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European Journal of Pharmacology 532 (2006) 115–127
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Functional inhibition of intestinal and uterine muscles by non-permeant triphenylethylene derivatives

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Received 7 July 2005; received in revised form 7 November 2005; accepted 14 November 2005
Available online 7 February 2006

Abstract

We have previously shown that the triphenylethylene antiestrogen tamoxifen reversibly inhibited spontaneous contractile activity in isolated duodenal muscle. Now, we have synthesized different quaternary ammonium salts of tamoxifen by changing the substituents on the nitrogen of the alkylaminoethoxy side-chain, to obtain plasma membrane impermeable compounds. Synthesized molecules were *N*-desmethyl-tamoxifen-hydrochloride, ethylbromide-tamoxifen and butylbromide-tamoxifen, which differed in the size of their ionic side-chain. All compounds rapidly and reversibly inhibited spontaneous and $CaCl_2$-induced contractions in mouse duodenum and uterus. Dose-response analyses revealed a structure-activity relationship where the larger the side-chain the higher the inhibitory potency. Fourier analyses on triphenylethylene-relaxed duodenal tissues showed that harmonic components of contractile activity were readily recovered upon exposure to the L-type calcium channel agonist 1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)phenyl]-pyridine-3-carboxilic acid methyl ester (BAY-K644). Likewise, BAY-K644 completely reversed triphenylethylene-induced effects on uterine tonic tension. Our experiments suggest that impermeant tamoxifen derivatives relax visceral smooth muscle through a membrane-mediated non-genomic mechanism that involves inhibition of L-type calcium channels.

Keywords: Tamoxifen derivatives; Impermeant triphenylethylene salts; Non-genomic effects; Uterine muscle; Intestinal muscle; Ca^{2+} channels

1. Introduction

The non-steroidal triphenylethylene antiestrogen tamoxifen has been proven to be a useful clinical adjuvant in the treatment of estrogen receptor-positive breast cancer in postmenopausal women. Its ability to displace estrogens from their intracellular receptor is the generally accepted antagonistic mode of action of tamoxifen. However, there is now considerable evidence to suggest that tamoxifen may act through estrogen receptor-independent processes. Indeed, it has been reported that tamoxifen is able to modify the function of a number of plasma membrane proteins. These include neurotransmitters dopamine, histamine and muscarinic receptors (Hiemke and Ghraf, 1984; Brandes et al., 1987; Batra, 1990) multidrug resistance P-glycoprotein (Kirk et al., 1994), ligand-gated cationic channels (Allen et al., 1998), voltage-dependent Na^+ and K^+ currents (Hardy et al., 1998, Smitherman and Sontheimer, 2001), Ca^{2+} channels (Song et al., 1996; Dick et al., 1999) and volume-sensitive chloride currents (Zhang et al., 1994) and Maxi-Cl channels (Díaz et al., 2001). In addition, crucial enzymes involved in cellular transduction, like protein kinase C (PKC, Horgan et al., 1986), calmodulin (Lopes et al., 1990), calmodulin-dependent cAMP-phosphodiesterase (Lam, 1984) or Ca^{2+}-ATPase (Malva et al., 1990) have also been shown to be inhibited by tamoxifen.

Although many of the cellular proteins inhibited by tamoxifen play crucial roles in the excitation–contraction coupling from contractile cells, it is remarkable the low number of clinical acute side effects reported for antiestrogens (Jordan and Murphy, 1990; Trump et al., 1992). This is particularly relevant on smooth muscle cells, which appear to be especially sensitive to triphenylethylene antiestrogens. Thus, in vitro studies on isolated

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doi:10.1016/j.ejphar.2005.11.031

uterine (Lipton et al., 1984; Cantabrana and Hidalgo, 1992; Kostrzewska et al., 1997), vascular (Song et al., 1996; Figtree et al., 2000), detrusor (Ratz et al., 1999) and recently on isolated duodenal smooth muscle (Díaz, 2002) from different species, including human, have shown that tamoxifen rapidly inhibits agonist-induced contractile activity, although the precise mechanisms of action are still poorly understood. Though tamoxifen is a highly lipophilic molecule, most of its acute effects on smooth muscle have been explained by interaction with targets other than estrogen receptors that might be located at the plasma membrane; such is the regulatory β1 subunit of the Maxi K^+ channel (Dick et al., 2001) or the L-type calcium channels (Díaz, 2002; Song et al., 1996). The aim of the present work was twofold: first, to synthesize different halide salts of quaternary derivatives of tamoxifen that are unable to cross the plasma membrane. These compounds have proven a powerful tool to unravel whether the rapid effects of tamoxifen on smooth muscle cells involve plasma membrane targets or if, alternatively, are due to modulation of intracellular signalling molecules such as calmodulin or protein kinase C. The second objective of our work was to assess the effects of these triphenylethylene derivatives on the contractile properties of uterine and duodenal muscles and explored their putative mechanism of action.

2. Materials and methods

2.1. Tissue preparation and solutions

Duodenal segments were dissected from male mice weighing 26–30 g following diethyl ether anaesthesia. Strips of duodenal smooth muscle (1.0 cm long) were immediately placed in cold physiological salt solution (PSS), containing (in mM) NaCl, 126; KCl, 4.5; $MgSO_4.7H_2O$, 1.0; $CaCl_2$, 2.0; NaH_2PO_4, 0.56; Na_2HPO_4, 1.44; (adjusted to pH 7.4) and glucose 15.0. Duodenal strips were incubated in aerated PSS at 37 °C in a water-jacketed 25 ml organ bath.

Uterine tissues were obtained from female mice (weighing 23–27 g) one week after being subjected to bilateral ovariectomy. Female mice were ovariectomized under ketamine (100 mg/kg) and xilazine (10 mg/kg) anaesthesia. Once isolated, both uterine horns were cleaned of adherens and placed in tyrode solution, containing (in mM), NaCl, 120; KCl, 4.7; $MgSO_4.7H_2O$, 1.2; $CaCl_2$, 1.6; NaH_2PO_4, 1.0; $NaHCO_3$, 25.0 (pH 7.4) and glucose 12.0. Uterine tissues were incubated in tyrode at 37°C in a water-jacketed 25 ml organ bath and continuously bubbled with a 95% O_2/5% CO_2 gas mixture.

Ca^{2+}-free solutions and Ca^{2+}- and K^+-free solutions were prepared by replacing $CaCl_2$ (supplemented with 25 μM EGTA) and KCl from the corresponding saline solution.

All experimental procedures were performed in accordance with the European Community and Local guidelines for the care of laboratory animals.

2.2. Recording of isometric tension

The isometric tension of isolated tissues was measured using isometric force transducers (TRI110, Letica, Spain) through a DC amplifier (Letica, Spain) connected to a multichannel polygraph (S460, Goerz Metrawatt. Germany) and to the A/D interface (LabPC+, National Instruments). Voltage signals were digitised at a sampling rate of 20 Hz (duodenal tissues) or 4 Hz (uterine tissues) using the A/D card and visualised on a computer screen using a data acquisition and analysis program written by one of the authors (MD, PHYSCAN software). Data was low-pass filtered at 1 Hz (uterine) or 5 Hz (duodenum) and analysed using computer routines included in the acquisition software.

2.3. Synthesis of tamoxifen derivatives

The *N*-desmethyl-tamoxifen hydrochloride was obtained from tamoxifen with vinyl chloroformate (Olofson et al., 1977). The free base, *N*-desmethyl-tamoxifen, was obtained from the suspended salt in sodium hydroxide aqueous solution by extraction with hexanes. The ethyl- and butyl-bromide salts were obtained by refluxing tamoxifen in acetone in the presence of a fourfold molecular excess of the corresponding bromide (ethyl- or butyl-), therefore following a different method to that reported by Jarman et al. (1986), for the ethylbromide salt. All synthesized compounds exhibited melting points, ultraviolet, infrared nuclear magnetic resonance and high resolution mass spectroscopy spectra consistent with the structures assigned to them. The microanalyses results were within ±0.4% of the calculated values for molecular formulas. At the time of submitting the present article, both the procedures and the molecules were subjected to patenting processes.

2.4. Experimental procedures

Once mounted in the organ baths, tissues were equilibrated at a resting tension of 0.5 g (duodenum) or 0.5 g (uterine). Muscle resting tension was readjusted every 5 min during the 30 min equilibration period. The maximum contraction produced by addition of 60 mM KCl and the minimal tension produced by calcium removal were recorded for each muscle preparation at the beginning of each experiment. These values were used to check the dynamic contractile response (100% RA) and to adjust the amplifier gain. Bath solutions were replaced every 20 min and muscle tissues were washed at least three times after application of drugs.

