A New Isoxazolic Compound Acts as α7 Nicotinic Receptor Agonist in Human Umbilical Vein Endothelial Cells

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Dedicated to the memory of Dr. Luis Astudillo

Recent evidence suggests that the α7 nicotinic acetylcholine receptors (α7 nAChRs) participate in the development of angiogenesis and could be a new endothelial target for revascularization in therapeutic angiogenesis. It has been shown that in human umbilical vein endothelial cells (HUVECs) α7 nAChR agonists increase the intracellular calcium concentration ($[Ca^{2+}]_i$), thus inducing proliferation and vessel formation which are important stages of angiogenesis. In the present study we evaluated the effect of new isoxazole compounds on the cytosolic Ca^{2+} signal in HUVECs using the fluorescent Ca^{2+} indicator Fluo-3AM and probing the involvement of α7 nAChR by means of pharmacological tools. HUVECs expressed mainly α7 nAChR, since there was no significant difference in the increase in $[Ca^{2+}]_i$ induced by nicotine, a non-selective nicotinic agonist, in relation to choline, a selective α7 nAChR agonist. The increase in $[Ca^{2+}]_i$ induced by 1 mM choline was inhibited significantly ($p = 0.014$) in cells which had been pre-incubated for 15 min with methyllycaconitine (MLA), a selective α7 nAChR antagonist. The studied compounds **1**, **2**, and **3** induced an increase in $[Ca^{2+}]_i$ in a dose-dependent manner. Compound **1** at 10 μM induced a greater increase in $[Ca^{2+}]_i$ than compounds **2** and **3**. The increase in $[Ca^{2+}]_i$ induced by compound **1** was significantly inhibited by MLA ($p = 0.013$) and completely inhibited by mecamylamine, a non-selective nAChR antagonist, indicating that the isoxazolic compound **1** acts as an α7 nAChR agonist.

Key words: α7 Nicotinic Receptor, Cytosolic Calcium Signal, Isoxazole

Introduction

Nicotinic acetylcholine receptors (nAChRs) are expressed in non-neuronal tissues, such as epithelial cells, endothelial cells (ECs), and immune cells, thereby proving the existence of the cholinergic system beyond neurons (Wessler and Kirkpatrick, 2008). Of all the nAChRs identified, the homomeric α7 has been an important target of investigations due to its

high permeability to calcium ions (Ca^{2+}) (Castro and Albuquerque, 1995).

In ECs, the α7 homomeric nAChRs are believed to predominate as the mediators of angiogenesis (Pillai and Chellapan, 2012; Heeschen *et al*., 2002). This process is defined as the dynamic process of neovascularization, representing the formation of new blood vessels from pre-existing ones (Risau, 1997; Folkman, 2006). Angiogenesis is a complex process that is regulated by a balance between pro- and antiangiogenic molecules. The complex interactions among

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these molecules and how they affect the vascular structure and function in different environments are the objects of research (Carmeliet and Jain, 2000).

By acting on nAChRs, acetylcholine (ACh) can regulate vascularization and remodeling (Cooke, 2007; Arias *et al.*, 2009). In the same way, nicotine has been defined as a proangiogenic factor (Costa and Soares, 2008), which modulates angiogenesis via *α*7 nAChRs in several types of human ECs (Wu *et al.*, 2009), and promotes EC migration, proliferation, survival, and tube formation *in vitro* (Kiuchi *et al.*, 2008). Nicotine has proved to be experimentally angiogenic in human umbilical vein endothelial cells (HUVECs) by increasing the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Wang *et al.*, 2006).

Indeed, the *α*7 nAChRs play an important role in cholinergic angiogenesis, and thus may be a target for therapeutic modulation in disorders of pathological or insufficient angiogenesis (Carmeliet and Jain, 2000). Currently, there is an intensive search for such specific agonists as potential agents in a process called "therapeutic angiogenesis". This process aims at the stimulation and growth of new blood vessels from pre-existing ones in order to re-supply blood flow to affected ischemic tissues (Deveza *et al.*, 2012).

