17β-Aminoestrogens induce guinea pig airway smooth muscle hyperresponsiveness through L-type Ca2+ channels activation

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Edgar Flores-Soto [a], Inocencio Martínez-Villa [a], Héctor Solís-Chagoyán [c], Bettina Sommer [b], Cristina Lemini [a], Luis M. Montaño [a,*]

[a] Departamento de Farmacología, Facultad de Medicina, Universidad Nacional Autónoma de México, México, DF, Mexico
[b] Departamento de Investigación en Hiperreactividad Bronquial, Instituto Nacional de Enfermedades Respiratorias, México, DF, Mexico
[c] Laboratorio de Neurofarmacología, Instituto Nacional de Psiquiatría Ramón de la Fuente Muñiz, México, DF, Mexico

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ABSTRACT

Therapy with estrogens is frequently used in menopausal women and as hormonal contraception. Because of its thrombotic effects, long term estrogen administration used in hormonal replacement therapy (HRT) and contraception could represent a health hazard. In this regard, 17β-aminoestrogens such as aminoestrol, butolame and pentolame have shown promising HRT potential, because they have a weak agonist estrogenic action and antithrombotic activity. Additionally, estrogens play a protective role in airway smooth muscle, but the effect of 17β-aminoestrogens on the airway smooth muscle has not been tested yet. In guinea pig tracheal smooth muscle pentolame and butolame induced hyperresponsiveness to histamine (His), carbachol (Cch) and KCl. Interestingly, aminoestrol did not show this effect at the highest concentration studied, it even lowered the contraction induced by Cch. The hyperresponsiveness induced by pentolame to His was abolished by nifedipine. In single tracheal myocytes, KCl induced an increment in the intracellular Ca^{2+} concentration $[Ca^{2+}]_i$, pentolame also showed an increase in $[Ca^{2+}]_i$ and the addition of KCl in the plateau of this rise further significantly augmented the $[Ca^{2+}]_i$ response. Additionally, in patch clamp experiments pentolame increased the L-type Ca^{2+} currents. Thus, 17β-aminoestrogens such as pentolame and butolame, but not aminoestrol, activate L-type Ca^{2+} channel to induced hyperresponsiveness to Cch, His and KCl in guinea pig tracheal smooth muscle. Due to its lack of effect on airways and to its anticoagulant characteristics, aminoestrol seems to be the best alternative in the HRT among the 17β-aminoestrogens studied.

1. Introduction

In menopausal women, hormonal replacement therapy (HRT) with estrogens is frequently used to diminish clinical symptoms of the climacterium: vasomotor symptoms, sweats, insomnia, hot flushes, and depressive moods. Nevertheless, this HRT used in high doses like those used in pharmacological formulations, even during a short period, might increase two to four times the possibility of thromboembolic venous diseases [1], by inducing immediate effects such as hepatic protein synthesis of coagulation factors, producing significant alterations on blood coagulation [2].

Interestingly, the 17β-aminoestrogens (AEs) such as pentolame [17β-(5-hydroxy-1-pentylamino)-1,3,5(10)-estratrien-3-ol], butolame [17β-(-4-hidroxi-1-butilamino)-1,3,5(10) estratrien-3-ol] and aminoestrol [(17β-amino-1,3,5(10)estratrien-3-ol)] have been proposed as an alternative of the HRT because they have shown low estrogenic and antithrombotic activity in contrast to other estrogens [3–6].

It has been well studied that asthma is linked to gender and it is more frequent in females than in males [7–10]. Furthermore, asthma exacerbations in women are related to life period and its severity to their hormonal status, i.e., puberty, menstrual cycle, pregnancy and menopause [11–15]. Thus, all these evidences point out the role of female sex steroids in airway smooth muscle reactivity. However, almost all the experimental data in mice clearly indicate that estrogens play a protective role by diminishing airway hyperresponsiveness [16–18]. In this context, in human airway smooth muscle (ASM) cells, 17β-estradiol (E_2) acting via

* Corresponding author at: Departamento de Farmacología, Edificio de Investigación, sexto piso, laboratorio 3, Facultad de Medicina, Universidad Nacional Autónoma de México, Ciudad Universitaria, CP 04510 México, DF, Mexico. Tel./fax: +52 (55)56232280.
E-mail address: lmmr@unam.mx (L.M. Montaño).

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estrogen receptors induces a non-genomic relaxation through cAMP [19].

