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Ethylbromide Tamoxifen, a Membrane-Impermeant Antiestrogen, Activates Smooth Muscle Calcium-Activated Large-Conductance Potassium Channels from the Extracellular Side

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ABSTRACT

Smooth-muscle calcium-activated large-conductance potassium channels (BK channels) are activated by tamoxifen and 17-β-estradiol. This increase in NP_o, the number of channels, N, multiplied by open probability, depends on the presence of the regulatory β1-subunit. Furthermore, a previous study indicated that 17-β-estradiol might bind an extracellular site on the β1-subunit. Because tamoxifen and 17-β-estradiol may share a common binding site, we hypothesized that tamoxifen activates BK channels through a site on the extracellular surface of the membrane. A membrane-impermeant analog of tamoxifen, ethylbromide tamoxifen, was synthesized and used to test this hypothesis in whole-cell, outside-out, cell-attached, and inside-out patches from canine colonic smooth muscle cells. Ethylbromide tamoxifen is positively charged and is therefore membrane-impermeant. In whole-cell experiments, ethylbromide tamoxifen increased K^+ current at potentials positive to +40 mV, which has previously been attributed to BK channels. Unlike tamoxifen, ethylbromide tamoxifen did not inhibit delayed rectifier current. In outside-out patches, ethylbromide tamoxifen increased BK channel NP_o with an EC_{50} value of 1 μM. Ethylbromide tamoxifen did not increase BK channel NP_o in cell-attached or inside-out patches; however, subsequent addition of equimolar tamoxifen did. Both drugs diminished BK channel unitary conductance to a degree that paralleled the effect on NP_o, suggesting an additional interaction with the pore-forming α-subunit. An interaction of tamoxifen with the pore was supported by a right shift in the concentration-response curve for tetraethylammonium; similar results were evident with iberiotoxin and charybdotoxin block. Our data suggest that ethylbromide tamoxifen does not easily traverse the plasma membrane and that tamoxifen binding responsible for activation of BK channels is at an extracellular site. The tamoxifen binding site may be within the extracellular loop of the BK channel β1-subunit or, alternatively, on an as-yet-unidentified mediator that has an extracellular binding site.

Tamoxifen ([*Z*]-1-[*p*-dimethylaminoethoxyphenyl]-1,2-diphenyl-1-butene) is a commonly used chemotherapeutic agent for the treatment and prevention of estrogen receptor-dependent cancers (Early Breast Cancer Trialists' Collaborative Group, 1998). In addition to binding the nuclear estrogen receptor (Jordan, 1976), tamoxifen has nongenomic effects including inhibition of protein kinase C (O'Brian et al., 1985) and calmodulin (Lam, 1984) activity. Tamoxifen also affects a variety of ion channels (Dick et al., 1999). For example, tamoxifen inhibits volume-sensitive Cl^-, voltage-activated Ca^{2+}, nonselective cation, and voltage-gated K^+ channels. In contrast to these inhibitory effects, tamoxifen increases the NP_o of BK channels that contain the regulatory β1-subunit (Dick et al., 2001; Dick and Sanders, 2001). The β1-subunit is expressed highly in, and may be limited to, smooth muscle cells (Tseng-Crank et al., 1996; Jiang et al., 1999). β1-Subunits combine with pore-forming α-subunits in a 1:1 ratio (Garcia-Calvo et al., 1994; Knaus et al., 1994a,b) and dramatically alter channel properties, including Ca^{2+}/voltage-sensitivity (McManus et al., 1995; Meera et al., 1996). The β1-subunit and its effects on Ca^{2+}/voltage-sensitivity make BK channels physiologically important modulators of smooth muscle tone (Brenner et al., 2000; Pluger et al., 2000). The β1-subunit also affects the pharmacological profile of BK channels (McManus et al., 1995; Hanner et al., 1997) including sensitivity to tamoxifen and 17-β-estradiol (Valverde et al., 1999; Dick et al., 2001; Dick and Sanders, 2001).

