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Triphenylethylene antiestrogen-induced acute relaxation of mouse duodenal muscle. Possible involvement of Ca^{2+} channels

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Abstract

The nonsteroidal antiestrogens tamoxifen, 4-OH-tamoxifen and toremifene rapidly inhibited spontaneous contractile activity and reduced basal tone in isolated mouse duodenal muscle. Inhibition was rapid in onset (~ 2 min) and was not mimicked by the pure steroidal antiestrogen 7α-[9-[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]-estra-1,3,5(10)-triene-3,17β-diol (ICI182,780) indicating the involvement of non-genomic mechanisms. Inhibition by tamoxifen and 4-OH-tamoxifen were observed at concentrations comparable to those reached in antiestrogen adjuvant therapy. Antiestrogen-relaxed tissues showed no response to KCl depolarisation or K^+ channel blockade but displayed clear transient responses to acethylcholine or to the muscarinic receptor agonist carbachol. Frequency analysis showed that spontaneous activity could be readily restored in antiestrogen-relaxed tissues by the exposure to the L-type Ca^{2+} channel agonist 1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)phenyl]-pyridine-3-carboxilic acid methyl ester (BAY K8644). Our experiments suggest that triphenylethylene antiestrogens relax duodenal intestinal muscle via a mechanism that involves inhibition of L-type Ca^{2+} channels but not activation of K^+ channels.

Keywords: Tamoxifen; Tamoxifen metabolite; Non-genomic; Intestinal muscle; Spasmolytic effect; Ca^{2+} channel

1. Introduction

Nonsteroidal triphenylethylene compounds such as tamoxifen and toremifene possess strong antiestrogenic activity and are widely used as valuable adjunct in the treatment of estrogen receptor-positive breast cancer by their ability to displace estrogens from their intracellular receptor. However, evidence accumulated in the last two decades has revealed that these antiestrogens may act through other non-genomic mechanisms. Indeed, tamoxifen has been reported to modify the function of a number of plasma membrane proteins including dopamine and histamine receptors (Hiemke and Ghraf, 1984; Brandes et al., 1987) 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 proteins and enzymes involved in cellular transduction, like protein kinase C (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, the low number of clinical acute side effects reported for antiestrogens is remarkable (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 uterine, vascular and detrusor smooth muscle 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 (Lipton et al., 1984; Cantabrana and Hidalgo, 1992; Kostrzewska et al., 1997; Song et al., 1996; Figtree et al., 2000; Ratz et al., 1999). Concerns over the clinical side effects of tamoxifen treatment, including nausea and gastrointestinal disturbances (Trump et al., 1992; Wada et al., 1981), suggest that intestinal smooth muscle may also be targeted by antiestrogens but, however, to date, no indication exists that these

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antiestrogens could directly modify gastrointestinal activity. The aim of the present work was to address the effects of different structurally unrelated antiestrogens on the contractile activity of duodenal muscle.

2. Materials and methods

2.1. Tissue preparation

Duodenal segments were dissected from male mice weighing 20–30 g following diethyl ether anaesthesia. Longitudinal strips of duodenal smooth muscle (1.5 cm long) were immediately placed in cold physiological salt solution (PSS), containing (in mM) NaCl, 120; KCl, 4.7; $MgSO_4.7H_2O$, 1.2; $CaCl_2$, 1.6; KH_2PO_4, 1.2; $NaHCO_3$, 25.0 (adjusted to pH 7.4); and glucose, 12.0. Ca^{2+}-free solutions were made by replacing all $CaCl_2$ from the standard saline solution and supplemented with 25 μM EGTA. Duodenal strips were incubated in aerated PSS at 37 °C in a water-jacketed 40 ml organ bath. Tissues were tied both ends with silk suture and secured to the bottom of the organ bath with a tissue holder, while the other end was attached to an isometric force transducer (TRI110, Letica, Spain).

2.2. Recording of isometric tension

The isometric tension of isolated duodenal strips was measured using a DC amplifier (Letica) 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 using the A/D card and visualised on a computer screen using a data acquisition and analysis program written by the author (PHYSCAN). Data were stored onto the hard disk for later analyses. Data were low-pass filtered at 10 Hz and analysed using computer routines included in the acquisition software.

2.3. Experimental procedure

After the duodenal strips were mounted in the bath, tissues were equilibrated at a resting tension value of 0.5 g. Muscle resting tension was readjusted every 5 min during the 30-min equilibration period. Bath solution was replaced every 20 min and the muscle strips were washed at least three times following the application of the drugs. The maximum contraction produced by 40 mM KCl at time 0 was recorded for each muscle strip at the beginning of each experiment to test the maximal contractile response and used to adjust the amplifier gain.

2.3.1. $CaCl_2$-induced contractions

In these experiments, tissues were first incubated in the presence of Ca^{2+}-free bathing solutions for 5 min. Progressive additions of small volumes of 1 M $CaCl_2$ were added to the bath at different times while the isometric tension responses were continuously recorded. To obtain the concentration–response curves for Ca^{2+} dependence, transient peak contractions were measured in response to graded concentrations of $CaCl_2$ added to tissues bathed under Ca^{2+}-free conditions. After each concentration was tested, the tissues were washed three times with Ca^{2+}-free solutions and left for 5 min before the next Ca^{2+} concentration was assayed.