Since AEs are structurally related to E_2, varying only in the C17 of the steroid nuclei where an OH group is substituted for an amino alcohol group, it can be expected that AEs have the same protective effect as E_2 on the airways. Therefore, we explored in guinea pig tracheal smooth muscle the effect of aminoestrol, butolame and pentolame in the carbachol, histamine and KCl induced-contraction.

2. Material and methods

2.1. Experimental animals

We used Hartley male guinea pigs weighing 400–600 g bred in our institutional animal facilities (filtered conditioned air, 21 ± 1 °C, 50–70% humidity, sterilized bed), fed with Harlan® pellets and sterilized water. The experimental protocol was approved by the Scientific and Bioethics Committees of the Facultad de Medicina, Universidad Nacional Autónoma de México. The Guiding Principles for the Care and Use of Vertebrate Animals in Research and Training published by the American Physiological Society were closely followed during the experimentation. All experimental studies were conducted in accordance to the Mexican National Protection Laws on Animal Protection and the General Health Law Related to Health Research (NOM-062-Z00-1999).

2.2. Organ baths

Guinea pigs were anesthetized with pentobarbital sodium (35 mg/kg, i.p.) and exsanguinated. Tracheas, dissected and cleaned of connective tissue, were cut in eight rings with intact epithelium and each was hung in a 5 ml organ bath containing Krebs solution (in mM): 118 NaCl, 25 $NaHCO_3$, 4.6 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 11 glucose, and 2 $CaCl_2$ at 37 °C. These preparations were continuously bubbled with 5% CO_2 in oxygen to maintain pH at 7.4. Indomethacin (1 μM) was added to the Krebs solution to block prostanoids formation. The tracheal rings were tied with silk thread to an isometric force transducer (model FT03; Grass Instruments, West Warwick, RI, USA) connected to a signal conditioner (CyberAmp 380, Axon Instruments, Foster City, CA, USA) and to an analog-to-digital interface (Digidata 1440A; Axon). An acquisition and analysis software (AxoScope version 10.2; Axon) was employed to record and analyze data. At the beginning of the experiments, tracheal rings were submitted to a resting tension of 1 g during 30 min. Afterwards, they were stimulated three times with KCl (60 mM) to allow tissue conditioning and optimization of the contractile apparatus. A cumulative concentration response-curve to carbachol (Cch, 10 nM to 32 μM), histamine (His, 100 nM to 100 μM) or KCl (10, 20, 40 and 80 mM) was constructed in the absence (control) or presence of 1, 10 and 32 μM of pentolame, butolame or aminoestrol. Some tissues were incubated with 1 μM nifedipine (an L-type Ca^{2+} channel blocker) before the cumulative concentration response-curves to agonists.

2.3. Intracellular Ca^{2+} measurements in tracheal myocytes

Guinea pig tracheal smooth muscle dissected free of epithelium and connective tissue was placed in 5 ml Hanks solution containing 2 mg cysteine and 0.04 U/ml papain. In all preparations, pH was adjusted to 7.4 using 1 M $NaHCO_3$ and were then incubated for 10 min at 37 °C. The enzyme excess from the tissues was removed with Leibovitz's solution, and the airway smooth muscle was then placed in Hanks solution with 1 mg/ml collagenase type I and 0.5 mg/ml protease during ~10 min at 37 °C. Myocytes were gently dispersed by mechanical agitation until detached cells were observed. The enzymatic activity was stopped by adding Leibovitz's solution; cells were centrifuged at 600 rpm, 20 °C during 5 min and the supernatant was discarded. This last step was repeated once.

To load myocytes with 2.5 μM fura 2-AM, they were kept in low Ca^{2+} (0.1 mM) at room temperature (~21 °C) for 1 h. Then, cells were settled down into a heated perfusion chamber with a glass cover at the bottom and the chamber was mounted on an inverted microscope (Diaphot 200, Nikon, Tokyo, Japan). The myocytes adhered to the glass were continuously perfused at a rate of 2–2.5 ml/min with Krebs solution at 37 °C, bubbled with 5% CO_2 in oxygen, pH 7.4.

Fura 2-AM loaded cells were given alternating pulses of 340 and 380 nm excitation light and emission light was collected at 510 nm using a microphotometer from Photon Technology International model D-104 (PTI, Princeton, NJ, USA). Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was calculated according to the Grynkiewicz formula [20] and fluorescence was obtained at a rate of 0.5 s. The Kd of fura 2-AM was assumed to be 386 nM [21]. The mean 340/380 fluorescence ratios R_{max} and R_{min} were 6.99 and 0.35 respectively.