It is not known whether the tamoxifen and 17-β-estradiol binding site that regulates BK channels is on the β1-subunit or

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ABBREVIATIONS: BK channels, calcium-activated large-conductance potassium channels; NP_o, number of channels multiplied by open probability; BAPTA, 1,2-bis[2-aminophenoxy]ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEEDTA, *N*-2[2-hydroxyethyl]ethylenediaminetriacetic acid; ANOVA, analysis of variance; TEA, tetraethylammonium.

on an unidentified molecule that interacts the β1-subunit. Furthermore, it is unknown whether ethylbromide tamoxifen interacts with this putative binding site. A previous study suggested that the 17-β-estradiol binding site is extracellular, because a membrane-impermeant conjugate of 17-β-estradiol activated BK channels only from the extracellular side (Valverde et al., 1999). Ethylbromide tamoxifen and tamoxifen both antagonize the nuclear estrogen receptor; however, only tamoxifen, which can cross the plasma membrane, inhibits the estrogen-dependent proliferation of MCF-7 cells (Jarman et al., 1986). We synthesized ethylbromide tamoxifen ([*Z*]-1-[*p*-dimethylammoniumbromide ethoxyphenyl]-1,2-diphenyl-1-butene) and used it to determine at which side of the plasma membrane tamoxifen activates BK channels. Our data indicate that the regulatory β1-subunit, or an as-yet-unidentified mediator, functions as an extracellular receptor for estrogen and xenoestrogens (i.e., agents that have estrogenic properties but are not steroid hormones).

Materials and Methods

Canine colonic myocytes were isolated by digestion with collagenase as described previously (Dick et al., 1999, 2001). The care and use of animals followed the recommendations and guidelines of the National Institutes of Health and were approved by the University of Nevada Animal Care and Use Committee. Mongrel dogs of either sex were anesthetized with 20 mg/kg ketamine and 55 mg/kg nembutol and their abdomens were opened. The proximal colon was removed and placed in Krebs buffer that contained 125 mM NaCl, 5.9 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 15.5 mM $NaHCO_3$, 1.2 mM Na_2HPO_4, and 11.5 mM glucose, pH 7.4, when bubbled with 95% O_2/5% CO_2. Salts for Krebs and other solutions were purchased from Sigma (St. Louis, MO) and Fisher Scientific Co. (Fairlawn, NJ). In Ca^{2+}-free Hanks solution, the smooth muscle layer was dissected free of mucosa, submucosa, and longitudinal muscle. Ca^{2+}-free Hanks solution contained 125 mM NaCl, 5.36 mM KCl, 15.5 mM $NaHCO_3$, 0.336 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 10 mM glucose, 2.9 mM sucrose, and 11 mM HEPES, pH adjusted to 7.4 with NaOH. The circular muscle layer was treated with collagenase (345 units/ml; Worthington Biochemical, Freehold, NJ) for 30 min in Ca^{2+}-free Hanks at 37°C to produce suspensions of single cells. Dispersed myocytes were placed in a recording chamber atop an inverted microscope for electrophysiological studies.

For whole-cell experiments, smooth muscle cells were suffused with a solution containing 135 mM NaCl, 5 mM KCl, 2 mM $MnCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, and 5 mM Tris (tris-[hydroxymethyl]-aminomethane), pH 7.4. The pipette solution for whole-cell experiments contained 120 mM K-aspartate, 20 mM KCl, 5 mM Mg-ATP, 0.1 mM Na-GTP, 10 mM BAPTA, 10 mM HEPES, and 5 mM Tris, pH 7.1. Whole-cell pipettes were fabricated and heat-polished to tip resistances of 1 to 3 MΩ when filled with pipette solution. After forming a high resistance (GigaOhm) seal and then rupturing the membrane, series resistance and whole-cell capacitance were compensated >70% using the circuitry of the amplifier. Command voltages were adjusted for a 12-mV junction potential between the bath and pipette. Currents were recorded before and after the addition of ethylbromide tamoxifen.

For single-channel experiments, the bath solution contained 140 mM KCl, 10 mM HEPES, and 5 mM TRIS, pH 7.1. Ca^{2+} (0.24 mM) was added to this bath solution buffered with 1 mM EGTA to obtain 100 nM free Ca^{2+} (Bers, 1982) (MaxChelator, Pacific Grove, CA). One set of experiments (those in Fig. 7) was performed in outside-out patches dialyzed with a pipette solution containing 10 μM free Ca^{2+} (buffered with 1 mM HEEDTA) and suffused with physiological saline containing 5 mM K^+. The bath solution for these experiments contained 0.01% fatty acid-free albumin to prevent nonspecific binding of iberiotoxin and charybdotoxin (all from Sigma). GigaOhm seals were made on the membranes of myocytes with heat-polished pipettes that had tip resistances of 5 to 10 MΩ when filled with the KCl solution. BK channel currents were recorded from inside-out and outside-out patches in symmetrical 140 mM K^+. BK channel currents were also recorded in cell-attached patches, where the intracellular K^+ concentration is unknown but is assumed to be near 140 mM. BK channel NP_o was allowed to reach steady state after patch excision, and then NP_o was determined before and after the addition of ethylbromide tamoxifen (and tamoxifen). The brief representative traces in the figures show just 30 s of data; however, the average length of recording in each condition [i.e., control or drug(s)] was 3.0 ± 0.3 min (n = 50), totaling 411 min of recording.