2.3.2. Effects of antiestrogens on spontaneous activity

For this kind of assays, tissues were incubated in PSS and spontaneous activity was recorded for 10 min after the equilibration period. Antiestrogens were then added in small volumes (5–30 μl) directly to the bath solution and the time-course of muscle activity was recorded. Comparison of duodenal activity was assessed by frequency analysis of data segments taken just before the addition of the drug and also after the steady state of drug effects. In some experiments, the effects of carbachol or acetylcholine on contractile activity were assessed by adding 1 μM of the each agonist to the bath. This concentration was chosen as it elicited maximal contractile response in preliminary experiments (not shown).

2.3.3. Effect of antiestrogens on $CaCl_2$-induced contraction

Duodenal preparations were incubated in Ca^{+2}-free solutions for 10 min and then exposed to 3 mM $CaCl_2$. The resulting peak contraction (T_{test}) was used as a control value for subsequent effects. Afterwards, the solution was replaced with a Ca-free solution and left for 5 min to stabilise. Tissues were then exposed to different concentrations of antiestrogens at the desired concentration for another 5 min. At the end of this period, the peak contraction (T_{drug}) elicited by a second application of 3 mM $CaCl_2$ was measured. Bath solutions were then replaced and washed three times with fresh PSS (in which spontaneous activity was checked) and then left for another 10 min in the Ca^{2+}-free solution. Each drug concentration tested was preceded by a T_{test} measurement.

2.4. Statistics and mathematical analyses

One-way analysis of variance (ANOVA) and Student–Newman–Keuls *t*-test were used to determine differences between sample means. Values of $P < 0.05$ were considered significant. Results are expressed as mean ± S.E.M. Dose–response curves were fitted to logistic equation using non-linear regression analysis tools provided in SigmaPlot software (Jandel Scientific, San Rafael, CA). Frequency analysis was assessed using the FFT algorithm implemented in the acquisition software (PHYSCAN). Analyses were performed on 512 data segments taken from data windows of 3.41 min 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 smoothed to reduce their variance, and the ensemble average power spectrum was obtained.

2.5. Drugs

Tamoxifen, carbachol, EGTA 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). ICI182,780 (7-α-[9-[(4,-4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]-estra-1,3,5(10)-triene-3,17β-diol) and toremifene were provided from Astra Zeneca Pharmaceuticals (Madrid, Spain) and Farmos (Torku, Finland), respectively. 4-OH-Tamoxifen was purchased from Calbiochem (Bionova, Spain) and Iberiotoxin was obtained from Alomone Labs (Israel). Tamoxifen, 4-OH-tamoxifen and ICI182,780 were dissolved in ethanol and stored at 4 °C as 20 mM stock. Toremifene was dissolved in dimethylsulfoxide as 10 mM solution and freshly prepared for each experiment. Solvent concentrations in the bath never exceeded 0.1%.