A 60 mM KCl stimulation was given twice to guinea pig myocytes. Afterwards, cells were perfused with 32 μM pentolame during 2 min and stimulated again with the same KCl concentration. Some cells were incubated with 30 μM D-600 and stimulated with 32 μM pentolame.

2.4. Patch-clamp studies

Guinea pig tracheal myocytes were isolated as described above. The cells were cultured as follows: the cell pellet was re-suspended in minimum essential medium containing 5% fetal bovine serum, 2 mM L-glutamine, 10 U ml^{-1} penicillin, 10 μg ml^{-1} streptomycin and 15 mM glucose, and plated on rounded cover slips coated with sterile rat tail collagen. Cells were cultured at 37 °C in a 5% CO_2 in oxygen during 24–48 h.

In the bottom of a 0.7 ml cover glass submerged in a perfusion chamber, myocytes were allowed to settle down. The chamber was perfused by gravity (~1.5–2.0 ml min^{-1}) with an external solution containing Ba^{2+} to replace Ca^{2+} as the inward charge carrier to measure Ca^{2+} currents, in mM: 136 NaCl, 6 CsCl, 5 $BaCl_2$, 11 glucose, 10 HEPES, and 0.1 niflumic acid, pH 7.4, adjusted with CsOH. All experiments were performed at room temperature (~21 °C).

The standard whole-cell configuration was used to record Ca^{2+} currents activated by depolarizing voltage steps (i.e., voltage clamp) through an Axopatch 200A amplifier (Axon Instruments). 1B200F-6 glass (Word Precision Instruments, Sarasota, FL) was used to make patch pipettes using a horizontal micropipette puller (P-87, Sutter Instruments Co, Novato, CA). The pipettes had a resistance ranging from 2 to 4 MΩ. The internal solution was (mM): 130 CsCl, 2 $MgCl_2$, 10 HEPES, 10 EGTA, 3.6 ATP disodium salt, and 1.9 GTP sodium salt, pH 7.3, adjusted with CsOH. Whole cell currents were filtered at 1–5 KHz, digitized (Digidata 1440A, Axon) at 10 KHz, and stored in a computer for later analysis through specialized software (pClamp v10.2, Axon).

To observe Ca^{2+} currents, tracheal myocytes were subjected to series of conditioning hyperpolarizing and depolarizing pulses of potentials ranging from −60 to +50 mV in 10 mV increments from a holding potential of −60 mV during 100 ms, 1 Hz. After the control protocol, pentolame (32 μM, $n = 5$) was added and the same experimental procedure was followed. Changes in the currents were evaluated as maximal current peak to each voltage tested.