Compounds with the isoxazole ring exhibit several important biological activities such as antibacterial (Ali *et al.*, 2011), antiplatelet (Vrzheshch *et al.*, 1994), and antioxidant (Kalirajan *et al.*, 2012). The isoxazole analogue of Nicotine, ABT-418, has become a standard nAChR ligand (Briggs *et al.*, 1995). Similarly, PNU-120596 has an isoxazole-based structure that has proven to be a positive allosteric modulator of *α*7 nAChRs (Hurst *et al.*, 2005). Many methods have been employed in the synthesis of isoxazoles (Melo, 2005), including sequential (3 + 2) cycloaddition/silicon-based cross-coupling reactions (Denmark and Kallemeyn, 2005), reactions of hydroxylamine with aldehydes in acid medium (Kaffy *et al.*, 2004), and reactions of 2-alkyn-1-one-*ortho*-methyl oximes with ICl, I_2, Br_2, or PhSeBr via electrophilic cyclization (Waldo and Larock, 2007). In this paper, we present the synthesis of three new isoxazolic compounds and the *in vitro* evaluation of their effects on $[Ca^{2+}]_i$ in a primary culture of HUVECs.

Materials and Methods

General aspects

^{1}H and ^{13}C NMR spectra were recorded on a Bruker AM-400 spectrometer (Rheinstetten, Germany), using CDCl$_3$ as solvent. Tetramethylsilane (TMS) was used as an internal standard. Chemical shifts (δ) and J values are reported in ppm and Hz, respectively, relative to the solvent peak – CHCl$_3$ in CDCl$_3$ at 7.24 ppm for protons and 77 ppm for carbon atoms. Signals are designated as follows: s, singlet; d, doublet; dd, doublet of doublets; t, triplet; m, multiplet; br.s, broad singlet. In DEPT-135 spectra, the signals of CH$_3$, CH$_2$, and CH carbon atoms are shown as positive ($+$), negative ($-$), and positive ($+$), respectively. Quaternary carbon atoms are not shown.

ESI-MS (MS) data were collected using a high-resolution hybrid quadrupole (Q) and orthogonal time-of-flight (TOF) mass spectrometer (Q-Tof; Micromass, Milford, MA, USA) with constant nebulizer temperature of 100 °C. The ESI source and the mass spectrometer were operated in the positive ion mode; the cone and extractor potentials were set to 40 and 5 V, respectively, with a scan range of m/z 80 – 1000. Samples were infused into the ESI source at flow rates of ca. 5 μL/min via a microsyringe pump. ESI-MS/MS experiments were carried out by selection of a specific ion in Q1 and by performing its collision-induced dissociation (CID) with argon in the collision chamber. The values expressed are average masses and correspond to the $[M + H]^+$ ions.

Reaction progress was monitored by means of thin-layer chromatography (TLC) using Merck silica gel 60 (Darmstadt, Germany). All reagents, including nicotine, methyllycaconitine (MLA), and mecamylamine were purchased from either Merck or Sigma Aldrich (St. Louis, MO, USA) and used without further purification. Final purification of all products for analysis was carried out by recrystallization. Acetonitrile was distilled from CaH and dried over 4-Å molecular sieves.

Chemical synthesis

Isoxazoles **1** and **3** were obtained using the method described by Hansen *et al.* (2005), starting from the aldehyde (20 mmol), hydroxylamine hydrochloride (21 mmol), chloramine-T trihydrate (5.9 g, 21 mmol), and the respective alkyne (21 mmol). Compound **2** was obtained by hydrogenation of **1**.

3-(4-Nitrophenyl)-5-phenylisoxazole (**1**): Yellow powder. – M.p. 225 °C. – Yield 79%. – ^{1}H NMR (400 MHz, CDCl$_3$): δ = 8.37 (2H, d, J = 8.84 Hz), 8.07 (2H, d, J = 8.84 Hz), 7.87 (2H, dd, J = 7.83 and 1.77 Hz), 7.53 (3H, m), 6.92 (1H, s). – ^{13}C NMR (101 MHz, CDCl$_3$): δ = 161.17, 157.10, 148.67,

135.20, (+) 130.74, (+) 129.19, (+) 127.69, (+) 125.95, (+) 124.28, (+) 97.45. – MS (EI): $m/z = 267$ $[M + 1]^+$. – $C_{15}H_{10}N_2O_3$: calcd. C 67.67, H 3.79, N 10.52, O 18.03; found C 66.52, H 3.80, N 10.63, O 18.12.