2.5. $CaCl_2$-induced contractions

In these experiments, tissues were first incubated in the presence of Ca^{2+}-free bathing solutions for 4 min. For uterine tissues, bath solution was made depolarizing by adding 60 mM KCl. Small volumes of 1 M $CaCl_2$ were added to the bath to yield the desired calcium concentration while the isometric tension was continuously recorded. To obtain the concentration–response curves for calcium dependence, transient peak (duodenum) or tonic (uterus) contractions were measured in response to graded concentrations of $CaCl_2$. After each concentration was tested, tissues were washed three times with Ca^{2+}-free solutions and left for 4 min before the next calcium concentration was assayed.

2.6. Effects of tamoxifen derivatives on spontaneous activity

Muscle preparations were incubated in PSS (duodenum) or tyrode solution (uterus) and spontaneous activity was recorded for 10 min after the equilibration period. Tamoxifen and derivatives were then added in small volumes (10–12 μl) directly to the bath solution while the time-course of muscle activity was recorded. In the case of duodenum, comparison of contractile activity was assessed by linear frequency analysis (see below) of data segments taken just before the addition of the drug and also during the steady-state of drug effects. In some experiments, the effects of carbachol, acetylcholine, tetraethylammonium chloride, nifedipine, verapamil and BAY K8644, on contractile activity were assessed by adding small volumes of appropriate stock solutions directly to the bath.

2.7. Effect of triphenylethylene derivatives on $CaCl_2$-induced contraction

Smooth muscle preparations were incubated in Ca^{2+}-free (duodenum) or Ca^{2+}- and K^+-free (uterus) solutions for 4 min, and then exposed to 2.0 mM $CaCl_2$ (duodenum) or 3.0 mM $CaCl_2$ + 60 mM KCl (uterus). Both the resulting peak (duodenum) and tonic (uterus) contractions were used as control values (T_{test}) for subsequent effects. Afterwards, the solution was replaced with a Ca^{2+}-free solution and left for 4 min to stabilise. Tissues were then exposed to different concentrations of triphenylethylene derivatives at the desired concentration for another 5 min. At the end of this period, the peak (duodenum) and tonic (uterus) contraction (T_{drug}) elicited by a second application of $CaCl_2$ was measured. Bath solutions were then replaced with fresh PSS or tyrode (in which spontaneous activity was checked) and then left for another 10–20 min until complete recovery.

2.8. Statistics and mathematical analyses

One-way analysis of variance (ANOVA) and *t* Student–Newman–Keuls test were used to determine differences between sample means. Values of $P<0.05$ were considered significant. Dose-response curves were fitted to logistic equation using non-linear regression analysis tools provided in SigmaPlot software (Jandel Scientific, San Rafael, CA). Frequency analyses of duodenal activity were assessed using the fast Fourier transform (FFT) algorithm implemented in the acquisition software (PHYSCAN). Analyses were performed on 512 data segments taken from data windows of 25.6 s from the steady-state phases of each experimental condition. Linear trends were removed from each data segment and the spectral coefficients of the power spectra were then smoothed to reduce their variance and assembled to obtain the average power spectrum.

2.9. Drugs

Tamoxifen, carbachol, acetylcholine, EGTA, tetraethylammonium chloride (TEA-Cl), verapamil and 1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl) phenyl]-pyridine-3-carboxilic acid methyl ester (BAY-K8644) were obtained from Sigma (Biosigma, Spain). 7α-[9-[(4,4,5,5,5,-pentafluoropenty)sulphinyl]nonyl]-estra-1,3,5(19)-triene-3,17β-diol (ICI182,780) was a gift from Astra Zeneca (Madrid, Spain). Nifedipine was purchased from Alomone Labs (Israel). Tamoxifen and their derivatives were dissolved in ethanol and stored at 4 °C as 20 mM stock. BAY-K8644, verapamil and nifedipine were dissolved in dimethyl sulfoxide (DMSO) and stored as stock solutions at −20 °C. Solvent concentrations in the bath never exceeded 0.12%.

3. Results

3.1. Spontaneous activity and $CaCl_2$-induced contraction

Duodenal tissues studied displayed characteristic spontaneous peristaltic activity that was entirely dependent on the presence of extracellular calcium, i.e., calcium removal completely abolished spontaneous activity and relaxed duodenal muscle (Fig. 1A). Spontaneous activity was not affected by addition of maximal volumes of vehicles ethanol (Fig. 1B) or DMSO (not shown). Despite some variability between tissues, analyses using the fast Fourier transform revealed that spontaneous activity was consistent with a principal harmonic component corresponding to frequencies between 0.49–0.61 Hz, which were preserved in the presence of vehicles (Fig. 1B and D). The frequency spectrum obtained remained similar all along the experiment with little variations over the time. In the absence of calcium, the power density spectrum was notably affected, and all frequency components were suppressed (Fig. 1B). These observations are well in agreement with our previous findings on this same tissue (Díaz, 2002; Díaz et al., 2004). Progressive addition of calcium to the incubation bath resulted in increased basal tone and wider peristaltic amplitude (Fig. 1E). Using non-linear fitting of experimental data to the logistic equation, we have previously shown that half-maximal activity was obtained at 196 μM and maximal activity observed around 1.0 mM (Fig. 1F).

Uterine tissues often displayed spontaneous transient contractions which were irregular in both amplitude and frequency (Fig. 2A). However, under depolarizing conditions (high-potassium, 60 mM), uterine tissues always responded with an initial transient peak contraction which was followed by a plateau (Fig. 2A, right panel). The plateau (tonic) phase was stable and maintained with little or no decay for up to 15 min as long as sufficient calcium ions were present in the bathing solution (shown in Fig. 2A, right panel). Concentration-dependence experiments for uterine isometric contraction against calcium and potassium concentrations are illustrated in Fig. 2B. The results showed that in the presence of 60 mM K^+ and 3 mM Ca^{2+} tissues displayed reproducible plateau phases. Fitting of data from steady-state plateau tension to logistic equations demonstrated an EC_{50}'s value of 2.17 mM for calcium (Fig. 2C). Given that the response to KCl was complex and only triggered a plateau phase at high concentrations (Fig. 2B, lower panel), dose-response analysis could not be performed.