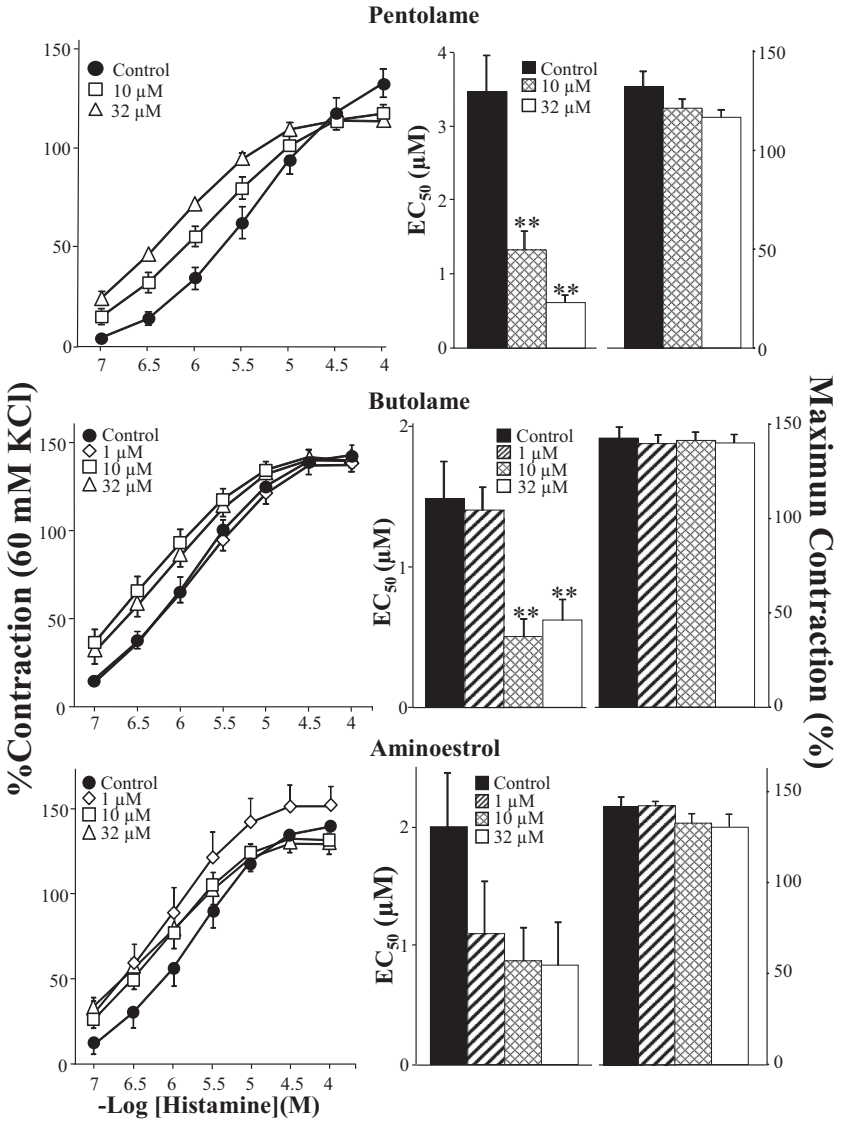

Fig. 1. Effect of 17β-aminoestrogens on the guinea pig tracheal smooth muscle contraction induced by histamine. Pentolame and butolame induced a leftward shift of the histamine concentration response curve. However, aminoestrol at different concentrations had no effect on the histamine concentration response curve. Statistical significance was found when curves were evaluated as EC_{50} for pentolame ($n = 5$) and butolame ($n = 7$) but not for aminoestrol ($n = 6$–7). Maximum contraction was not modified. Symbols and bars represent mean ± SEM. $^{**}p < 0.01$ compared to control group.

2.5. Drugs and chemicals

The AEs: aminoestrol, pentolame and butolame were synthesized from estrone [3-hydroxy-1,3,5(10)-estratrien-17-one] (Syntex, Mexico) according to reported methods. Chemical purity of all AEs was defined by comparing its spectral (IR, NMR, MS) and chromatographic (HPLC and TLC) properties with those previously reported [6,22].

Carbamylcholine chloride, histamine, nifedipine, D-600 and indomethacin, were all purchased from Sigma Chem. Co. (St. Louis, MO, USA).

2.6. Statistical analysis

Reactivity to Cch or His was evaluated through the effective concentration 50% (EC_{50}) and maximum contraction for each aminoestrogen tested. The EC_{50} was calculated from the cumulative concentration–response curve by straight-line regression as −Log using the ED50plus v1.0 software (http://www.softlookup.com/display.asp?ID=2972) and is expressed as μM concentration. Responses to KCl were evaluated point by point for each concentration used in the presence of different concentrations of the AEs. Data values were analyzed through a one-way analysis of variance followed by Dunnett's or Tukey–Kramer multiple comparison tests. Results for $[Ca^{2+}]_i$ and patch clamp experiments were evaluated with paired student's t test. Data along the manuscript and figures are expressed as mean ± SEM. In the isolated myocytes experiments, each n corresponds to a cell from a different animal. Statistical significance was set at $p < 0.05$ bimarginally.

3. Results

In guinea pig tracheal smooth muscle, histamine induced a contraction that was dependent on the concentration used. This concentration response-curve to histamine was shifted to the left when tissues were incubated with 10 and 32 μM pentolame or