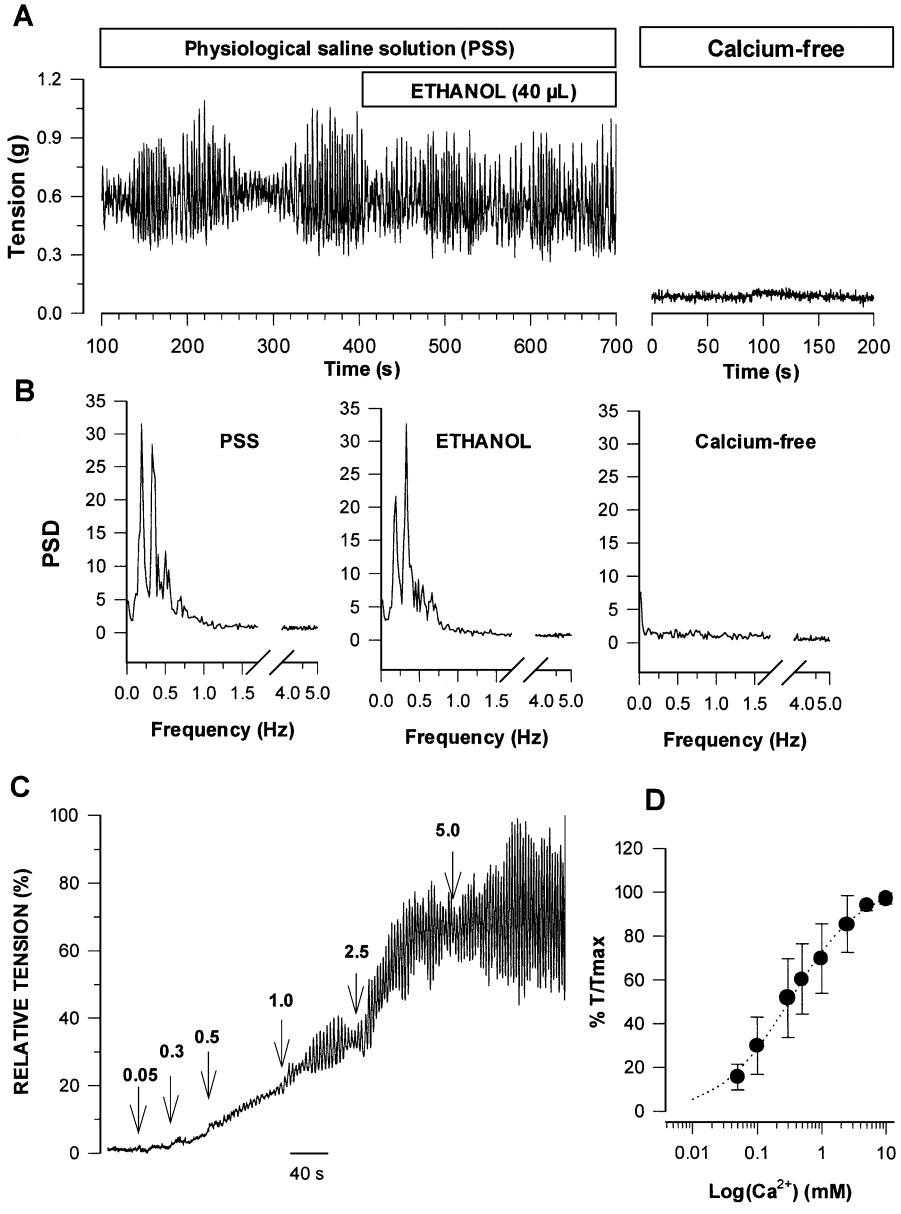

Fig. 1. Characterisation of duodenal contractile activity. (A) Recording of isometric tension in mouse duodenal muscle incubated in physiological saline solution (PSS). Spontaneous activity was not affected by the addition of maximal vehicle volume (ethanol, 40 µl) but was completely abolished in the absence of extracellular Ca^{2+}. (B) Fourier analysis of duodenal spontaneous activity determined under control conditions (left panel), in the presence of ethanol (middle panel) and after replacement of the normal PSS with a Ca^{2+}-free solution (right panel). Ordinates represent power spectral density (PSD) and abscises the frequency components (Hz). Abscises have been scaled to illustrate frequencies associated with contractile activity. (C) Original recording showing contractile responses induced by cumulative addition of $CaCl_2$. (D) Concentration–response curve for $CaCl_2$-induced contraction in mouse duodenum incubated in Ca^{2+}-free solution. The values are expressed as mean ± S.E.M ($n = 5$) for percentage fraction of 100% maximal activity.

3. Results

3.1. Spontaneous activity and $CaCl_2$-induced contraction

When bathed with standard PSS, all tissues studied displayed spontaneous peristaltic activity. Spontaneous activity was not affected by the addition of maximal volumes of vehicles dimethylsulfoxide or ethanol (Fig. 1A). Though some variability was evident between tissues, Fourier spectral analyses revealed that spontaneous activity was consistent with 1–3 main harmonic components corresponding to frequencies between 0.19 and 0.41 Hz, which were preserved in the presence of vehicles (Fig. 1B). The frequency spectrum obtained remained similar all