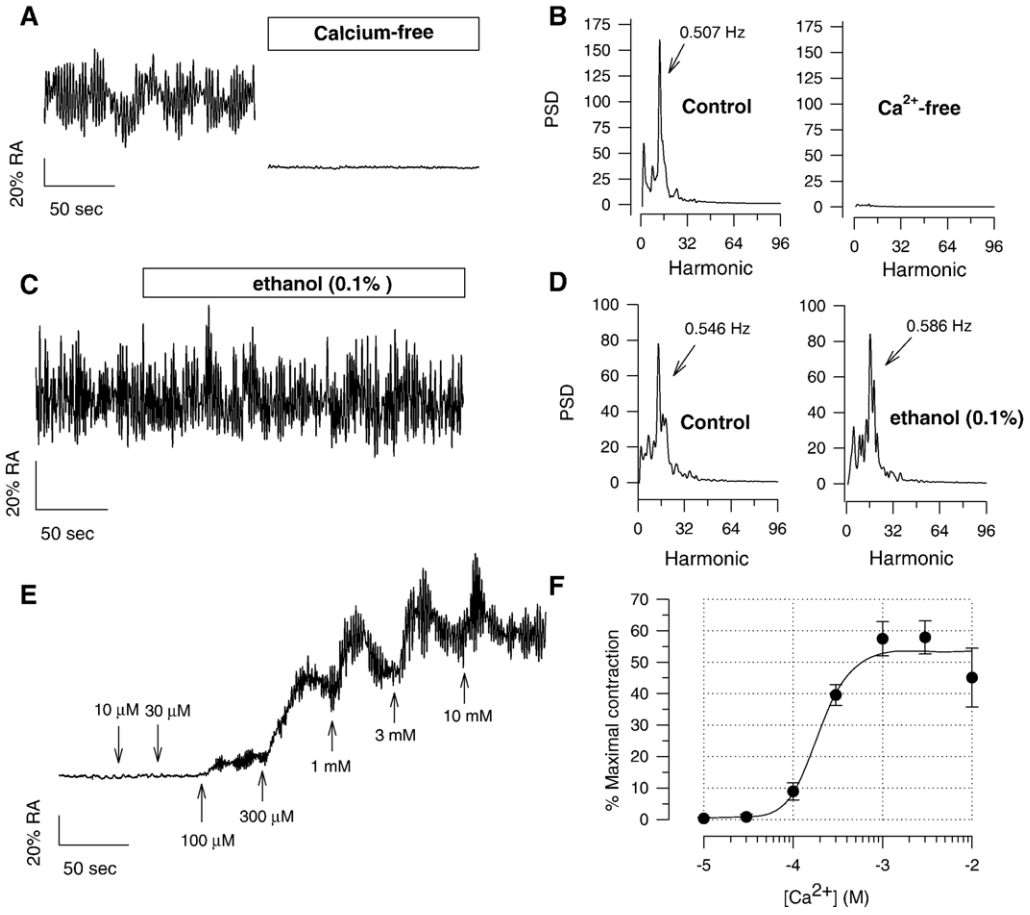

Fig. 1. Characterisation of duodenal contractile activity. (A) Recording of isometric tension in mouse duodenal muscle incubated in physiological saline solution (left trace) and following replacement of calcium ions from the extracellular solution (right trace). (B) Fourier analysis of duodenal spontaneous activity determined under control (left panel) and calcium-free conditions (right panel). Ordinates represent power spectral density (PSD) and abscises the harmonic components. Note that the single dominant component observed at 0.507 Hz under control conditions vanishes upon calcium replacement. (C) Spontaneous isometric signals were not substantially affected by addition of maximal vehicle volume (ethanol, 0.1%) either in time or frequency domains (D). (E) Original recording showing contractile responses induced by cumulative addition of $CaCl_2$. (F) Concentration–response curve for $CaCl_2$-induced contraction for mouse duodenum. Values are expressed as mean ± S.E.M. ($n = 3$).

3.2. Synthesis of tamoxifen derivatives

We have previously shown that tamoxifen and its relevant physiological metabolite 4-OH-tamoxifen exerted a potent relaxing effect on duodenal peristaltic activity by an estrogen-receptor independent mechanism likely involving an interaction with a plasma membrane target (Díaz, 2002). In order to gain a better understanding of the mechanism of tamoxifen action on smooth muscle cells, we have proceeded to synthesize different membrane-impermeant derivatives of tamoxifen, namely *N*-desmethyl-tamoxifen hydrochloride, ethylbromide-tamoxifen and butylbromide-tamoxifen. Within these molecules, the alkylaminoethoxy side chain of tamoxifen has been modified to hold quaternary nitrogen atoms. The rationale for the synthesis of the different derivatives was threefold. First, the generation of non-permeable molecules, either negatively or positively charged in aqueous solution, therefore rendering them impermeant to cell membranes. Second, suppression of the unshared pair of electrons in the nitrogen of the side chain, which are non-available in quaternary salts, to determine the absolute requirement of this nitrogen for tamoxifen effects. And third, the modification of the size of substituents on the nitrogen of the aminoethoxy side chain (*N*-desmethyl-, *N*-ethyl- and *N*-butyl-) to perform structure-activity analyses. Molecular structures of tamoxifen and all synthesized molecules, i.e., *N*-desmethyl-tamoxifen, *N*-desmethyl-tamoxifen hydrochloride, ethylbromide-tamoxifen and butylbromide-tamoxifen are shown in Fig. 3.

3.3. Effects of tamoxifen and membrane-impermeant derivatives on duodenal contractile activity

We have explored the effects of different chemically related membrane-impermeant tamoxifen derivatives on the spontaneous peristaltic activity of duodenal tissues. As can be seen in Fig. 4A, tamoxifen, *N*-desmethyl-tamoxifen hydrochloride, ethylbromide-tamoxifen and butylbromide-tamoxifen, all inhibited spontaneous peristaltic activity (Fig. 4A). Measurements of basal tone in response to tamoxifen and its derivatives showed a significant reduction ($P < 0.01$) compared to respective control

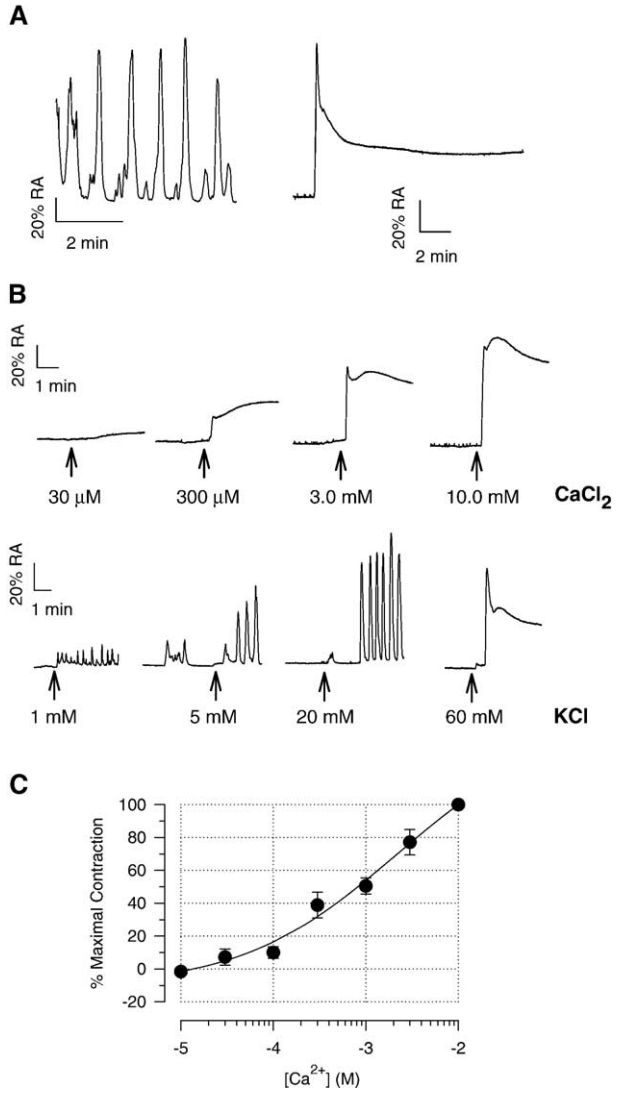

Fig. 2. Characterisation of uterine contractile activity. (A) Illustrative recordings of isometric tension from mouse uterine muscle incubated in tyrode saline solution (spontaneous activity, left trace) and following depolarization with KCl in the presence of high calcium to induce tonic contractile response (right trace). (B) Original traces showing the calcium-dependence (upper traces, bath KCl concentration was 60 mM) and depolarization-dependence (lower traces, bath $CaCl_2$ concentration was 3.0 mM) of uterine contractile activity. (C) Concentration–response curve for $CaCl_2$- and depolarization-induced contraction in mouse uterus. Values are expressed as mean±S.E.M. (n=3) for percentage fraction of 100% maximal activity.

periods (Fig. 4A, lower right panel). The inhibitory effects were rapidly achieved within 10–180 s after exposure to micromolar concentrations of the different triphenylethylenes derivatives reversible upon washout in PSS (Fig. 4B). Spectral analyses using the fast Fourier transform algorithm revealed that exposure to tamoxifen derivatives abolished all frequency components in the bandwidth of peristaltic activity observed in the corresponding control periods which, in turn, reappeared with minor changes after washout (shown for butylbromide-tamoxifen in Fig. 4C). These effects are specific for tamoxifen and its derivatives since the pure steroidal antiestrogen 7α-[9-[(4,4,5,5,5,-pentafluoropenty)sulphinyl]nonyl]-estra-1,3,5(19)-triene-3,17β-diol (ICI182,780, 10 μM) was unable to produce any appreciable change on either peak-to-peak amplitude (98.72±9.32% vs 100% in control tissues) or basal tone (92.34±11.03% vs 100% in control tissues), paralleling our previous findings on duodenal tissues (Díaz, 2002; Díaz et al., 2004).

In order to quantify the response of intestinal muscle to the molecules under study, we have assessed their effects on calcium-induced contractions. As can be seen in Fig. 5A, ethylbromide-tamoxifen produced a concentration-dependent reduction of peak contractions, with maximal inhibition being reached around 3 μM. Similar results were observed in response to preincubation with the other tamoxifen derivatives (not illustrated). Dose-response curves for non-permeant triphenylethylene derivatives (Fig. 5B) showed that butylbromide-tamoxifen was more potent (IC_{50}=0.099 μM) than ethylbromide-tamoxifen (IC_{50}=0.37 μM), while *N*-desmethyl-tamoxifen hydrochloride was the least effective triphenylethylene (IC_{50}=1.42 μM) towards inhibition of calcium-induced contractions. Under this same experimental paradigm, *N*-desmethyl-tamoxifen and tamoxifen exhibited IC_{50} values of 2.46 μM (this study) and 0.85 μM (Díaz, 2002). Accordingly the inhibitory potency of triphenylethylene molecules (Table 1) follows a sequence: butylbromide-tamoxifen > ethylbromide-tamoxifen > tamoxifen > *N*-desmethyl-tamoxifen hydrochloride > *N*-desmethyl-tamoxifen, which in turn, matches the molecular size of side chains (see Fig. 3).