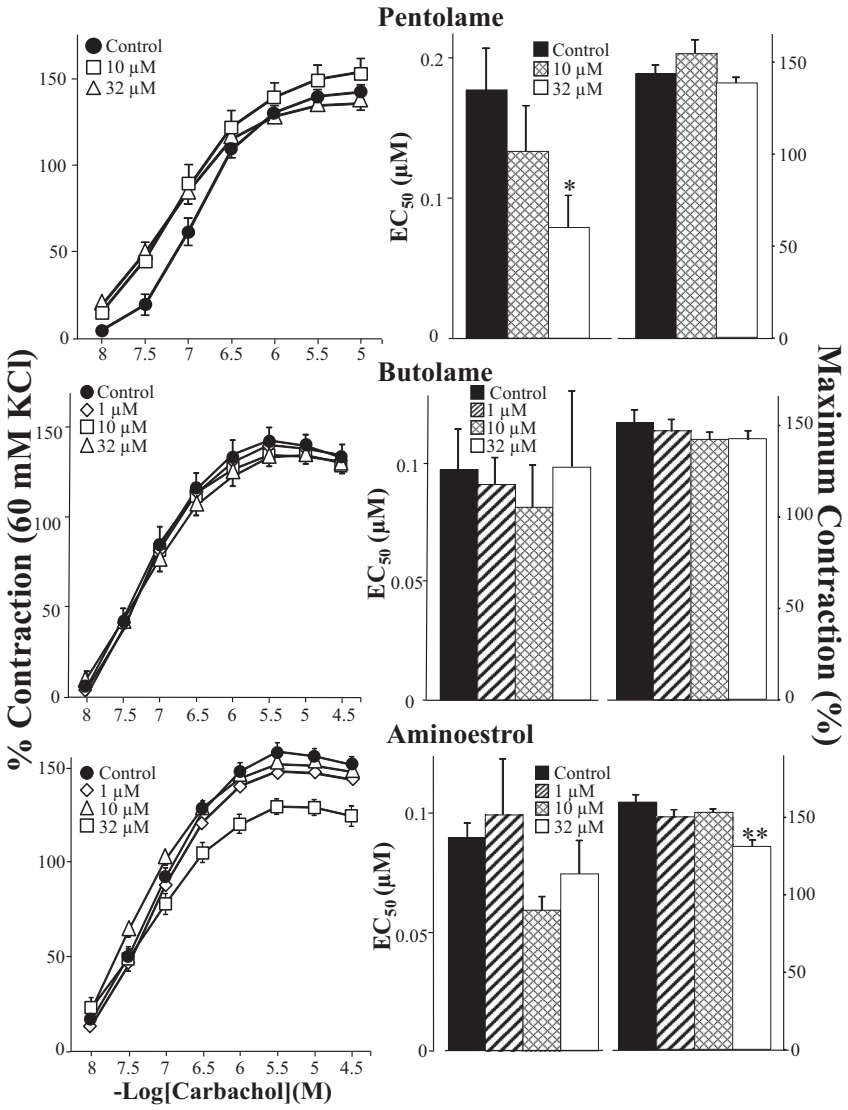

Fig. 2. Effect of 17β-aminoestrogens on the guinea pig tracheal smooth muscle contraction induced by carbachol. Pentolame ($n = 8–9$) induced a leftward shift of the carbachol concentration response curve, but not butolame ($n = 7$) or aminoestrol ($n = 7$). Statistical significance was only found for EC_{50} at 32 μM pentolame. Maximum contraction was not modified for pentolame and butolame, but was significantly diminished at 32 μM aminoestrol. Mean ± SEM were graphed. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to control group.

butolame. Both EC_{50} showed statistical significance when compared to control group, i.e., the concentration required to produce a similar response (EC_{50}) was lower, but maximal contraction was not modified (Fig. 1). These results showed that pentolame and butolame are inducing hyperresponsiveness to histamine, meanwhile aminoestrol had no effect on the histamine concentration response curve (Fig. 1).

Similar results were observed with Cch but significance was only reached for the EC_{50} of 32 μM pentolame (Fig. 2). However, butolame and aminoestrol did not modify the concentration response curve to Cch, nor the EC_{50}. However, 32 μM aminoestrol significantly diminished the maximum response to this agonist (Fig. 2).

KCl induced a contraction which was concentration dependent. Increased responsiveness to KCl 10 and 20 mM was significantly different when tissues were incubated with pentolame. Difference was only significant for 20 mM KCl when butolame was tested. Aminoestrol did not change the concentration response curve to KCl (Fig. 3). These results indicate that pentolame is the most potent aminoestrogen inducing hyperresponsiveness to His and Cch. The increased responses to KCl induced by this aminoestrogen suggested the participation of L-type Ca^{2+} channels (LVDCC).

The hyperresponsiveness induced by 32 μM pentolame to histamine (EC_{50}: control, 2.24 ± 0.31 μM vs pentolame, 0.98 ± 0.15 μM, $p < 0.01$) was abolished when tissues were incubated with nifedipine, a well-known L-type Ca^{2+} channel blocker (EC_{50}: 2.47 ± 0.58 μM, Fig. 4) pointing out that, in this phenomenon, LVDCC are involved.