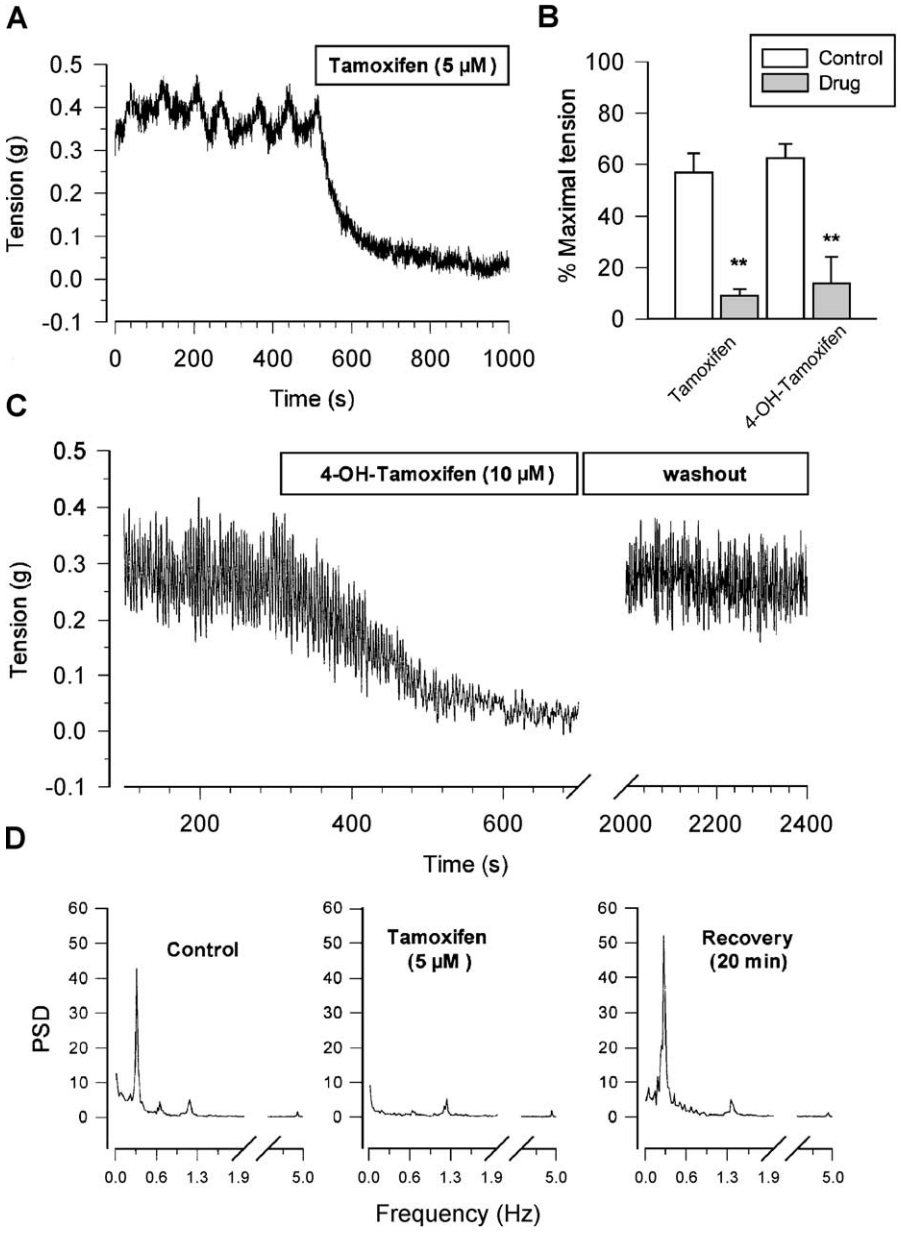

Fig. 2. Effects of triphenylethylene antiestrogens on duodenal activity. Representative traces for tamoxifen inhibition of spontaneous activity in isolated mouse duodenum. (B) Effects of antiestrogens (applied at 5 µM) on basal contractile tone. Results are expressed as mean ± S.E.M. of at least three preparations. ** Statistically different from control conditions with a probability value of $P < 0.01$. (C) Illustrative traces showing the reversibility of spontaneous activity inhibition by 4-OH-tamoxifen. (D) Spectral density of duodenal spontaneous activity determined under control conditions (left panel), 5 min after tamoxifen application to the bath (middle panel) and 20 min after tamoxifen washout (right panel). Ordinates represent 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 six preparations.

along the experiment with little variations over the ~ 3 h of each experiment. Both spontaneous activity and basal tone were entirely dependent on the presence of extracellular Ca^{2+} since calcium removal from the bath completely abolished spontaneous activity and relaxed duodenal muscle (Fig. 1A, right panel). In the absence of Ca^{2+}, the power density spectrum was notably affected and all frequency components were suppressed (Fig. 1B, right panel). Progressive addition of Ca^{2+} to the incubation bath resulted in increased basal tone and wider peristaltic amplitude (Fig. 1C). Experiments aimed at characterising $CaCl_2$-induced contractions were performed as stated in Materials and methods and the experimental data were fitted to a logistic function. These approach revealed that half-maximal activity (50% T/T_{max}) was obtained at 0.33 mM ($n=4$) and maximal activity observed around 3.0 mM. (Fig. 1D, right panel). From these analyses, a concentration of 3.0 mM was chosen as to achieve maximal Ca^{2+}-induced contraction.

3.2. Effects of antiestrogens on spontaneous activity

The effects of different chemically unrelated antiestrogens were assayed to explore their ability to alter spontaneous doudenal peristaltis. Nonsteroidal triphenylethylene antiestrogens tamoxifen, its hydroxy derivative, 4-OH-tamoxifen, and toremifene, inhibited spontaneous peristaltic activity (see Fig. 2A and C). Measurements of basal tone in response to tamoxifen and 4-OH-tamoxifen showed a significant reduction ($P<0.01$) compared to controls (Fig. 2B). The inhibitory effect was rapidly achieved within the next ~ 120 s after the exposure to micromolar concentration of the antiestrogen and was maintained for as long as 20 min at the least. The inhibitory effect of tamoxifen and derivatives was clearly reversible upon washout in PSS (Fig. 2C). Spectral analyses revealed that tamoxifen abolished all frequency components in the bandwidth of peristaltic activity observed in the control period which, in turn, reappeared after tamoxifen washout (Fig. 2D). Similar effects were observed for 4-OH-tamoxifen and toremifene. However, the pure steroidal antiestrogen ICI182,780 used at concentrations as high as 10 μM was unable to produce any appreciable change on duodenal contractile activity, but subsequent application of tamoxifen or 4-OH-tamoxifen to the same preparations readily relaxed duodenal muscle (Fig. 3).

3.3. Effect of antiestrogens on $CaCl_2$-induced contraction

Experiments designed to quantify the response of intestinal muscle to antiestrogens included the measuring of the response of tissues incubated in Ca^{2+}-free conditions to the addition of 3.0 mM $CaCl_2$. Maximal response was measured in control (T_{test}) and after 5-min incubation with the corresponding antiestrogen (T_{drug}). As can be seen in Fig. 4A, tamoxifen produced a concentration-dependent reduction of maximal response to Ca^{2+} addition, with maximal inhibition reached around 5 μM. The analyses of dose–response curves for triphenylethylene antiestrogens showed that tamoxifen was slightly more potent than 4-OH-tamoxifen, which displayed IC_{50} values of 0.85 and 1.60 μM, respectively (Fig. 4B). Toremifene was the least effective antiestrogen towards inhibition of Ca^{2+}-induced contraction and failed to completely relax duodenal muscle at concentrations as high as 7.5 μM.