An Axopatch 1D amplifier and CV-4 headstage were used for patch-clamp data acquisition (Axon Instruments, Union City, CA). Data were acquired in pCLAMP 5.5.1 (Clampex and Fetchex; Axon Instruments) by an IBM-compatible computer interfaced via TL-1 analog-digital converter. The digitization rate was four times greater than the low pass cut-off frequency for filtration (1 KHz). Data were analyzed using pCLAMP 6 (Clampfit; Axon Instruments) and ASCD (University of Leuven, Belgium) and expressed as mean ± S.E. of n cells. NP_o and single-channel conductance were determined by all-points amplitude histograms. Statistical analyses were made by paired or unpaired Student's t test, one-way repeated measures analysis of variance (ANOVA), and two-way repeated measures ANOVA (with post hoc analyses) as appropriate. The threshold for statistical significance was set at $p < 0.05$.

Tamoxifen was quaternized by the addition of ethyl bromide. The melting point of 167°C was consistent with the literature value (166–168°C; Allen et al., 2000), as determined on an electrothermal melting point apparatus and uncorrected. Thus, the purity of ethylbromide tamoxifen was estimated near 98%. Details of the ^{1}H NMR spectrum were also similar (Allen et al., 2000) where δ_H (360 MHz; $CDCL_3$): 0.94 (3H, t, J 7.4 Hz, CH_3CH_2C), 1.41 (3H, t, J 7.4 Hz, CH_3CH_2N), 2.47 (2H, q, J 7.4 Hz, CH_3CH_2C) 3.42 [6H, s, $N(CH_3)_2$], 3.77 (2H, q, CH_3CH_2N), 4.16 (2H, brs, OCH_2CH_2N), 4.35 (2H, brs, OCH_2CH_2N), 6.55 (2H, Abq, J 8.5 Hz, $C_6H_4OCH_2$, *ortho* H), 6.83 (2H, Abq, J 8.5 Hz, $C_6H_4OCH_2$, *meta* H), and 7.11–7.39 (10H, m, 2Ph). The ^{1}H NMR spectrum was determined in deuteriochloroform with tetramethylsilane as the internal standard at 360 MHz using a Bruker WM 360 spectrometer (Newark, DE).

Results

Two types of voltage-dependent K^+ currents were observed in canine colonic myocytes studied in the whole-cell patch-clamp configuration with physiological ion gradients (5 mM K^+ outside and 140 mM K^+ inside; Fig. 1A). Depolarizations to 0 mV activated delayed rectifier current, whereas greater depolarizations additionally activated BK current (Cole and Sanders, 1989). Myocytes were dialyzed with a pipette solution containing 10 mM BAPTA and no added Ca^{2+}. In addition, the bath was nominally Ca^{2+}-free. The calculated free intracellular Ca^{2+} concentration was approximately 2 nM (assuming 50 μM Ca^{2+} contamination from water and reagents), and the free BAPTA concentration was greater than 9.9 mM, providing a large buffer against Ca^{2+} changes. Thus, any potential effects of ethylbromide tamoxifen on K^+ current observed under these conditions probably would not be caused by changes in Ca^{2+}. Cells were held at −80 mV and stepped to +80 mV in 20-mV increments before and after the addition of 1 μM ethylbromide tamoxifen. Ethylbromide tamoxifen increased K^+ currents at potentials positive to +40 mV (Fig. 1A). Current at +80 mV was increased to 352 ± 44% of control (n = 6; $p < 0.005$ by paired Student's t test). This current, also activated by tamoxifen, is mediated by BK