KCl (60 mM) induced an increment in $[Ca^{2+}]_i$ of tracheal myocytes. Cells perfused with 32 μM pentolame also showed an increase in $[Ca^{2+}]_i$ and the addition of KCl in the plateau of this increment further significantly augmented the $[Ca^{2+}]_i$ response (Fig. 5). The pentolame-induced $[Ca^{2+}]_i$ increment was abolished by D-600, another well-known L-type Ca^{2+} channel blocker. These results support that L type Ca^{2+} channels are being opened by pentolame. Furthermore, this finding was corroborated by patch clamp results where L-type channel Ca^{2+} currents were

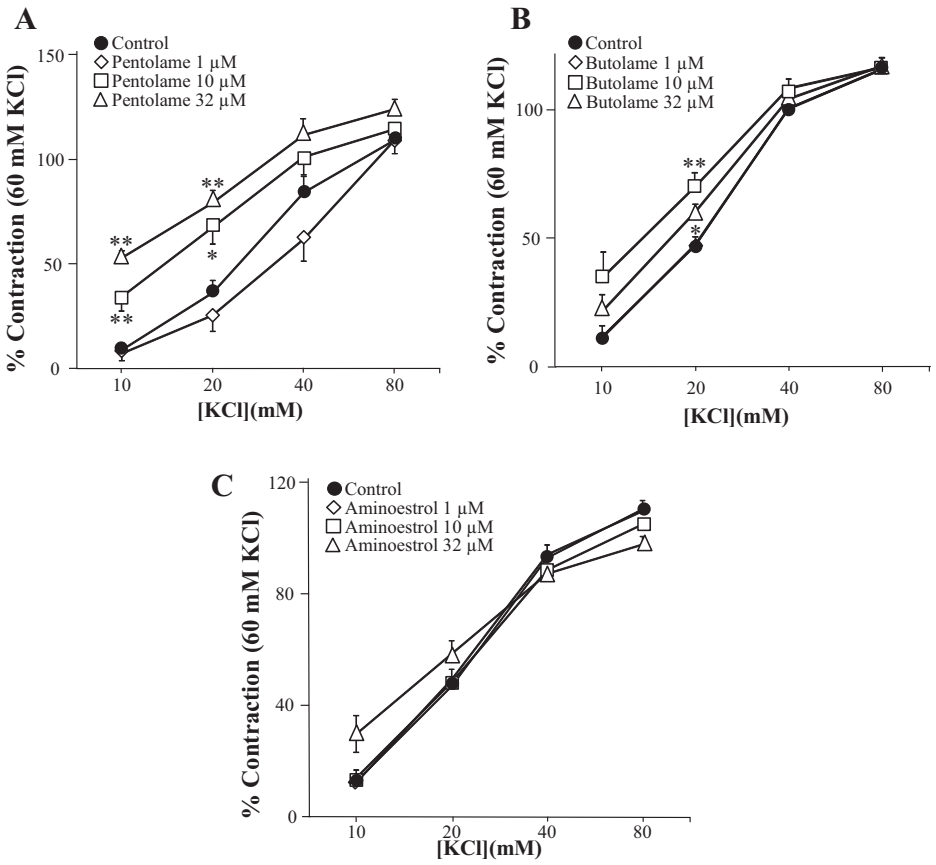

Fig. 3. Effect of the different 17β-aminoestrogens on the KCl induced contraction in guinea pig tracheal smooth muscle. (A) Pentolame ($n = 8$) and (B) butolame ($n = 6$) at 10 and 32 μM produced an increment of the KCl response curve reaching statistical significance at 10 and 20 mM KCl for pentolame, and at 20 mM for butolame. (C) Aminoestrol ($n = 8$) did no modify KCl responses. Mean ± SEM were graphed. $^{*}p < 0.05$, $^{**}p < 0.01$.