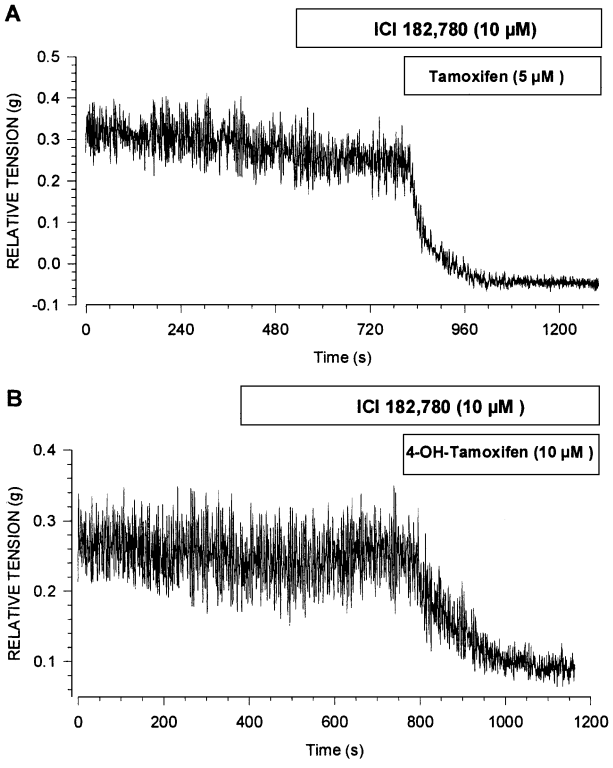

Fig. 3. Effects of steroidal antiestrogen ICI182,780. Typical recordings showing the absence of effects of steroidal antiestrogen ICI182,780 on the spontaneous activity. ICI182,780 failed to prevent the relaxation induced by tamoxifen (5 μM) (in A) or 4-OH-tamoxifen (10 μM) (in B).

3.4. Effects of KCl, K^+ channel blockers, acetylcholine and carbachol on antiestrogen-relaxed duodenum

In an attempt to elucidate the mechanisms underlying the relaxing action of triphenylethylene antiestrogens on duodenal smooth muscle, several experiments were designed to determine the effects of several agents known to affect smooth muscle activity on antiestrogen-relaxed tissues. Under control conditions, addition of 50 mM KCl induced a fast contraction followed by a slow decline leading to a contractile plateau. All phases in the response to KCl were blocked by the L-type Ca^{2+} channel antagonists verapamil or nifedipine (not shown). However, in the presence of tamoxifen, addition of KCl did not cause any appreciable

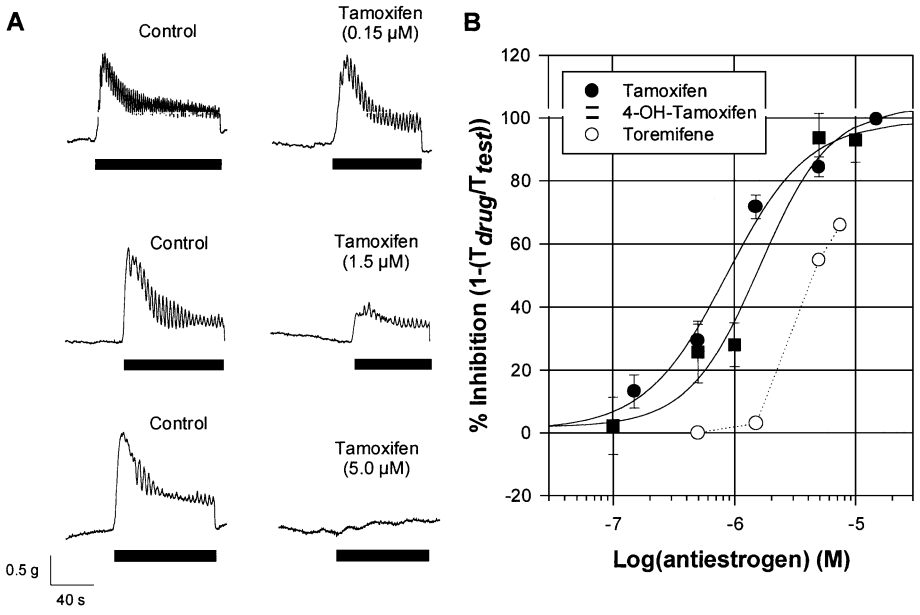

Fig. 4. Effects of nonsteroidal antiestrogens on $CaCl_2$-induced contractions. (A) Representative traces showing the inhibitory effect of tamoxifen on $CaCl_2$-induced contractions in isolated duodenal muscle strips. All traces were obtained from the same animal. (B) Cumulative concentration–response curves for tamoxifen, 4-hydroxi-tamoxifen and toremifene. Values are expressed as mean ± S.E.M. for percentage of reduction compared to 100% maximal tension in the test pulse. At least three different preparations were used for each concentration.

change of isometric tension (Fig. 5A). Similarly, addition of 5 mM barium chloride, a general K^+ channel blocker, or the Maxi K^+ channel blocker Iberiotoxin (50 nM), failed to reverse tamoxifen-induced relaxation (Fig. 5B). On the contrary, application of acetylcholine (1 μM) or the muscarinic receptor agonist carbachol (1 μM) to tamoxifen-treated duodenum was followed by a phasic peak contraction that returned to baseline after 10–30 s after the neurotransmitter pulse (Fig. 6A). This effect of CCH was similar to that elicited by CCH in tissues incubated in Ca^{2+}-free solutions (Fig. 6B,C).