3.4. Effects of tamoxifen and membrane-impermeant derivatives on uterine contractile activity

Tamoxifen and its derivatives also reduced spontaneous activity and tonic contraction of uterine tissues observed under depolarizing conditions (see methods). Results illustrated in Fig. 6 shows that micromolar concentrations of tamoxifen or its impermeant derivatives rapidly and reversibly reduced the tonic contraction of uterine tissues induced by calcium in the presence of high potassium. The relaxing effect of triphenylethylenes was fastest for butylbromide-tamoxifen (t_{50}≈5 s) and slowest for *N*-desmethyl-tamoxifen hydrochloride (t_{50}≈50 s) and was not mimicked by the steroidal antiestrogen 7α-[9-

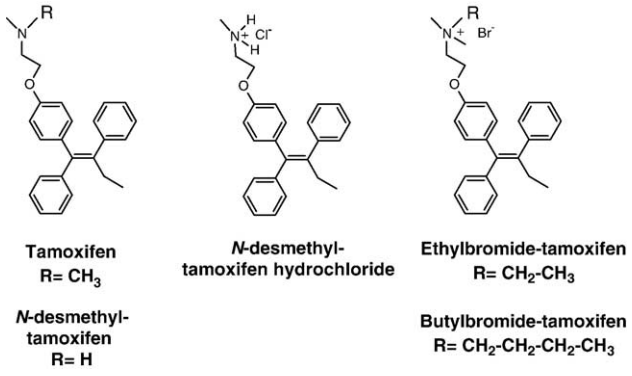

Fig. 3. Molecular structures of tamoxifen, *N*-desmethyl-tamoxifen and non-permeant derivatives.

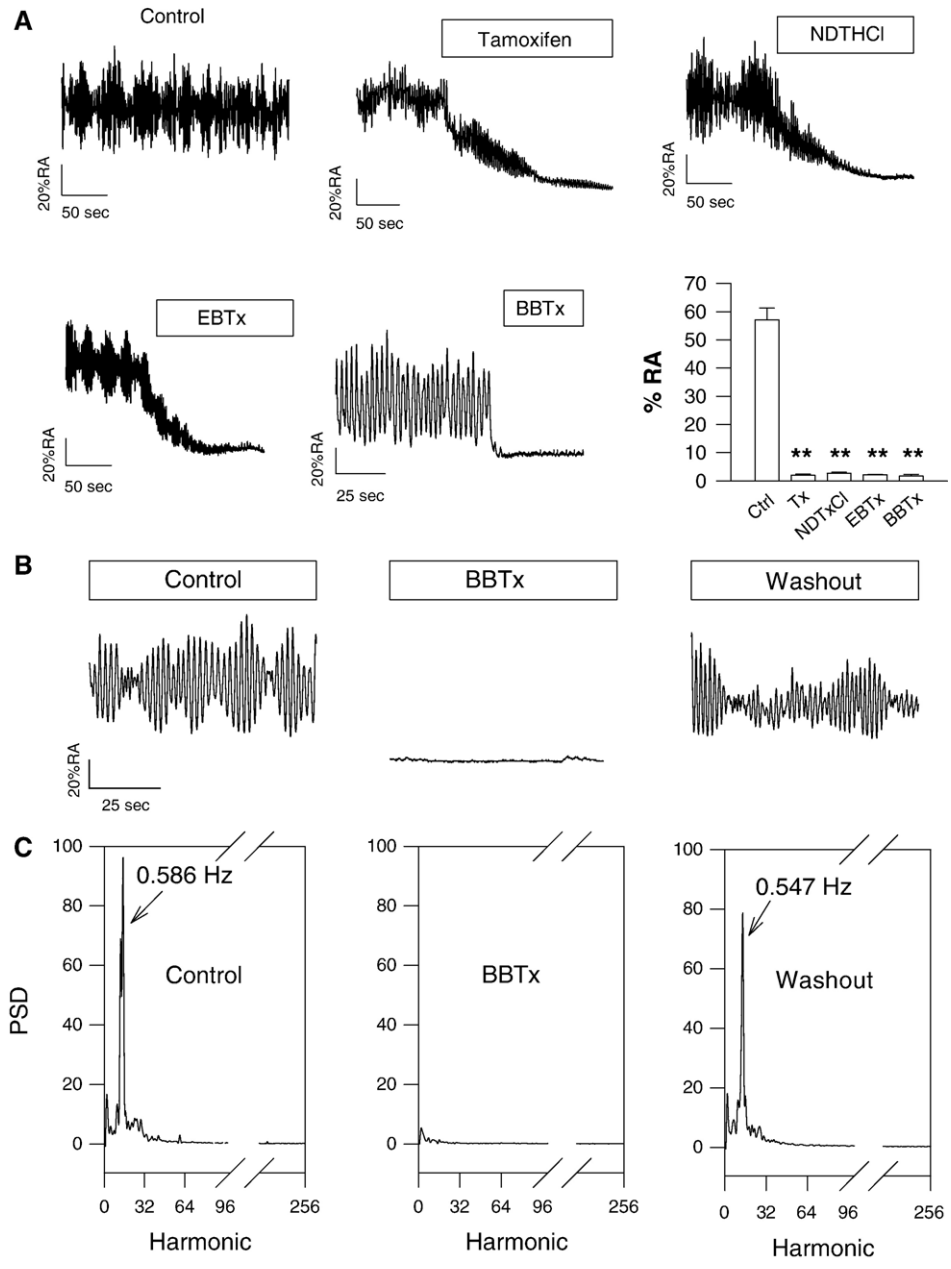

Fig. 4. Effects of triphenylethylene derivatives on duodenal activity. (A) Representative traces for the effects of tamoxifen and non-permeant tamoxifen derivatives on spontaneous activity of isolated mouse duodenum. All compounds were applied at 10 μM. The lower right panel shows the effect of tamoxifen and non-permeant tamoxifen derivatives on basal tone. Results are expressed as mean ± S.E.M. of, at least, three different preparations. ** Statistically different from control conditions with a probability value of $P<0.01$. (B) The inhibitory effect of tamoxifen and derivatives was reversible upon washout (shown here for 5 μM butylbromide-tamoxifen, BBTx). Tissues recovered basal tone and peristaltic activity once triphenylethylene molecules were removed from the bath. (C) Spectral density spectrum of duodenal spontaneous activity determined under control conditions (left panel), 5 min after butylbromide-tamoxifen (BBTx) application to the bath (10 μM, middle panel) and 10 min after butylbromide-tamoxifen (BBTx) washout (right panel). Ordinates represent the power spectral density (PSD) and abscises the frequency components (Hz). Abscises have been scaled to illustrate frequencies associated with contractile activity. Similar responses were obtained for another 6 preparations.

[(4,4,5,5,5,-pentafluoropenty)sulphinyl]nonyl]-estra-1,3,5(19)-triene-3,17β-diol (ICI182,780, not shown). In general, the slower the inhibition by triphenylethylenes the longer the recovery time, suggesting a certain degree of toxicity.