1-(3-(Benzo[d][1,3]dioxol-5-yl)isoxazol-5-yl)-N,N-dimethylmethanamine (**3**): Amorphous solid. – Yield 48%. – ^{1}H NMR (400 MHz, CDCl$_3$): $\delta = 7.33$ (1H, s), 7.27 (1H, d, $J = 8.0$ Hz), 6.87 (1H, d, $J = 8.0$ Hz), 6.42 (1H, s), 6.02 (2H, s), 3.66 (2H, s), 2.35 (6H, s). – ^{13}C NMR (101 MHz, CDCl$_3$): $\delta = 170.46$, 162.05, 149.16, 148.30, 123.24, (+) 121.20, (+) 108.69, (+) 107.06, (−) 101.54, (+) 100.99, (−) 54.53, (+) 45.32. – MS (EI): $m/z = 247$ $[M + 1]^+$. – $C_{13}H_{14}N_2O_3$: calcd. C 63.40, H 5.73, N 11.38, O 19.49; found C 63.52, H 5.65, N 11.42, O 19.47.

4-(5-Phenylisoxazol-3-yl) aniline (**2**): Amorphous solid. – Yield 98%. – ^{1}H NMR (400 MHz, CDCl$_3$): $\delta = 7.83$ (2H, dd, $J = 8.08$ and 1.26 Hz), 7.68 (2H, d, $J = 8.40$ Hz), 7.49 (3H, m), 7.76 (2H, d, $J = 7.60$ Hz), 6.75 (1H, s), 3.89 (2H, s, −NH$_2$). – MS (EI): $m/z = 237$ $[M + 1]^+$. – $C_{15}H_{12}N_2O$: calcd. C 74.97, H 6.71, N 11.66, O 6.66; found C 74.99, H 6.67, N 11.68, O 6.66.

Primary culture and incubation conditions

All umbilical cords were collected after delivery from full-term normal pregnancies of 40 weeks (Ethics Committee's approval and informed patient consent were obtained at Hospital Carlos Van Büren, Valparaiso, Chile). The ECs were isolated as described by Jaffe *et al.* (1973) and were identified by acetylated low-density lipoprotein (Voyta *et al.*, 1984) and von Willebrand factor (Jaffe *et al.*, 1973) according to the validated methodologies developed in our laboratory (Cortés *et al.*, 2013). In summary, endothelium was isolated by collagenase-I (0.5 mg/mL) digestion from human umbilical veins and cultured in a 199 medium, supplemented with 2.5 mM L-glutamine, 14 mM Hepes, 200 UI/L penicillin, 400 UI/L streptomycin, 10% fetal calf serum, 10% newborn calf serum, at pH 7.42. Experiments were performed in confluent primary cultures (at a density of $25 \cdot 10^4$/plate) on days three to five of culture.

Determination of [Ca^{2+}]$_i$

As described for other ECs (Vinet *et al.*, 2009), HUVECs were washed twice with phosphate-buffered saline (PBS) (pH 7.4) and subsequently loaded for 20 min with 7 μM Fluo-3AM (Molecular Probes, Eu-

gene, OR, USA) and 0.094% pluronic acid in Locke's solution consisting of (in mM): NaCl (135), KCl (5.6), CaCl$_2 \cdot$ 2H$_2$O (2.5), Hepes (10), MgCl$_2 \cdot$ 6H$_2$O (1.2), D-glucose (5.5), pH 7.4. Light emitted by Fluo-3AM, resulting from changes in [Ca^{2+}]$_i$ in a group of 6 – 10 cells placed in a superfusion chamber of an epifluorescence microscope (Nikon Eclipse E600 FN; Nikon Corp., Tokyo, Japan), was continuously measured with a photomultiplier (Hamamatsu Photonics, Hamamatsu, Japan) and appropriate filters (excitation, 490 nm; emission, 530 nm). Data were digitalized at 3 Hz by an analog converter using the Axotape software (Axon Instruments, Foster City, CA, USA), analysed, and plotted by the OriginPro software (OriginLab Corp., Northampton, MA, USA). The magnitude of the fluorescent signal was presented as a ratio according to the equation $\Delta F/F_b = (F_t - F_b)/F_b$, where F_t is the fluorescent value of Fluo-3AM at time t and F_b is the basal fluorescence, as described by Takahashi *et al.* (1999).

