 100 nM  $\alpha$ -bungarotoxin (non- $\alpha$ 7 response- Trace B) from the total response (PNU 282987 + PNU 120596- Trace A). Variables of Trace A and Trace B were performed in parallel in the same batches of cell cultures.

**Figure S2** Comparison of the contribution of the ER to the  $\alpha$ 7 and non- $\alpha$ 7 nACh-mediated responses. Intracellular Ca<sup>2+</sup> was measured in bovine chromaffin cells loaded with Fluo-4AM using a fluorescence microplate reader as described in the Materials and

Methods section. Blockade of the ER was achieved by treating the cells for 15 min with thapsigargin 1  $\mu$ M prior to the addition of the nicotinic agonists. Data correspond to the mean and S.E.M of five experiments. \*\*\* $P < 0.001$  in comparison to control response. \*\*\* $P < 0.001$  comparing the  $\alpha$ 7 and non- $\alpha$ 7 response in thapsigargin-treated cells.

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