In order to quantify the relaxing effect of tamoxifen and derivatives under study on uterine tissues, measurements were obtained for peak and plateau contractions elicited by $CaCl_2$ under depolarizing conditions (60 mM KCl), both in control (vehicle) and after 5 min preincubation with the corresponding molecule. The results demonstrated that tamoxifen and its derivatives produced a dose-dependent reduction of both peak and tonic contractions, with maximal inhibitions being reached around 10 μM (shown in Fig. 7A for ethylbromide-tamoxifen) except for *N*-desmethyl-tamoxifen hydrochloride that caused maximal inhibition above 30 μM. Dose-response curves for triphenylethylene derivatives showed that butylbromide-

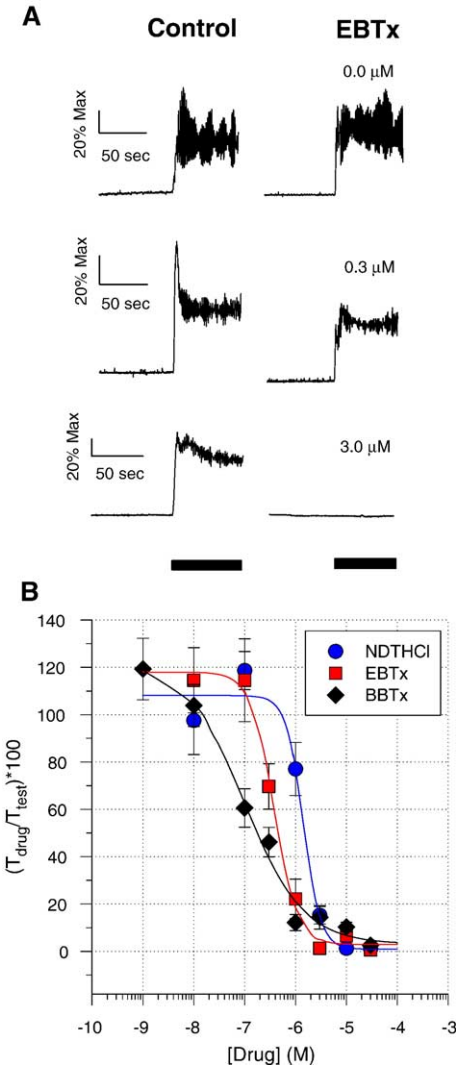

Fig. 5. Effects of non-permeant tamoxifen derivatives on duodenal $CaCl_2$ −induced contraction. (A) Illustrative recordings showing the inhibitory effect of ethylbromide-tamoxifen (EBTx) on $CaCl_2$-induced contractions in isolated duodenal muscle strips. Calcium pulses are indicated by black rectangles under the traces. (B) Cumulative concentration–response curves for *N*-desmethyl-tamoxifen hydrochloride (NDTHCl), ethylbromide-tamoxifen (EBTx) and butylbromide-tamoxifen (BBTx). Values are expressed as mean±S.E.M of, at least, 4 different preparations for each concentration.

tamoxifen was more potent (IC_{50}=0.40 μM) than ethylbromide-tamoxifen (IC_{50}=0.58 μM), while *N*-desmethyl-tamoxifen hydrochloride was the least effective triphenylethylene (IC_{50}=23.42 μM) towards inhibition of calcium-induced contractions. Interestingly, tamoxifen and *N*-desmethyl-tamoxifen were poor inhibitors towards relaxation of uterine muscle and, even at the highest concentration used, maximal inhibition (as ascertained from the logistic minimal asymptote) were never above 48% (Table 1), therefore were not used in the structure-activity assessments. Similarly to the aforementioned results for duodenal tissues, the inhibitory potency sequence (Table 1) for non-permeant derivatives was butylbromide-tamoxifen>ethylbromide-tamoxifen>*N*-desmethyl-tamoxifen hydrochloride.

3.5. Effects of KCl, K^+ channel blockers, acetylcholine and carbachol on triphenylethylene-relaxed tissues

In an attempt to elucidate the mechanisms underlying the relaxing action of triphenylethylene derivatives on duodenal smooth muscle, several experiments were designed to determine the effects of several agents known to affect smooth muscle activity on triphenylethylene-relaxed tissues. In the presence of tamoxifen derivatives, addition of KCl (33 mM) did not cause any appreciable change on isometric tension of duodenal tissues (Fig. 8A, left panel), which is indicative that relaxed tissues have lost the ability to respond to depolarization. Similarly, addition of 5 mM tetraethylammonium chloride (TEA-Cl), a general Maxi K^+ channel blocker failed to reverse triphenylethylene-induced relaxation (Fig. 8A, right traces). On the contrary, application of the muscarinic agonist carbachol (1–10 μM) or acetylcholine (10 μM, not shown) to triphenylethylne-treated duodenum was followed by a phasic peak contraction that returned to baseline after 10–30 s after the agonist pulse (Fig. 8B).

Similar experiments were performed on uterine tissues. Application of TEA-Cl (10 mM) to triphenylethylene-relaxed uteri failed to induce any significant change on isometric tension (Fig. 8C). Conversely, addition of acetylcholine (10–50 μM) to triphenylethylene-relaxed uteri brought about an increase of isometric tension, (Fig. 8C).

3.6. Effects of triphenylethylene derivatives on calcium-dependence curves

Following similar protocols to those used in Fig. 1F for duodenal tissues and Fig. 2C for uterine tissues, we have assessed the effects of triphenylethylene derivatives on the concentration–response curves for $CaCl_2$-induced contractions. All compounds, used at concentrations around the IC_{50} values shown in Table 1, caused a right shift in the calcium-dependence curves that was accompanied by a considerable reduction of the maximal asymptotes in both duodenal and uterine tissues (Fig. 9). The analyses also showed that the larger the inhibitory potency the wider the displacement of the calcium-dependence curve to the right.

3.7. Effects of BAY K8644 on triphenylethylene-relaxed tissues

Finally, we explored the effects of the L-type calcium channel agonist BAY K8644 on the relaxation induced by tamoxifen derivatives. The relaxing effect of all tamoxifen derivatives could be significantly counteracted by the addition of 0.5–1 μM BAY K8644 to the bath and, both basal tone and peristaltic amplitude returned to control values (Fig. 10A–Bd). Interestingly, this same response was obtained when tissues were relaxed with the L-type calcium channel antagonist nifedipine (1 μM, not shown), a finding that may be interpreted as the result of competition between dihydropyridines. Frequency analysis using the fast Fourier transform algorithm revealed that even in the continuous presence of ethylbromide-tamoxifen, application of BAY K8644 readily restored the main

Table 1
Pharmacological comparison of the effects of tamoxifen and its derivatives on the contractile activity of visceral smooth muscles

Tissue		Compound				
		Tx	NDTx	NDTHCl	EBTx	BBTx
Duodenum	IC_{50}	0.85±0.25	2.46±0.18	1.42±0.30	0.37±0.04	0.099±0.03
	RP	1.00	0.34	0.59	2.29	8.58
	Asymptote	2.11±5.67	−0.81±2.84	0.86±8.00	2.81±3.49	3.06±5.68
	% Maximal inhibition	97.89	100.81	99.14	97.19	96.94
Uterus	IC_{50}	1.17±0.12	2.15±1.00	23.44±2.73	0.58±0.18	0.40±0.19
	RP	1.00	0.54	0.05	2.01	2.92
	Asymptote	64.90±6.28	51.88±6.19	4.71±37.2	25.01±4.58	21.34±3.84
	% Maximal inhibition	35.1	48.12	95.29	74.99	78.66

IC_{50} values are given in μM. IC_{50} and Asymptote values are expressed as mean±S.E.M. RP: relative potency compared to tamoxifen (Tx). NDTx: *N*-desmethyl-tamoxifen; NDTHCl: *N*-desmethyl-tamoxifen hydrochloride; EBTx: ethylbromide-tamoxifen; BBTx: butylbromide-tamoxifen.

frequency components in the bandwidth of spontaneous activity featuring the corresponding control period (Fig. 10B). Similar results were obtained for triphenylethylenes butylbromide-tamoxifen and *N*-desmethyl-tamoxifen hydrochloride (not shown) and, as we have previously reported, for tamoxifen (Díaz, 2002). All these data strongly suggest a crucial involvement of L-type calcium channels in triphenylethylene-induced duodenal muscle relaxation.

With regard to uterine tissues, experiments performed on triphenylethylene-relaxed tissues showed that exposure to BAY K8644 (0.5 μM) effectively recover tonic contraction in a dose-dependent manner. Indeed, the L-type calcium channel agonist readily increases (and restores) tonic isometric tension of tissues relaxed with tamoxifen, *N*-desmethyl-tamoxifen hydrochloride, ethylbromide-tamoxifen and butylbromide-tamoxifen (illustrated for ethylbromide-tamoxifen and *N*-desmethyl-tamoxifen hydrochloride in Fig. 10C), suggesting that all compounds share a common relaxation mechanism. Similarly, exposure of precontracted uterine tissues to the dihydropyridine nifedipine (1 μM) or the phenothyacine verapamil (100 μM), well-known blockers of L-type calcium channels, both caused a dose-dependent inhibition of tonic isometric contraction (not shown), mimicking the effect observed here for triphenylethylene derivatives.