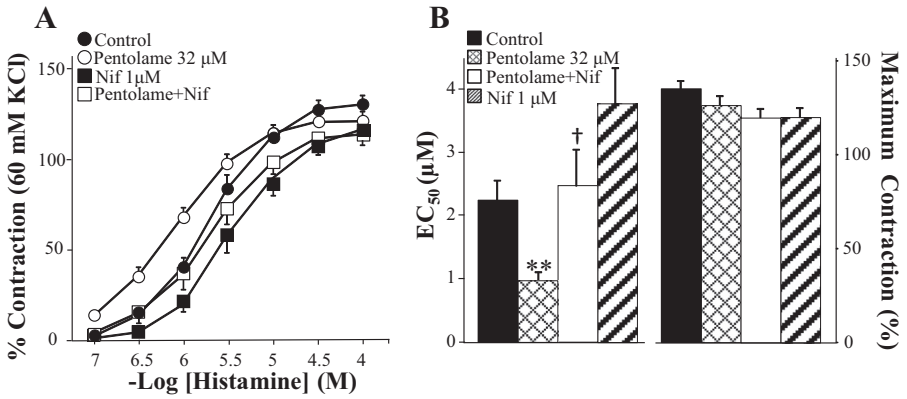

Fig. 4. L-type Ca^{2+} channels are involved in the pentolame induced hyperresponsiveness to histamine. (A) Effect of nifedipine (Nif) on pentolame induced hyperresponsiveness to histamine in the guinea pig tracheal smooth muscle. Pentolame (32 μM) induced a leftward shift of the histamine concentration response curve which was abolished by Nif. This dihydropyridine alone produced a displacement to the right of the concentration response curve to histamine. (B) Statistical significance between control and pentolame groups was found for the EC_{50} and for pentolame vs Nif + pentolame groups. Maximum contraction was not modified. Mean ± SEM of $n = 6$ experiments were graphed. $^{**}p < 0.01$ and $^{\dagger}p < 0.01$.

significantly increased in the presence of pentolame (Fig. 6). This Ca^{2+} current was previously characterized as an L-type because it was almost completely abolished by 1 μM nifedipine [23].

4. Discussion

Our data in the present study point out that in guinea pig airway smooth muscle, AEs like pentolame and butolame induce hyperresponsiveness to His, Cch and KCl through a mechanism favoring the opening of L-type voltage dependent Ca^{2+} channels. This is an interesting finding since it is the first time that L-type Ca^{2+} channels opening has been mentioned as inducing airway hyperresponsiveness to bronchoconstrictor agonist and certainly requires further research in an asthmatic model.

Even though asthma symptoms exacerbations in humans have been linked to hormonal status in females [11–15], airway hyperresponsiveness in estrogen receptor-α knockout mice augments, while it diminishes in mice treated with E_2 [16–18]. Because AEs

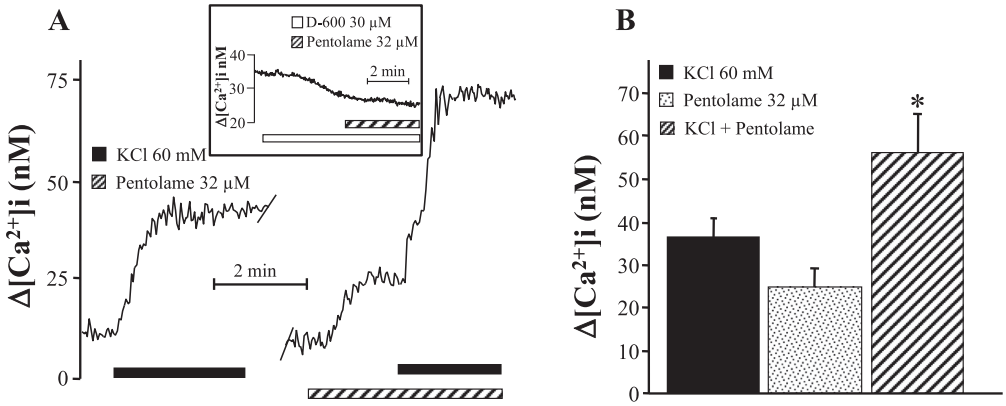

Fig. 5. Pentolame opens L-type Ca^{2+} channels increasing KCl responses in guinea pig tracheal myocytes. (A) KCl and pentolame induced an increment of $[Ca^{2+}]_i$. However, when KCl was added at the top of the pentolame response, its increase in $[Ca^{2+}]_i$ was significantly potentiated. Notice that the pentolame response was abolished when cells were incubated with 30 μM D-600 (inset, $n = 3$). (B) Statistical analysis of KCl induced $[Ca^{2+}]_i$ increases and the effect of pentolame. Mean ± SEM of $n = 4$ experiments were graphed. $^{*}p < 0.05$ when compared to control group.