In each experiment, HUVECs were acutely stimulated with the respective agonist (30 s), and then washed with Locke's solution. At the end of each experiment, a pulse of ATP was applied to verify the functional state of cells through Ca^{2+} influx via P$_2$X$_4$ receptors (Yamamoto *et al.*, 2000). Experiments that included antagonists were done according to the following protocol: HUVECs were loaded with Fluo-3AM and incubated for 15 min with the respective antagonist at room temperature. Then, the cells were washed with Locke's solution, and Ca^{2+} measurements were immediately recorded.

Statistical treatment

Results are given as means $\pm$ standard error (mean $\pm$ SEM), where *n* represents the number of glass slips examined per experiment. For statistical analysis, Satistix software for Windows (Analytical Software, Tallahassee, FL, USA) was used. Statistical analysis comparing multiple groups was performed by ANOVA. For comparison between two groups, the unpaired Student's *t*-test was applied. $p < 0.05$ was considered statistically significant.

Results

Synthesis and characterization of isoxazoles

The isoxazolic compounds **1** and **3** were synthesized according to the procedure described by Hansen *et al.* (2005). After conversion of an aldehyde to the corresponding aldoxime via the reaction with
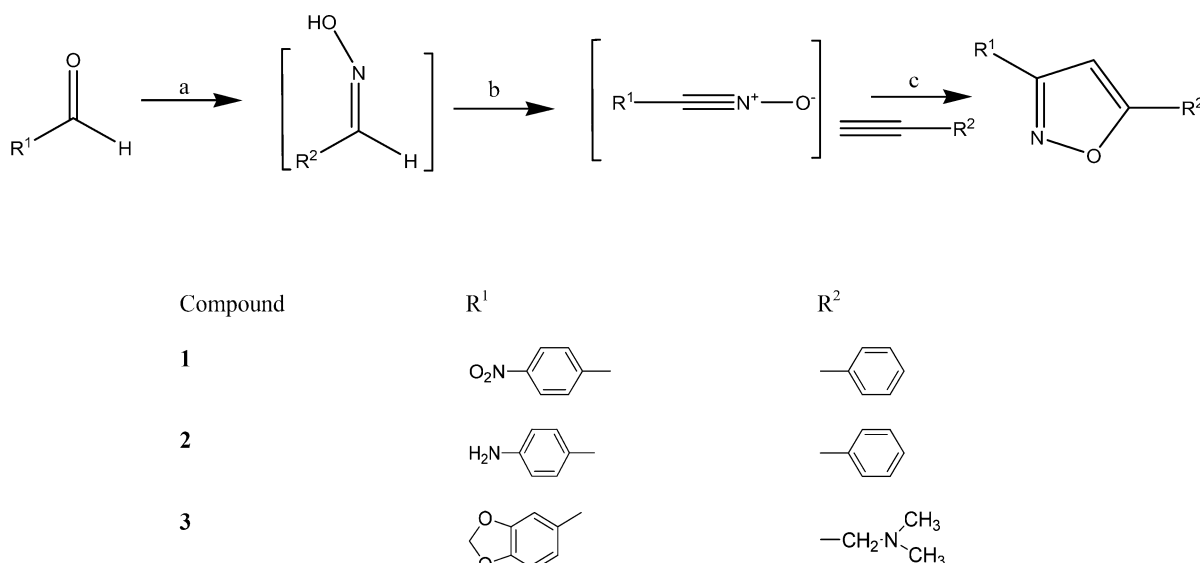
Fig. 1. Reagents and conditions for the synthesis of isoxazoles. a) NaOH, $NH_2OH \cdot HCl$. b) $TsN(Cl)Na \cdot 3H_2O$. c) Alkyne-R^2Cu/$CuSO_4$ (catalyst) in *t*-BuOH/H_2O (1:1), with ultrasound.

hydroxylamine hydrochloride, the aldoxime was transformed into the nitrile oxide with chloramine-T trihydrate, and in the presence of a catalytic amount of *in situ* copper(I) (comproportionation), finally in the 3,5-disubstituted isoxazole. The isoxazole **2** was prepared from a mixture of isoxazole **1** with 10 % Pd/C as catalyst and ethyl acetate (EtOAc), with stirring under H_2 (1 atm) at room temperature. The reaction mixture was filtered, concentrated *in vacuo*, and then purified by column chromatography (silica gel, petroleum ether/EtOAc). The schematic procedure is presented in Fig. 1.