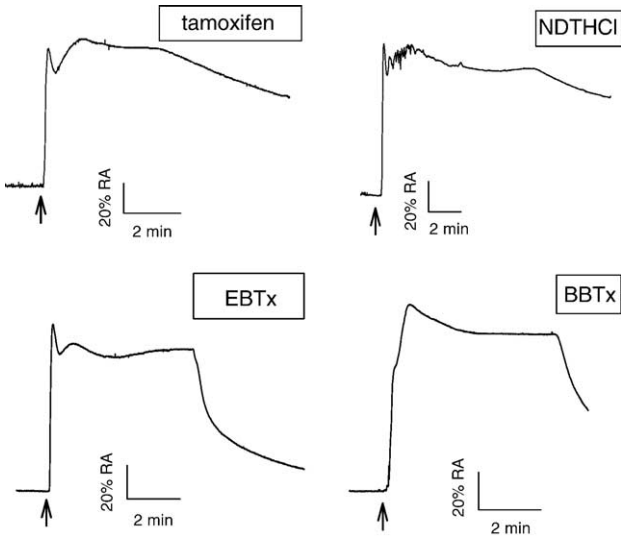

Fig. 6. Effects of tamoxifen and non-permeant tamoxifen derivatives on uterine $CaCl_2$−induced contraction. Representative traces showing the inhibitory effect of tamoxifen, *N*-desmethyl-tamoxifen hydrochloride (NDTHCl), ethylbromide-tamoxifen (EBTx) and butylbromide-tamoxifen (BBTx) (all applied at 10 μM) on $CaCl_2$- and depolarization-induced contraction in isolated uterine muscle strips. Recordings were obtained from four different animals. High potassium high calcium pulses were applied at the times indicated by the arrows under the traces.

4. Discussion

Data reported here show that tamoxifen derivatives, *N*-desmethyl tamoxifen hydrochloride, ethylbromide-tamoxifen and butylbromide tamoxifen, three non-permeable salts of tamoxifen, are able to inhibit spontaneous activity and calcium-induced contractions in mice duodenal and uterine muscles. These compounds share the central triphenylethylene core of tamoxifen but differ in that the nitrogen of the basic alkylaminoethoxy side chain contains a positively charged quaternary ammonium (see structures in Fig. 3). Unlike tamoxifen, which is a highly lipophilic molecule and can traverse cell membranes, the ionic nature of these tamoxifen derivatives makes them impermeable to plasma membrane.

We have observed that the effects of tamoxifen and derivatives on contractile activity were rapidly achieved within 10–180 s after exposure and completely reversible upon washout. On the contrary, steroidal antiestrogen 7α-[9-[(4,4,5,5,5,-pentafluoropenty)sulphinyl]nonyl]-estra-1,3,5(19)-triene-3,17β-diol (ICI182,780) had no discernible effect on either duodenal or uterine contractile activity, ruling out the involvement of either canonic estrogen receptor α or membrane-associated estrogen receptor α-like receptors, in the inhibitory effect of triphenylethylene compounds. These findings, together with the rapid onset of relaxation of duodenal and uterine smooth muscles by triphenylethylene derivatives, and the impermeable nature of these molecules rule out the involvement of classic genomic pathways for the effects reported here. In fact, tamoxifen and ethylbromide-tamoxifen have been reported to behave as estrogen receptor antagonists and to displace ^{3}H-estradiol binding to estrogen receptors in in vitro studies (Jarman et al., 1986). However, the inability of the

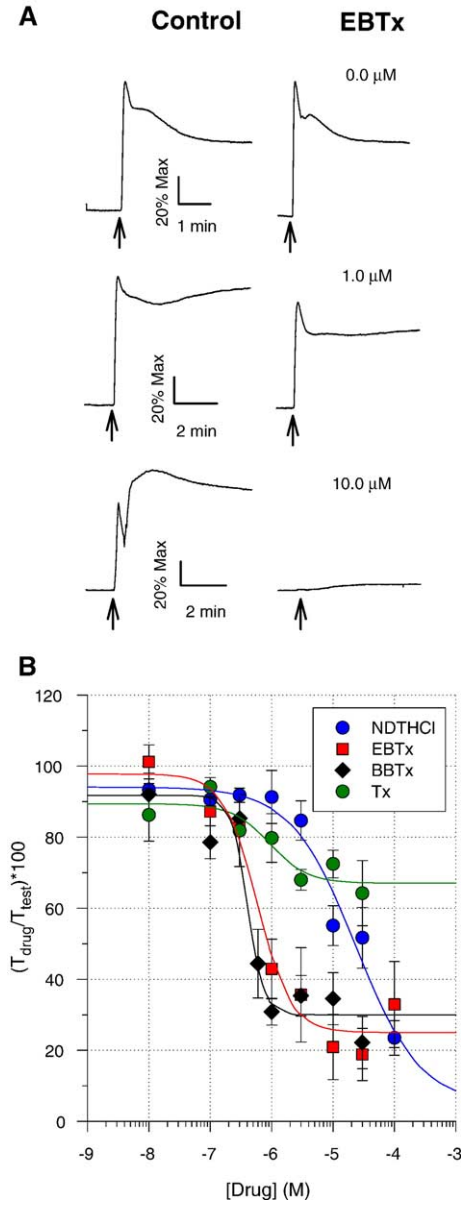

Fig. 7. Concentration-dependence curves for non-permeant tamoxifen derivatives on uterine $CaCl_2$-induced tonic contraction. (A) Recordings showing the inhibitory effect of 5 min preincubation of uterine tissues with ethylbromide-tamoxifen (EBTx) on tonic contractions induced by $CaCl_2$ under depolarizing conditions. Calcium pulses are indicated by the arrows under the traces. (B) Concentration–response curves for *N*-desmethyl-tamoxifen hydrochloride (NDTHCl), ethylbromide-tamoxifen (EBTx), butylbromide-tamoxifen (BBTx) and tamoxifen (Tx). Values are expressed as mean±S.E.M. At least four different preparations were used for each concentration.

quaternary derivative ethylbromide-tamoxifen to cross the cell membrane and binding intracellular targets prove that tamoxifen but not ethylbromide-tamoxifen can inhibit estrogen-dependent proliferation in MCF-7 cells (Jarman et al., 1986).

Dose-response analyses on $CaCl_2$-induced (and depolarization-induced for uterine tissues) contractions revealed an inhibitory potency sequence: *N*-desmethyl-tamoxifen hydrochloride < ethylbromide-tamoxifen < butylbromide-tamoxifen for both duodenal and uterine tissues, indicating that the inhibitory efficiency was related to the size of the alkylaminoethoxy side chain. Accordingly, the larger the ionic side chain of triphenylethylene derivatives, the higher the inhibitory potency. It is noteworthy the low IC_{50} value obtained for butylbromide-tamoxifen (0.099 μM in duodenum and 0.40 μM in uterus), which is nearly two orders of magnitude smaller than values reported for tamoxifen and ethylbromide-tamoxifen in the in vitro rat uterus (Cantabrana and Hidalgo, 1992). Our data also show that acute effects of tamoxifen on smooth muscle are largely independent on the presence of the unshared pair of electrons on the lateral chain of tamoxifen, which are required for its antiestrogenic action (Murphy and Jordan, 1989), since they were not prevented by formation of quaternary ammonium salts.

Evidence accumulated in the last decades has demonstrated that triphenylethylene antiestrogens are able to exert a number of non-genomic actions by modulating the function of several proteins, including key molecules of intracellular signalling cascades. Studies on rat uterine myometrium have shown that the spasmolytic effect of tamoxifen is due to inhibition of calmodulin (Lipton and Morris, 1986). In the case of duodenal muscle, this would produce a complete inhibition of peristaltic activity and muscle tone because of the impairment of myosin light chain kinase activation by the Ca^{2+}-calmodulin complex (Allen and Walsh, 1994). Also, because of its ability to bind calmodulin, tamoxifen can increase cyclic AMP levels by inhibiting cyclic AMP-dependent phosphodiesterase (Lam, 1984), which, in turn, would relax smooth muscle. However, this does not seem to be the case of tamoxifen derivatives on either duodenum or uterus. Besides their inability to traverse cell membrane, the fact that acetylcholine (and carbachol) induced contractile responses in triphenylethylene-relaxed tissues is indicative that the contractile machinery remained functionally active. If calmodulin were inhibited then all steps of the contractile machinery located downwards calmodulin activation would be impaired and the smooth muscle would remain in an inactive state. Clearly, these experiments rule out a possible inhibition of calmodulin or activation of protein kinase A by non-permeant tamoxifen analogues, and demonstrates that the mechanism of relaxation by triphenylethylenes is initiated at the plasma membrane.

It has been reported that tamoxifen, in the range of concentrations used here, negatively modulates different types of potassium channels in different excitable cells (Hardy et al., 1998; Smitherman and Sontheimer, 2001). However, it is well known that both K^+ channel blockade or depolarization by high extracellular potassium causes a considerable increase of contraction force generated by gastrointestinal muscles (Meiss, 1987). Hence, it cannot be expected a blocking effect of tamoxifen derivatives on K^+ channels since it would result in a depolarization of smooth muscle cells, which, in turn, would activate muscle contraction. In agreement with this hypothesis, comparative studies using tamoxifen and ethylbromide-tamoxifen have demonstrated that unlike tamoxifen, ethylbromide-tamoxifen fails to block voltage-dependent K^+ channels in NG108-15 neuroblastoma cells (Allen et al., 2000) and colonic myocytes (Dick et al., 2002).