channels (Dick et al., 1999, 2001; Dick and Sanders, 2001). Specifically, the current at potentials greater than +40 mV is mediated by large-conductance (>200 pS), Ca^{2+}/voltage-sensitive channels blocked by inhibitors of BK channels including 1 mM tetraethylammonium (TEA) and 50 nM charybdotoxin. Ethylbromide tamoxifen activated BK current whether cells were studied with the amphotericin-perforated (data not shown) or the conventional dialyzed patch technique, suggesting that the preservation of the intracellular signaling milieu was unnecessary. Unlike tamoxifen, however, ethylbromide tamoxifen had no effect on delayed-rectifier current (Fig. 1B). When cells were stepped from a holding potential of −80 to 0 mV, delayed-rectifier current was activated. Ethylbromide tamoxifen (1 μM) had no effect on current at this potential (99 ± 2% of control; $n = 6$). In contrast, 1 μM tamoxifen, added after ethylbromide tamoxifen was washed out, inhibited the delayed rectifier current to 24 ± 5% of the control value ($p < 0.001$ by one-way repeated measures ANOVA with Bonferroni post hoc analysis).

We tested the effect of 1 μM ethylbromide tamoxifen on BK channel NP_o in outside-out patches of canine colonic myocytes. Outside-out patches were obtained by making a GigaOhm seal, rupturing the membrane to gain whole-cell access, and then pulling the pipette from the cell. Both the bath and pipette contained 140 mM K^+ and 100 nM free Ca^{2+}. Positive voltages were applied to activate currents. BK channel currents were recorded before and after the addition of 1 μM ethylbromide tamoxifen. Ethylbromide tamoxifen increased NP_o and decreased single-channel conductance (Fig. 2A). Group data ($n = 13$) demonstrate a significant effect on both NP_o (Fig. 2B; 269 ± 42% increase; $p < 0.001$ by paired Student's t test) and single-channel conductance (Fig. 2C; 11 ± 1% reduction; $p < 0.001$ by paired Student's t test). The membrane-impermeant analog of tamoxifen activates BK channels and inhibits unitary conductance when the extracellular face is exposed.

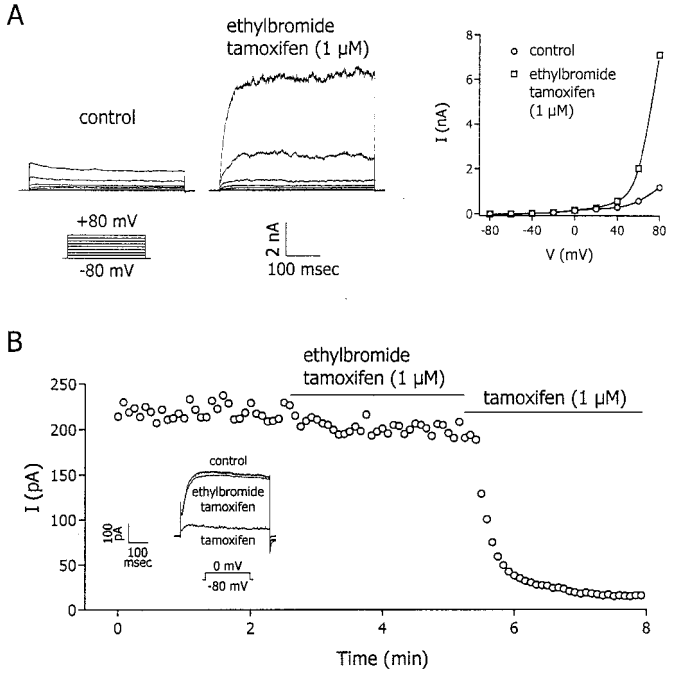


Fig. 1. Ethylbromide tamoxifen activates BK current but does not inhibit delayed rectifier current. A, whole-cell K^+ currents were measured in myocytes with a physiological K^+ gradient (5 mM K^+ outside and 140 mM K^+ inside). The pipette contained 10 mM BAPTA and no added Ca^{2+}, whereas the bath was nominally Ca^{2+}-free. Cells were held and −80 mV and stepped to +80 mV in 20-mV increments. The raw current traces on the left demonstrate that 1 μM ethylbromide tamoxifen activated outward current. The current-voltage relationship on the right shows that current at potentials positive to +40 mV was activated by ethylbromide tamoxifen. B, current at 0 mV is plotted versus time to demonstrate the effects of ethylbromide tamoxifen and tamoxifen on delayed rectifier current. Ethylbromide tamoxifen had little effect on current at 0 mV, whereas tamoxifen inhibited it. Representative current traces in the inset show little effect of ethylbromide tamoxifen on current at 0 mV, which is mediated predominantly by delayed rectifier channels. However, replacement of 1 μM ethylbromide tamoxifen with equimolar tamoxifen inhibited current.