The isoxazoles were characterized by ^{1}H NMR, ^{13}C NMR, and mass spectra. ^{1}H NMR spectra of the synthesized isoxazoles showed the characteristic signal of a singlet near 6.5 ppm, corresponding to a proton of the isoxazolic ring. The mass spectra showed similar fragmentation patterns among the compounds, always showing the molecular ion $[M + H]^+$.

Biological activity

In HUVECs loaded with Fluo-3AM, nicotine increased $[Ca^{2+}]_i$ after 10 s of acute perfusion, and $[Ca^{2+}]_i$ reached its maximum 10 s after the beginning of the response. In the wash-out period, the response induced by nicotine decreased up to 50 % of the maximum and no late response was observed. The maximum effect was achieved at 100 μM nicotine (Fig. 2A).

We selected the choline concentration according to data obtained for chromaffin cells (Maneu *et al.*, 2002), starting at 1 mM. After basal stabilization, HUVECs were acutely perfused with choline during 30 s. Choline induced a $[Ca^{2+}]_i$ increase in a concentration-dependent manner with a rapid onset at 10 s, and in the wash-out period $[Ca^{2+}]_i$ decreased to 50 % of the maximum, which was reached at 10 mM (Fig. 2B).

Figure 2C shows the respective maximum $[Ca^{2+}]_i$ peak in response to each agonist concentration (*E*), standardized with regard to the mean higher value of $[Ca^{2+}]_i$ among the evaluated concentrations (E_{max}). It is important to note that nicotine reached the maximum effect at lower concentrations than choline.

In order to demonstrate the implication of α7 nAChRs in the cholinergic agonist-induced $[Ca^{2+}]_i$ response, we used MLA, a selective α7 nAChR antagonist (Astles *et al.*, 2002). Preincubation of HUVECs with MLA significantly inhibited the $[Ca^{2+}]_i$ increase induced by choline ($p < 0.005$) and nicotine and partially inhibited the response to ACh (Fig. 3).