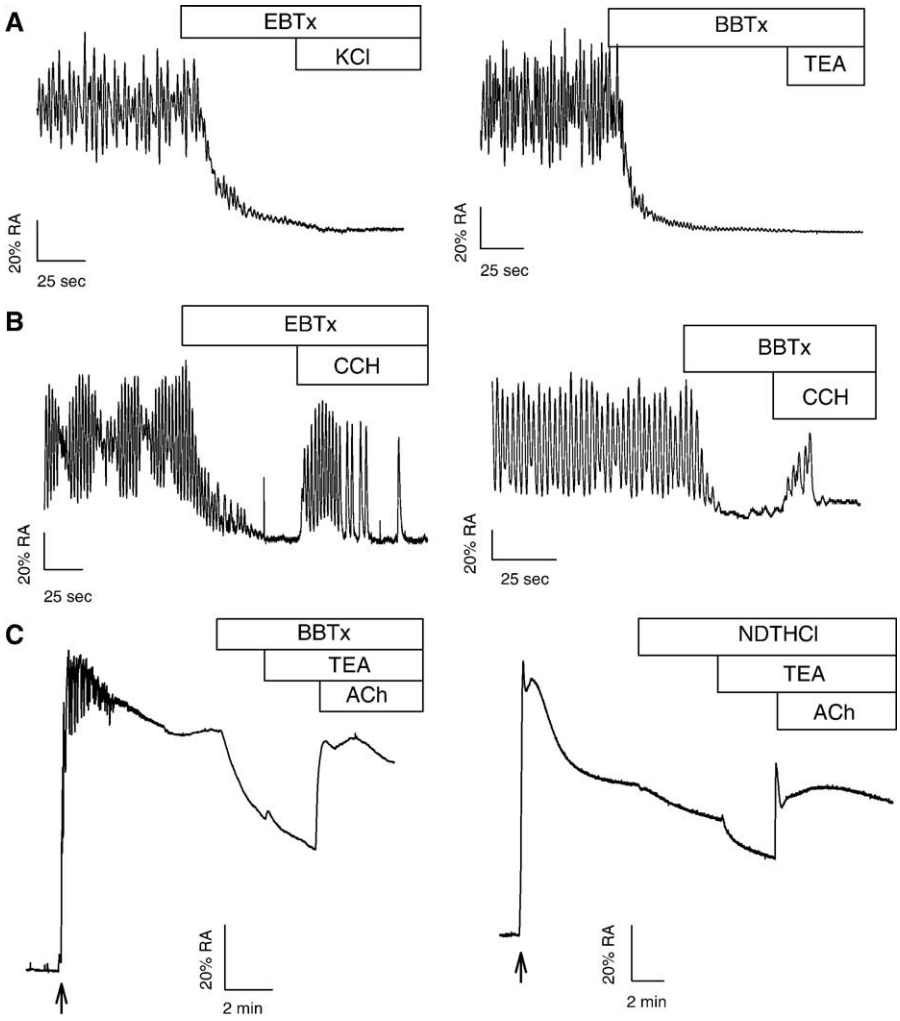

Fig. 8. Effects of KCl, K^+ channel blockers, acetylcholine and carbachol on triphenylethylene-relaxed muscles. (A) Illustrative recordings showing the effects of KCl (60 mM) and K^+ channel blockade with TEA-Cl (5 mM) on the relaxation induced by tamoxifen derivatives (10 μM). (B) Representative traces showing the effects of carbachol (CCH, 10 μM) on the relaxation induced by triphenylethylene derivatives (10 μM) on isolated duodenal muscle. Results are representative of another 4 experiments for each condition and tamoxifen derivative. (C) Sequential effects of TEA-Cl (5 mM) and acetylcholine (ACh, 10 μM) on triphenylethylene-relaxed uteri. Traces are representative of, at least, four different preparations. Concentration of tamoxifen derivatives was 10 μM in all records. Similar results were obtained for ethylbromide-tamoxifen (EBTx).

A putative activation of potassium channels and consequent hyperpolarization of smooth muscle cells would provide a feasible explanation for the mechanism of relaxation by triphenylethylene derivatives. Indeed, recent studies on the modulation of Maxi $K_{(Ca)}$ channels (BK) in smooth muscle cells have shown that tamoxifen and ethylbromide-tamoxifen increase the channel open probability by interacting with the regulatory β1 subunit (Dick et al., 2001, 2002), and it has been suggested that the activation of BK channels reduces the voltage-dependent calcium influx and $[Ca^{2+}]_i$ through tonic hyperpolarization of smooth muscle cells (Lohn et al., 2001). However, in our case, the fact that high KCl-induced depolarisation of duodenal muscle and the blockade of duodenal and uterine K^+ channels with TEA, did not induce any noticeable change in the recorded tension of duodenal or uterine tissues (see Fig. 7), strongly suggest that the effect of tamoxifen derivatives was not related to activation of K^+ channels. On the other hand, we have previously observed that Maxi K^+ β1 subunit was not expressed in mouse duodenum (Díaz et al., 2004). These observations indicate that the relaxing effect of tamoxifen derivatives must have been occurred on the coupling of the oscillating membrane potential and the voltage-dependent calcium influx.

This hypothesis was tested by using the dihydropiridine derivative BAY K8644, a well-known calcium channel agonist, which increases the current through the L-type calcium channels of nerve and muscle cells (Schramm et al., 1983; Coruzzi and Poli, 1985). Our present results demonstrate that inhibition by triphenylethylene derivatives could be counteracted by reactivating calcium influx through L-type calcium channel with the dihydropiridine BAY K8644. Interestingly, the isometric tension developed by duodenal tissues followed a peristaltic pattern, which exhibited a frequency spectrum similar to that present in control tissues before the addition of triphenylethylenes. This result strongly points to a blockade of L-type calcium channels by tamoxifen derivatives being

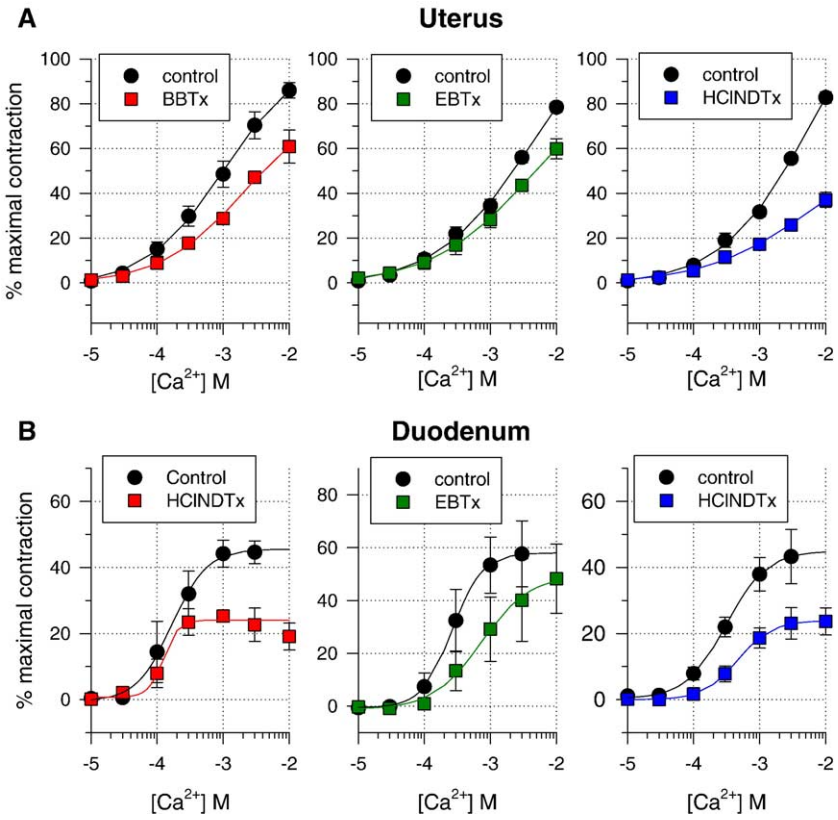

Fig. 9. Effects of non-permeant tamoxifen derivatives on calcium-dependence curves. Concentration–response curves for Ca^{2+}-induced contraction in uterine (A) and duodenal (B) tissues. Data were obtained in controls and after 5 min preincubation of duodenal and uterine tissues with butylbromide-tamoxifen (BBTx), ethylbromide-tamoxifen (EBTx) and *N*-desmethyl-tamoxifen hydrochloride (NDTHCl). In duodenal tissues the concentrations of triphenylethylene derivatives were 100 nM, 1.2 and 1.4 μM, for BBTx, EBTx and NDTHCl, respectively. In uterine tissues the concentrations of BBTx, EBTx and NDTHCl were 0.4 μM, 0.58 μM and 23.0 μM, respectively. Values are expressed as mean±S.E.M. At least four different preparations were used for each tissue and tamoxifen derivative.