We performed experiments to determine the concentration and time dependence for the effect of ethylbromide tamoxifen on BK channel NP_o. Currents were recorded from outside-out patches before and after the addition of five different concentrations of ethylbromide tamoxifen (from 0.1 to 10 μM). NP_o was steady after patch excision and increased rapidly with the addition of even low concentrations of ethylbromide tamoxifen (Fig. 2D). The effect of ethylbromide tamoxifen on NP_o was fully reversible upon washout (data not shown), as are the effects of tamoxifen and 4-OH tamoxifen we have reported previously (Dick et al., 2001; Dick and Sanders, 2001). The EC_{50} value for the effect of ethylbromide tamoxifen was 0.96 ± 0.24 μM ($n = 3$), very similar to the values we have reported previously for tamoxifen (0.65 μM) and 4-OH tamoxifen (0.87 μM) (Dick et al., 2001; Dick and Sanders, 2001).

The NP_o of BK channels under control conditions in outside-out patches was 0.38 ± 0.07 and increased to 1.18 ± 0.18 (Fig. 2, A and B; $n = 13$) with the addition of 1 μM ethylbromide tamoxifen. We also tested the effect of ethylbromide tamoxifen over a wider range of open probabilities (i.e., from zero to maximum) by constructing activation curves (Fig. 3). Outside-out patches were obtained from by the same methods and conditions described for Fig. 2. Membrane potential was held at 0 mV and stepped from +10 to +160 mV in 10-mV increments. Conductance was calculated from current and voltage, normalized, and fit with a Boltzmann sigmoidal function. Ethylbromide tamoxifen (1 μM) decreased conductance (Fig. 3A; 23 ± 5% reduction; $n = 4$; $p < 0.001$ by paired Student's t test). The activation curve under control conditions had a voltage of half-activation ($V_{1/2}$) of 101 ± 4 mV ($n = 4$; Fig. 3B). The addition of 1 μM ethylbromide tamoxifen shifted the $V_{1/2}$ to less positive potentials (86 ± 3 mV; $p = 0.01$ by Student's paired t test). These data indicate that ethylbromide tamoxifen activates BK channels in outside-out patches over a wide range of potentials and, thus, open probabilities.

We measured BK channels in cell-attached patches to determine whether 1 μM ethylbromide tamoxifen applied to the bath increased the NP_o of BK channels isolated inside the recording pipette. A GigaOhm seal was made on the membrane of myocytes and negative voltages were applied to activate BK channels. The bath contained 140 mM K^+ to nullify the membrane potential of the cell so that patch potential could be controlled. Ethylbromide tamoxifen did not activate BK channels in cell-attached patches (Fig. 4). The addition of 1 μM ethylbromide tamoxifen did not alter NP_o significantly (29 ± 12% increase; $n = 9$; $p > 0.50$ by one-way repeated measures ANOVA with Bonferroni post hoc analysis). Adding 1 μM tamoxifen to the bath did activate BK

channels significantly in cell-attached patches (324 ± 71% increase; $p < 0.001$ by one-way repeated measures ANOVA with Bonferroni post hoc analysis). Tamoxifen, but not ethylbromide tamoxifen, attenuated single-channel conductance (2 ± 1; $p > 0.70$ versus 10 ± 2%; $p < 0.001$ reduction for ethylbromide tamoxifen and tamoxifen, respectively; one-