We analysed the effect of the isoxazolic compounds on $[Ca^{2+}]_i$ following the same protocol as described for the agonists, *i. e.*, the compounds were first examined at 10 μM and then, according to the observed response, higher or lower concentrations were employed. The three compounds induced an increase in $[Ca^{2+}]_i$ in a concentration-dependent manner, highest in the case of compound **1** (Fig. 4). To exam-
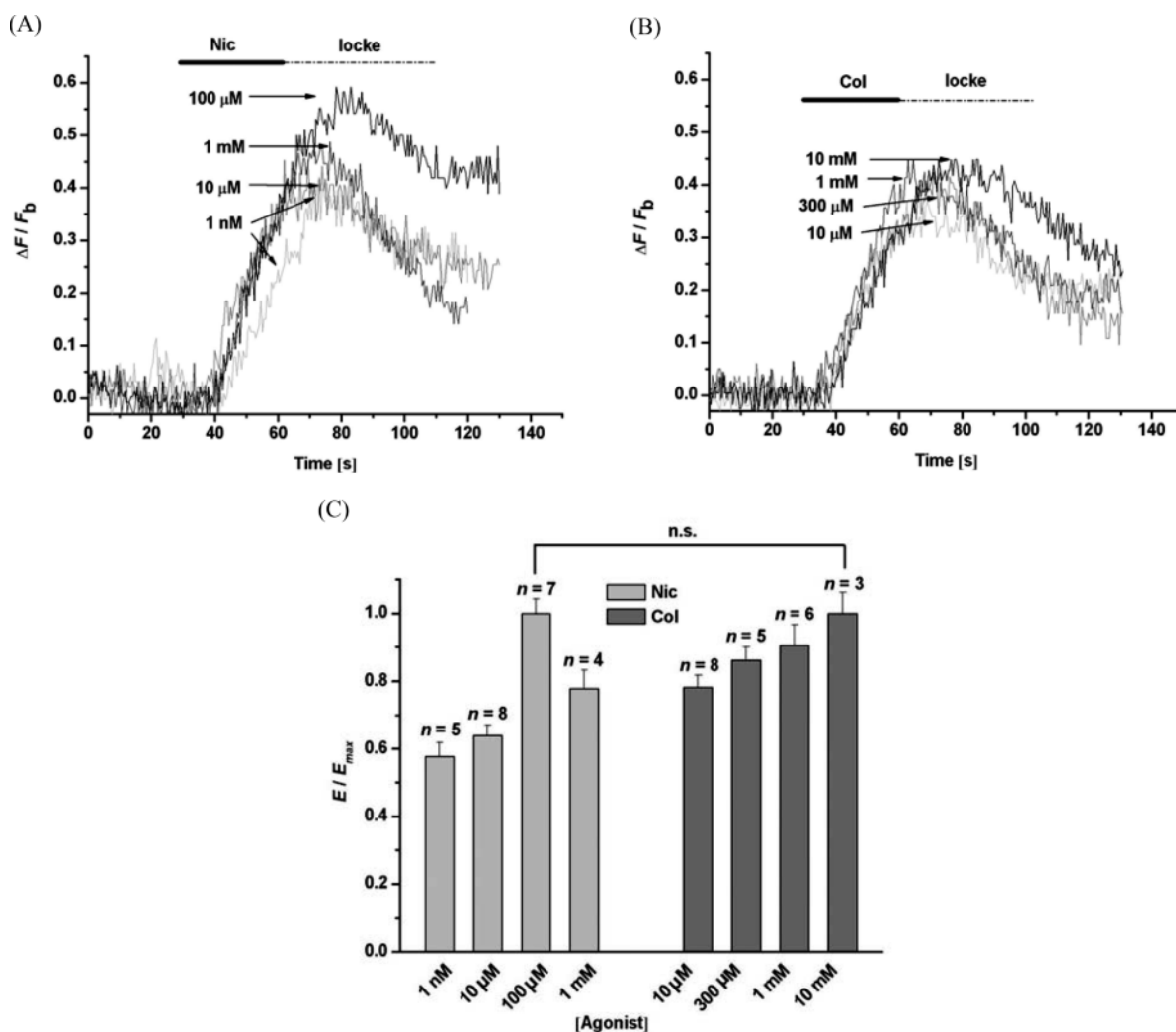
(A)

(B)

(C)

Fig. 2. Cytosolic Ca^{2+} increment in HUVECs in response to acute stimulation with nicotine (Nic) (A and C) or choline (Col) (B and C). (A, B) Original recordings of cytosolic Ca^{2+} signals from four individual experiments. The cells were loaded with Fluo-3AM during 20 min at 37 °C. The agonists nicotine (non-selective nAChR agonist) or choline (selective α7 nAChR agonist) were applied after a 30-s wash with Locke's solution. At least three experiments were done for each agonist at a defined concentration. (C) The bar graph shows comparative increments of cytosolic Ca^{2+} induced by the two agonists. Values are means $\pm$ SEM, and n represents the number of experiments at each concentration. Nicotine at 1 mM induces desensitization of nAChR. n.s., statistically not significant.

ine whether the effects of the isoxazole compounds were mediated by nAChR, we incubated HUVECs in individual experiments with MLA (selective α7 nAChR antagonist) or mecamylamine (non-selective nAChR antagonist), following the protocol described above. The action of compound **1** (100 nM) was blocked by both MLA ($p = 0.013$) and mecamylamine. On the other hand, the increase in $[Ca^{2+}]_i$, in-