3.5. Effects of BAY K8644 on antiestrogen-relaxed duodenum

Finally, we explored the effects of the L-type Ca^{2+} channel agonist BAY K8644 on the relaxation induced by tamoxifen. As can be observed in Fig. 7A, application of 1 μM BAY K8644 in the bath restored the peristaltic activity on tamoxifen-relaxed duodenum. The relaxing effect of tamoxifen could be completely counteracted by the addition of BAY K8644 since both basal tone and peristaltic amplitude returned to control values. This same response was observed for 4-OH-tamoxifen. Frequency analysis revealed that even in the continuous presence of tamoxifen, BAY K8644 restored main frequency components featuring spontaneous activity in the control period (Fig. 7B). The counteracting effects of BAY K8644 were inhibited and prevented by the addition of verapamil or nifedipine to the bath (not shown).

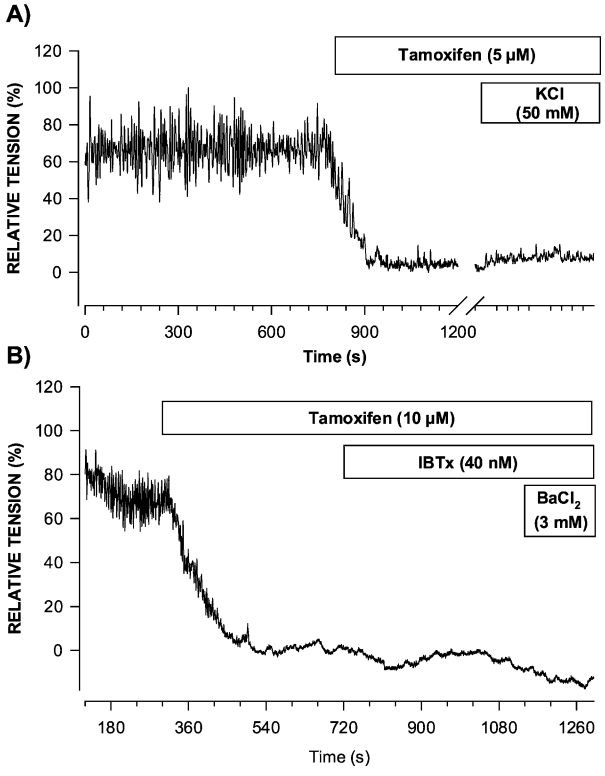

Fig. 5. Effects of KCl and K^+ channel blockers on tamoxifen-relaxed duodenal muscle. (A) Original recordings showing the effects of KCl-induced depolarization on tamoxifen-relaxed duodenal muscle. (B) Effects of K^+ channel blockade with iberiotoxin (IBTx, 40 nM) and $BaCl_2$ (3 mM) on antiestrogen-relaxed tissues.

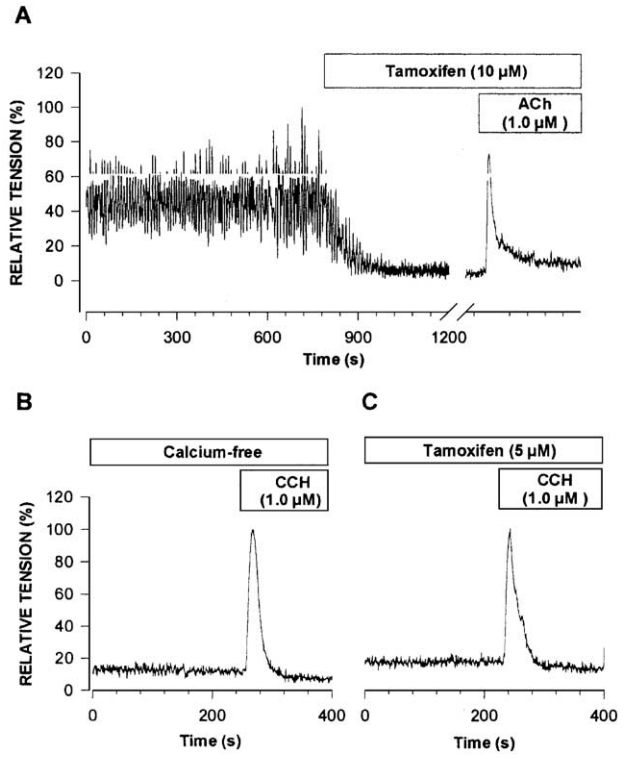

Fig. 6. Effects of acetylcholine and carbachol on tamoxifen-relaxed duodenal muscle. (A) Representative traces showing the effects of acethylcholine on tamoxifen-relaxed duodenal muscle. (B) Transient effects of carbachol (CCH, 1 μM) on muscle tension recorded under Ca^{2+}-free conditions. (C) Effects of CCH (1 μM) on antiestrogen-relaxed tissues. Traces are representative of four different preparations.

4. Discussion

The data presented here show that tamoxifen, 4-hydroxy-tamoxifen and toremifene, three nonsteroidal antiestrogens, are able to inhibit spontaneous activity and Ca^{2+}-induced contractions in mice duodenal muscle. Tamoxifen and 4-OH-tamoxifen are equally potent blocking Ca^{2+}-induced contraction while toremifene was one order of magnitude less potent and inhibited contraction incompletely at concentrations as high as 10 μM. The effects of tamoxifen and derivatives were rapidly achieved within the 2–4 min after antiestrogen exposure. The inhibition of contractile activity by antiestrogens was completely reversible upon washout of the drug. On the contrary, steroidal antiestrogens (ICI182,780) had no discernible effect on duodenal activity, this is noticeable since ICI182,780 has been long recognised as a pure antiestrogen by interacting with estrogen receptors (ER). This finding, together with the rapid relaxation of duodenal muscle by triphenylethylene antiestrogens, rule out the involvement of classic genomic pathways for the effects of tamoxifen reported here.