responsible for the spasmolytic action of tryphenylethylene derivatives on mouse duodenum. Likewise, the ability of BAY K8644 to fully recover the tonic contraction in triphenylethylene-relaxed uterine tissues demonstrates that L-type calcium channels play a central role in the mechanism of relaxation. Accordingly, analyses of calcium-dependence curves on duodenal and uterine tissues measured at the IC_{50} for each tryphenylethylene derivative, showed both a right shift in the concentration-dependence curve and a significant reduction of maximal contraction that was more pronounced for BBTx, paralleling its relative inhibitory potency. In agreement with our hypothesis, several studies have demonstrated that tamoxifen inhibits calcium entry through L-type calcium channels in A7r5 and aortic smooth muscle cells (Song et al., 1996), colonic myocytes (Dick et al., 1999), rabbit detrusor (Ratz et al., 1999), and non-muscle cells like clonal pituitary cells (Sartor et al., 1988) and PC12 neurosecretory cells (Greenberg et al., 1987). Furthermore, antiestrogens tamoxifen and clomiphene have been shown to compete ^{3}H-nitrendipine binding in membrane fractions of human and rabbit urinary bladder and myometrium (Batra, 1990).

Finally, two pieces of evidence suggest that the main target for the actions of tamoxifen derivatives are likely the smooth muscle cells themselves rather than the interstitial Cajal pacemaker cells (ICC). First, it has been shown that unlike inward calcium currents in small intestine smooth muscle cells, the depolarisation phase of ICC is insensitive to L-type calcium channel blockers and abolished by hyperpolarization (Lee et al., 1999). On the other hand, despite the existence of spontaneous contractile activity in uterine tissues, ICC-like pacemaking cells have not been found in uterus and the spontaneous activity appears to be a property of smooth muscle cells (Wray et al., 2001). Second, the power density spectrum in the presence of BAY K8644 after triphenylethylene relaxation showed similar frequency components in the control periods in the same tissues (see Fig. 10). This finding can only be explained if the mechanisms responsible for generating the pace within the ICC remained unaffected by quaternary tamoxifen analogues.

In summary, non-permeant tamoxifen derivatives relax duodenal and uterine smooth muscles in a rapid and reversible fashion by a common mechanism most likely involving inhibition of L-type calcium channels. Since quaternary derivatives studied here are unlikely to fully partition into a lipid bilayer because of its charged quaternary group, the binding site for non-steroidal quaternary compounds is most likely on the extracellular domain of the channel, likely the dihydropiridine binding site, rather than the intracellular hydrophobic membrane spanning region. Studies using fluorescent calcium probing and electrophysiological experiments

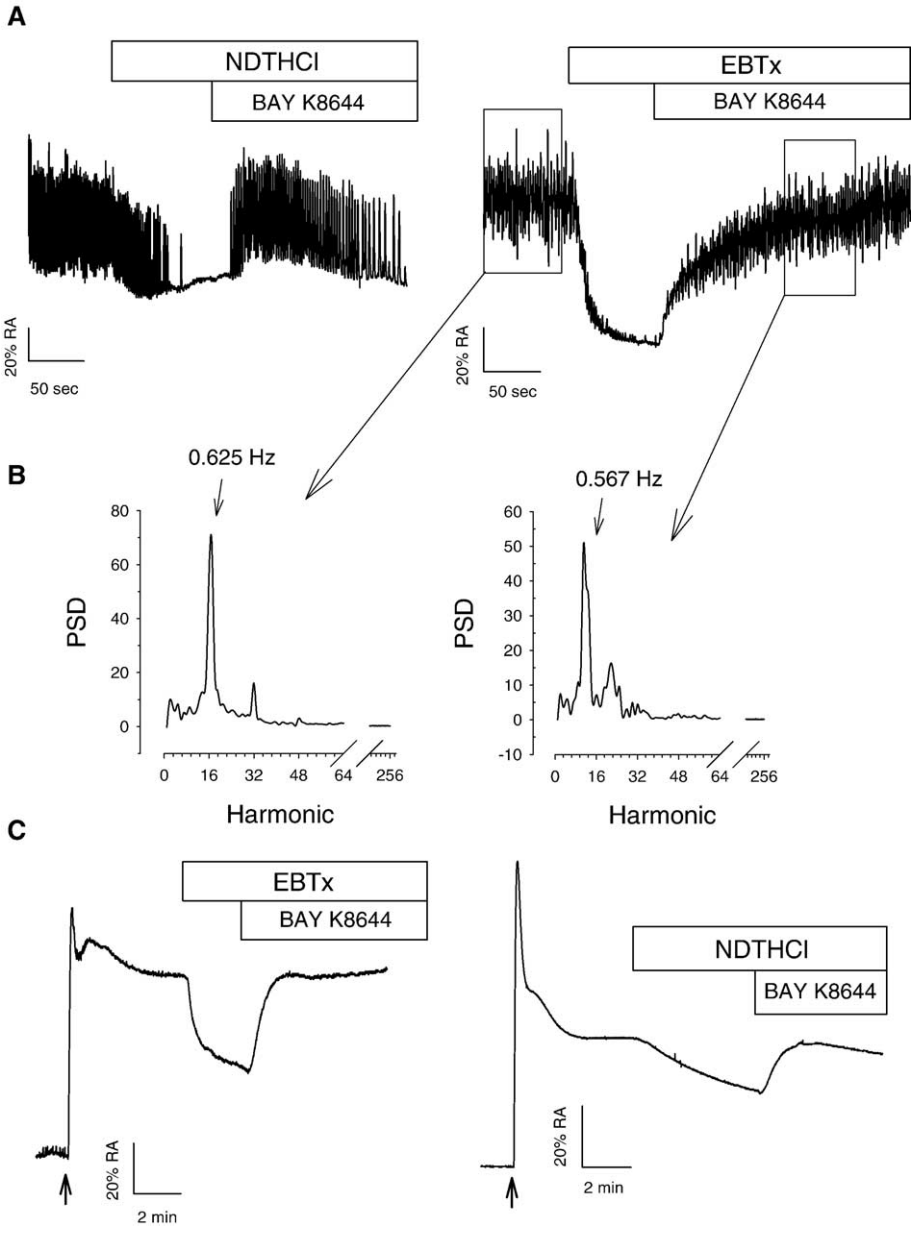

Fig. 10. Effect of BAY K8644 on tamoxifen derivatives-relaxed tissues. (A) Representative recording showing the effects of BAY K8644 (1 μM) on tamoxifen derivatives-relaxed duodenal muscles. Traces were obtained in different preparations and were mimicked by tamoxifen and butylbromide-tamoxifen. Similar results were obtained in another 4 preparations. (B) Frequency analysis of duodenal spontaneous activity determined under conditions indicated above. Ordinates represent power spectral density (PSD) and abscises the harmonic components. Tension traces and frequency spectra are representative of 3 different experiments for each compound. (C) Effects of application of BAY K8644 (0.5 μM) on triphenylethylene-induced inhibition of uterine tonic contraction. Concentration of non-permeant derivatives was 10 μM. Similar effects were produced in ethylbromide-tamoxifen-relaxed tissues. Data are representative of, at least, three different experiments.

on calcium channels in isolated duodenal and uterine smooth muscle cells are being carried out to ascertain such hypothesis. Overall, these data add new insights in the rationale for the development of a new generation of more specific and selective estrogen receptor modulators.

Acknowledgements

Supported by research grant numbers SAF2001-3614-C03-02 (Ministerio de Ciencia y Tecnología, Spain), PI042460 (FIS, Ministerio de Sanidad y Consumo, Spain) and the Spanish Network for Neurological Disorders (CIEN, Spain). JMA holds a fellowship from convenio ULL-CajaCanarias (Spain). We wish to thank Mari Carmen González-Montelongo for her collaboration in caring the animals and cooperation in some of the experiments and to Dr. Raquel Marín for critical reading of the manuscript.

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