duced by either compound **2** and **3** in the presence of the antagonist, was blocked neither by MLA nor by mecamylamine, while hexamethonium (HEXA), a non-selective nAChR antagonist, completely inhibited their effect (data not shown). This indicates that the elevation of $[Ca^{2+}]_i$ in response to compounds **2** and **3** is not due to their agonistic action on α7 nAChRs.
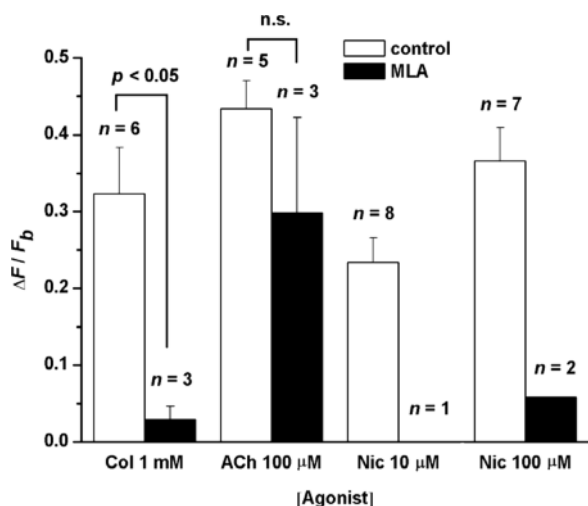
Fig. 3. Effect of cholinergic agonists on $[Ca^{2+}]_i$ in the presence or absence of MLA (selective α7 nAChR antagonist). Cells were loaded with Fluo-3AM as in Fig. 2 and then acutely stimulated by the agonist with or without a prior 15-min incubation with 10 nM MLA. The bars correspond to the mean of the maximum response obtained in the individual experiments. Values are means ± SEM. $p \leq 0.05$, statistically significant; n.s., statistically not significant.
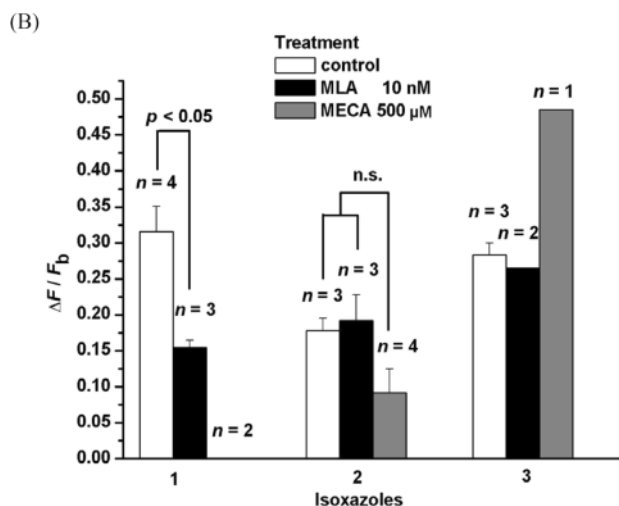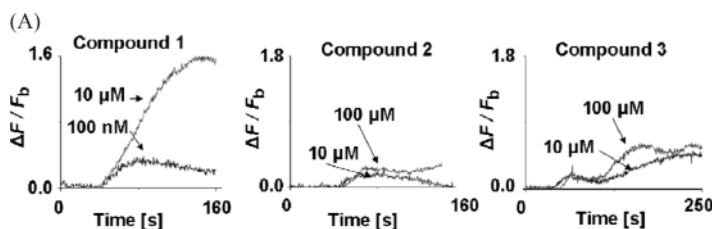
Discussion

The results obtained in this study demonstrate that the isoxazolic compound **1** behaves as an agonist of α7 nAChR. Precisely, based on the observation that it induced a concentration-dependent increment in $[Ca^{2+}]_i$, and furthermore, that the response induced at 100 nM concentration was inhibited by MLA, **1** is a selective α7 nAChR antagonist.

The study also validates the functionality of nAChRs in HUVECs obtained from primary cultures and grown in small groups. Our results indicate the same behaviour that was observed by Heeschen *et al.* (2002), who, by using immunohistochemistry, found that nAChRs were expressed in HUVECs – at 50% confluence preferentially the α7 type. Under similar conditions of confluence in our experiments, the $[Ca^{2+}]_i$ increment induced by nicotine and by choline was inhibited by MLA, an α7 nAChRs antagonist, suggesting the preferential presence of α7 nAChRs.