Cumulative evidence has unambiguously demonstrated that aside from their ability to bind estrogen receptor, triphenylethylenic antiestrogens are able to exert a number of non-genomic actions by modulating the function of several proteins, including signalling molecules and ion channels. In some cases, these interactions are considered to be responsible for some of its side effects in long-term thera-

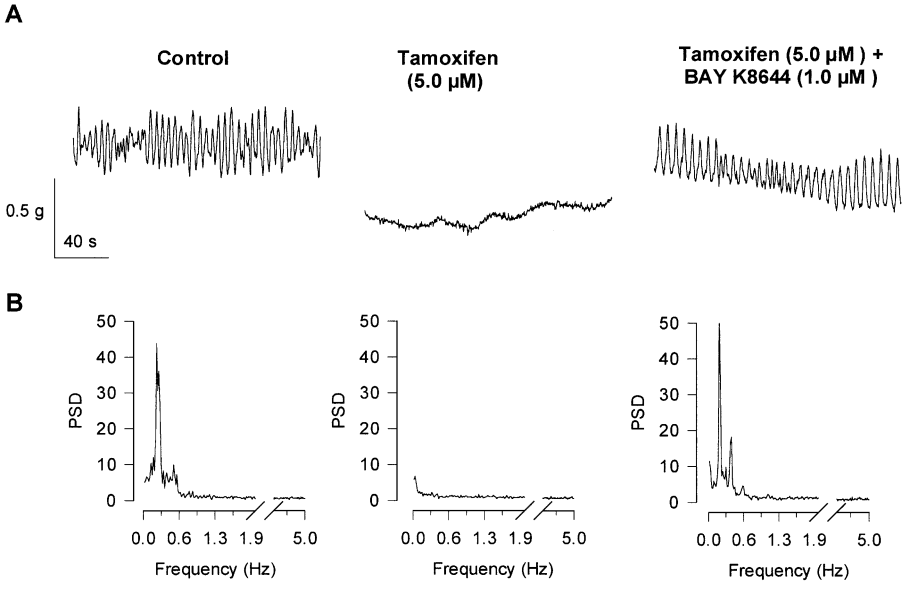

Fig. 7. Effect of BAY K8644 on tamoxifen-relaxed tissues. (A) Recording of the effects of BAY K8644 on tamoxifen-relaxed duodenal muscle. All traces were obtained in the same preparation in the control period (left), 5 min after the addition of tamoxifen to the bath (middle) and 3 min after the additive application of BAY K8644 (right). (B) Fourier analysis of duodenal spontaneous activity determined under conditions indicated above (in A). Ordinates represent power spectral density (PSD) and abscises the frequency components (Hz). Abscises have been scaled to illustrate frequencies associated with contractile activity. Traces are representative of three different experiments.

pies. Indeed, a cause–effect relationship for the interaction of tamoxifen with volume-regulated chloride channels is likely to underlie lens opacification in the development of cataracts (Zhang et al., 1994).

In order to investigate the mechanism(s) underlying the relaxing effect of tamoxifen and related antiestrogens, we used first an approach to explore the locus of tamoxifen action. Several studies have shown that some actions of tamoxifen are due to the inhibition of calmodulin (Lipton and Morris, 1986; Lopes et al., 1990). Thus, it has been demonstrated that the spasmolytic effect of tamoxifen on rat uterine myometrium 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 case of tamoxifen on mouse duodenum. The main evidence against a possible effect of tamoxifen through inhibition of calmodulin comes from the experiments using carbachol and acetylcholine. These experiments revealed that the contractile machinery remained functionally active after tamoxifen. Indeed, induction of Ca^{2+} release from intracellular stores by muscarinic stimulation of the duodenal muscle successfully elicited the peak contraction induced by IP_3 formation (Sims and Jansen, 1993), this response being identical to that obtained in the absence of extracellular Ca^{2+} in control tissues. Obviously, 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 tamoxifen.

The origin and propagation of excitation in gastrointestinal smooth muscle has been extensively investigated and it is now well established that a rhythmic electrical activity underlies synchronous intestinal muscle contraction (Szurszewski, 1987). This electrical activity consists of slow waves in membrane potential with superimposed action potentials (Szurszewski, 1987). In mouse small intestine, slow wave generation has been linked to the presence of a layer of interstitial cells of Cajal within the Auerbach's plexus (Thuneberg et al., 1995). Multiple electrode studies and visualisation of Ca^{2+} signals indicate that once excitation has been initiated, the slow waves and Ca^{2+} signals propagate rapidly through smooth muscle cells in the transverse (longitudinal muscle) and axial (circular muscle) directions (Stevens et al., 2000). The depolarisation–repolarisation phases of slow waves in smooth muscle cells reflect a balance between the activity of voltage-dependent Ca^{2+} channels and Ca^{2+}-activated K^+ channels (Lee et al., 1999).

Thus, given the fact that the duodenal contractile machinery remained operative after tamoxifen, we concentrated our experiments on a possible locus for tamoxifen actions on the plasma membrane. It has been reported that tamoxifen, at concentrations similar to those used in the present study, negatively modulates different types of K^+ channels in excitable cells (Hardy et al., 1998; Smitherman and Sontheirmer, 2001). However, it is well known that both K^+ channel blockade or depolarization by high extracellular K^+ causes a considerable increase of contraction force generated by gastrointestinal muscles (Meiss, 1987), hence, it can not be expected a blocking effect of tamoxifen on K^+ channels since it would result in a depolarization of the cells, which, in turn, would activate muscle contraction. The present data would be more compatible with a putative activation of K^+ channels and hyperpolarization of the smooth muscle cell.