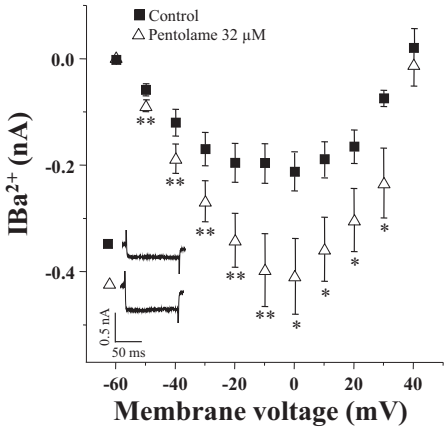

Fig. 6. Pentolame potentiates L type channel Ca^{2+} currents in guinea pig tracheal myocytes. Ba^{2+} currents induced by 10 mV voltage increments were significantly increased by pentolame. Inset depicts original recordings. Mean ± SEM of $n = 5$ experiments were graphed. $^{*}p < 0.05$, $^{**}p < 0.01$.

are structurally related to E_2, we expected them to have a protective effect on the airways, nevertheless our findings showed opposite results. We found that pentolame induced hyperresponsiveness to His, Cch and KCl and butolame only to His and KCl; the former aminoestrogen had the highest potency. Interestingly, aminoestrol did not show this effect, furthermore, at the maximum concentration used (32 μM) it lowered the contraction induced by Cch. The AEs pentolame, butolame and aminoestrol contain an amino alcohol group on the C17 of the steroid nuclei instead of OH group of the E_2 molecular structure. The difference between pentolame and butolame is one carbon of the intermediate chain between the alcohol and the amino group substituent in C17, which has an impact determining the hyperresponsiveness effect of these AEs on the tracheal smooth muscle. However, aminoestrol lacks the alky-alcohol substitution on the amino group which seems to be involved in this hyperresponsiveness. In this context, the potency sequence of the evaluated compounds was as follows: pentolame > butolame > aminoestrol.

AEs such as pentolame, butolame and aminoestrol have shown a lower estrogenic and anticoagulant activity in contrast to E_2 and other estrogens due to the change of $–NH_2$ instead of –OH in the E_2. In the context of its estrogenic effect, it has been reported that pentolame had the lowest potency to activate the estrogens receptors (ERα, ERβ) as compared with butolame, prolame and even aminoestrol. This might be due to the effect of the amino-alcohol side-chain of the AEs ($–NH(CH_2)_n–OH$ at C-17) on the ligand receptor, demonstrating how the effect of the insertion of each methylene into the amino-alcohol side-chain composition impacts the estrogen ligand-receptor interaction. However, pentolame and butolame activate mainly ERα while aminoestrol stimulates ERβ and to a lesser extent the ERα [3,24]. Aminoestrol lacks the substituent alkyl-alcohol on the amino group, the change of $–NH_2$ in this molecule for the –OH in C17 of E_2 produces a drop of its estrogenic properties *in vivo* and *in vitro* models, and confers to this steroid an increase of selectivity for the ERβ interaction [24]. In this regard, the role of estrogens on the ERα in human cervical cancer has been well documented [25] and therefore the main effect of aminoestrol on the ERβ may diminish deleterious consequences on the reproductive system, representing an advantage to HRT.

Furthermore, our results indicate that the side chain that pentolame and butolame possess, not only modifies ligand binding interactions to estrogen receptors. This structural change also influences the opening of the L-type voltage dependent Ca^{2+} channels, i.e., the longer the alkyl chain on NH_2 the stronger the effect on this channel. However the effect of aminoestrol in the present study, to some extent resembles E_2 because it did not induce hyperresponsiveness to any of the agonists used and even reduced the maximum contraction induced by Cch.

In recent reports the antidepressant, anxiolytic and neuroprotective effects on learning and memory processes of ovariectomized rats have been described with AEs such as prolame, in doses within the same range as those elicited by the natural hormone E_2 [26–28]. This fact might be another beneficial characteristic for AEs administered for HRT during menopause because they could diminish mood changes. However, their potential usefulness as HRT needs to be explored for each derivative, because pentolame or butolame could worsen asthmatic exacerbations in female patients, but not aminoestrol.

In conclusion, 17β-aminoestrogens such as pentolame and butolame, but not aminoestrol, activate L-type Ca^{2+} channel to induced hyperresponsiveness to Cch, His and KCl in guinea pig tracheal smooth muscle. Additionally, aminoestrol seems to be the best alternative fort HRT since it does not have this side effect.

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