The first phenomenon to take into account at the beginning of the investigation was the rapid desensitization of nAChRs, in particular of α7 nAChRs, in response to nicotinic modulators that had previ-

(A)

(B)

Fig. 4. Effect of isoxazolic compounds on cytosolic Ca^{2+} signal in HUVECs in the presence and absence of pharmacological tools. (A) Original recordings of $[Ca^{2+}]_i$ increase in HUVECs loaded with Fluo-3AM, induced by compounds **1** (100 nM, 10 μM), **2** (10 μM, 100 μM), and **3** (10 μM, 100 μM). (B) To determine the specific effect on α7 nAChRs, the isoxazolic compounds were acutely applied in the presence of MLA (selective α7 nAChR antagonist) or mecamylamine (non-selective nAChR antagonist). Values are means ± SEM of at least 3 experiments; *n* represents the number of experiments for each treatment. $p \leq 0.05$, statistically significant; n.s., statistically not significant.

ously been investigated in HUVECs by Xiao and Wang (2006). These authors measured $[Ca^{2+}]_i$ by loading the cells with Fluo-3AM and using confocal microscopy. They investigated the effects of nicotine, choline, mecamylamine, and α-bungarotoxin, a selective α7 nAChR antagonist, at different concentrations on intracellular Ca^{2+} and observed that acute stimulation of HUVECs with 10^{-12} M nicotine increased $[Ca^{2+}]_i$, whereas a second nicotine stimulus of 10^{-11}, 10^{-10}, or 10^{-9} M, respectively, applied after the first stimulus, had no effect on $[Ca^{2+}]_i$. Because of this characteristic of nicotinic receptors, we developed protocols with unique concentrations per experiment with single groups of cells. Under these conditions, we observed a sub-maximal response of the $[Ca^{2+}]_i$ increase only at 1 mM nicotine (see Figs. 2A, 2C). This effect has been also seen in electrophysiological studies in bovine chromaffin cells in which nicotine (1 mM) induced a higher current inactivation than other agonists (Rojo, 2006).

In our experiments, nicotine, a non-selective nicotinic agonist, and choline, a selective α7 nAChR agonist, induced similar $[Ca^{2+}]_i$ increments. In both cases, the basal $[Ca^{2+}]_i$ signal was restored after washing out of the agonist. These results agree with those of Xiao and Wang (2006), who stimulated HUVECs with choline and attributed the increase in $[Ca^{2+}]_i$ to the activation of α7 nAChRs. Additionally, the response induced by nicotine in HUVECs is similar to that described by Wang *et al.* (2006), who used Fura-2 to determine $[Ca^{2+}]_i$. These authors concluded that nicotine mainly affects α7 nAChRs in HUVECs, which agrees with our conclusions from the experiments in which nicotine was applied in the presence of 10 nM MLA given that the increment of $[Ca^{2+}]_i$ was completely inhibited at 10 μM nicotine and 85 % inhibited at 100 μM nicotine (see Fig. 3). The response induced by 1 mM choline was significantly inhibited by 10 nM MLA as well.

Compound **1** induced a concentration-dependent increment of $[Ca^{2+}]_i$. The response induced by 100 nM **1** was strongly inhibited by 10 nM MLA, demonstrating the agonistic effect of **1** on α7 nAChRs. Compounds **2** and **3** both increased $[Ca^{2+}]_i$ without significant differences between responses at 10 μM and 100 μM. In addition, the magnitude of the $[Ca^{2+}]_i$ response induced by these compounds was minor in comparison to that of **1** at the same concentration (10 μM), and, contrary to the response to **1**, these responses were unaffected by MLA. Thus, elevation of $[Ca^{2+}]_i$ must have been mediated by receptors other than α7 nAChRs.

According to a review by Grochot-Przeczek *et al.* (2013), therapeutic angiogenesis in peripheral artery disease based on stimulation of collateral vessel formation seems to be a good alternative for peripheral artery disease therapy. This implies that studies of new compounds, such as isoxazoles, should be conducted to develop a treatment for angiogenesis-associated diseases.

Conclusion

The isoxazolic compound **1** appears to act as an α7 nAChR agonist, given that it induced an increase in $[Ca^{2+}]_i$ in a concentration-dependent manner, which was inhibited by MLA. In our experiments, nicotine and choline exhibited similar responses which were both blocked by MLA, suggesting a preferential expression of α7 nAChRs in HUVECs.

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