In the human myometrium, tamoxifen induces a potent inhibition of spontaneous activity which was counteracted by either glibenclamide, an inhibitor of K_{ATP} channels, or by high external potassium chloride (Kostrzewska et al., 1997). Furthermore, recent studies on the modulation of Maxi $K_{(Ca)}$ channels in smooth muscle cells have shown that both estrogen and tamoxifen increase the channel open probability by interacting with the beta-1 subunit (Dick et al., 2001; Valverde et al., 1999), and it has been suggested that the activation of Maxi $K_{(Ca)}$ channels reduces the voltage-dependent Ca^{2+} influx and $[Ca^{2+}]_i$ through tonic hyperpolarization of smooth muscle cells (Lohn et al., 2001). However, in the present case, the fact that depolarisation of the duodenal smooth muscle using high KCl or the blockade of K^+ channels with barium and iberiotoxin did not induce any noticeable change in the recorded tension (see Fig. 5) strongly suggest that the effect of tamoxifen observed here was not related to activation of K^+ channels but rather to the coupling of the oscillating membrane potential and the voltage-dependent Ca^{2+} influx.

This hypothesis was tested by using the dihydropiridine derivative BAY K8644, a well-known Ca^{2+} channel agonist that increases the current through the L-type Ca^{2+} channels of nerve and muscle cells (Coruzzi and Poli, 1985; Schramm et al., 1983). Our present results demonstrate that inhibition by tamoxifen could be counteracted by reactivating Ca^{2+} influx through L-type Ca^{2+} channel with 1 μM BAY K8644 and, more interestingly, that the isometric tension developed by the isolated duodenum followed a peristaltic pattern, which exhibited a frequency spectrum similar to that present in control tissues before tamoxifen was added. This result strongly points to a blockade of L-type Ca^{2+} channels by tamoxifen being responsible for the spasmolytic action of triphenylethylene antiestrogens on mouse duodenum. Several other studies have also demonstrated that tamoxifen may inhibit Ca^{2+} entry through L-type Ca^{2+} channels in A7r5 and aortic smooth muscle cells (Song et al., 1996) isolated colonic myocytes (Dick et al., 1999), isolated rabbit detrusor (Ratz et al., 1999) and other

non-muscle cells like clonal pituitary cells (Sartor et al., 1988) and PC12 neurosecretory cells (Greenberg et al., 1987).

It has also been demonstrated in the isolated duodenal muscle that nifedipine and verapamil completely depressed the spasmogenic effect of BAY K8644 by competitively interacting with two different binding sites at the slow Ca^{2+} channel in duodenal muscle (Coruzzi and Poli, 1985). This is in agreement with our finding that verapamil (100 μM) completely blocked the activity induced by BAY K8644 in tamoxifen-relaxed tissues, which reinforces the hypothesis that triphenylethylene antiestrogens inhibit smooth muscle Ca^{2+} influx through L-type channels.

Although the precise site of action of triphenylethylene antiestrogens cannot be precisely ascertained from the present data, two pieces of evidence suggest that the main target for the actions of tamoxifen are the smooth muscle cells rather than Cajal's interstitial pacemaker cells. First, the fact that BAY K8644 restored basal tone and spontaneous activity shortly after exposure to the agonist and that this effect could be prevented with verapamil suggest the involvement of L-type Ca^{2+} channels being targeted by tamoxifen. It has been shown that unlike inward Ca^{2+} currents in small intestine smooth muscle cells, the depolarisation phase of Cajal's interstitial cells is insensitive to L-type Ca^{2+} channel blockers and abolished by hyperpolarization (Lee et al., 1999). Second, the analysis of the power density spectrum in the presence of BAY K8644 showed that the frequency components in the presence of the agonist were very similar to those observed in the control periods in the same tissues. This finding can only be explained if the mechanisms responsible for generating the pace within the Cajal's interstitial cells remained unaffected by tamoxifen. Nevertheless, further electrophysiological experiments on isolated duodenal Cajal's interstitial cells and smooth muscle cells will be required to ascertain such hypothesis.

Finally, the antagonistic properties of tamoxifen observed here may be of clinical significance. Chronic administration of therapeutic doses of tamoxifen (about 40 mg daily as adjuvant for breast cancer) give serum concentration increasing linearly with intake of tamoxifen averaging 4–6 μM at the higher dose levels (Trump et al., 1992). Moreover, tamoxifen is a lipophilic compound whereby its concentration in the plasma membranes may be even higher than in serum. In fact, tissue concentration of tamoxifen is approximately 10- to 60-fold higher than in serum (Lien et al., 1991). On the other hand, tamoxifen is metabolised in the liver and to produce the active metabolite 4-OH-tamoxifen, which according to our results is as effective as tamoxifen in relaxing duodenal smooth muscle. Thus, levels of tamoxifen and 4-OH-tamoxifen shown here to cause a significant reduction of Ca^{2+} channels are well within the range of tamoxifen concentration obtained in humans clinically and might provide a clue to explain the occurrence of gastrointestinal disorders in patients receiving high-dose tamoxifen therapies.

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