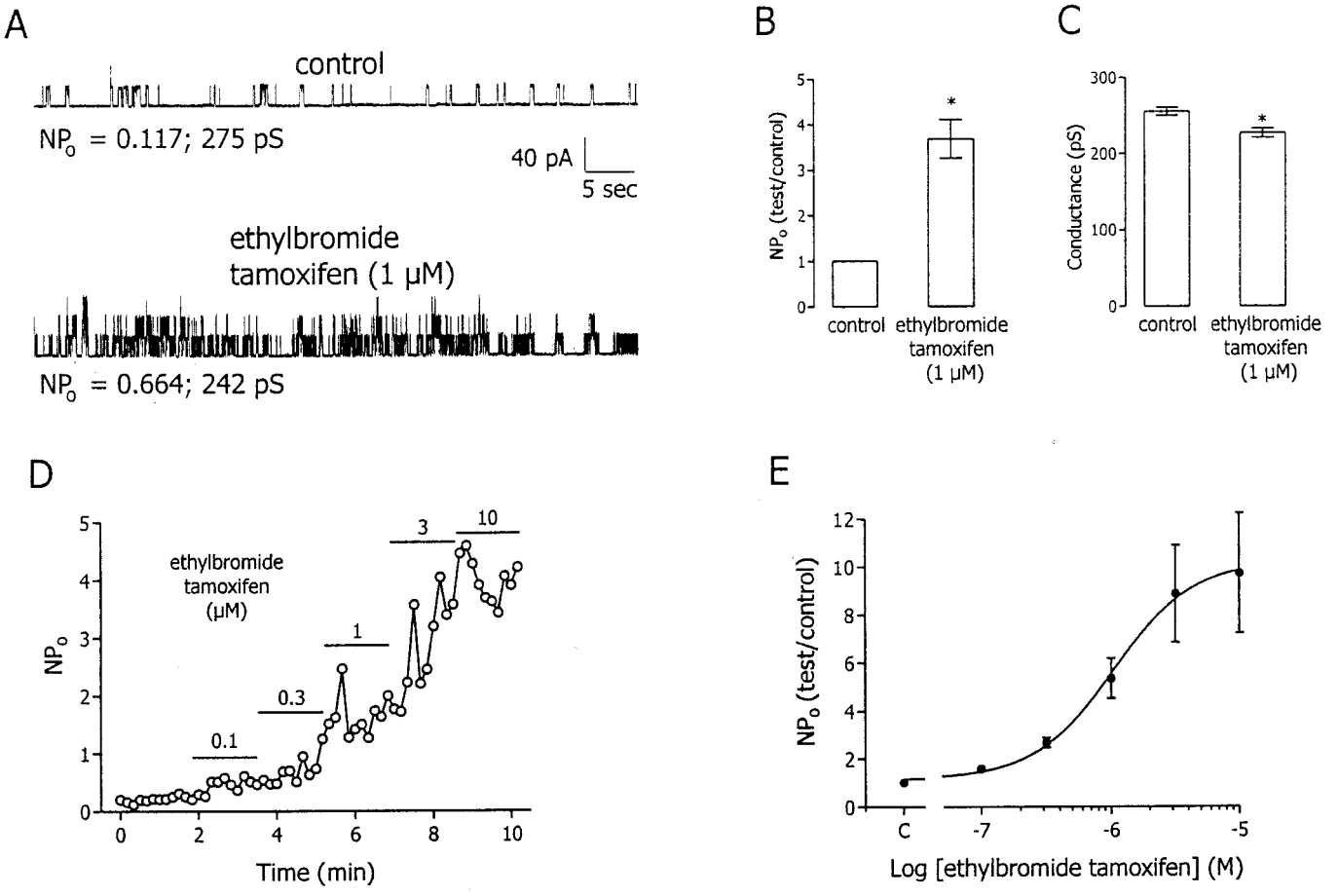


Fig. 2. Ethylbromide tamoxifen activates BK channels in outside-out patches. Single-channel BK currents were measured in outside-out patches in symmetrical 140 mM K^+ with 100 nM free Ca^{2+}. A, 30 s of representative channel activity at +60 mV. NP_o and single-channel conductance were measured before and after the addition of 1 μM ethylbromide tamoxifen. B, group data for the effect of 1 μM ethylbromide tamoxifen on NP_o ($n = 13$). *, significant increase in NP_o due to the addition of ethylbromide tamoxifen (paired Student's t test; $p < 0.0001$). C, 1 μM ethylbromide tamoxifen, added to outside-out patches, inhibited single-channel conductance ($n = 13$; $p < 0.0001$). D, time- and concentration-dependence of the effect of ethylbromide tamoxifen on BK channel NP_o. E, group data ($n = 3$) for the concentration-dependent effect of ethylbromide tamoxifen on BK channel NP_o ($EC_{50} = 0.96 \pm 0.24$ μM).

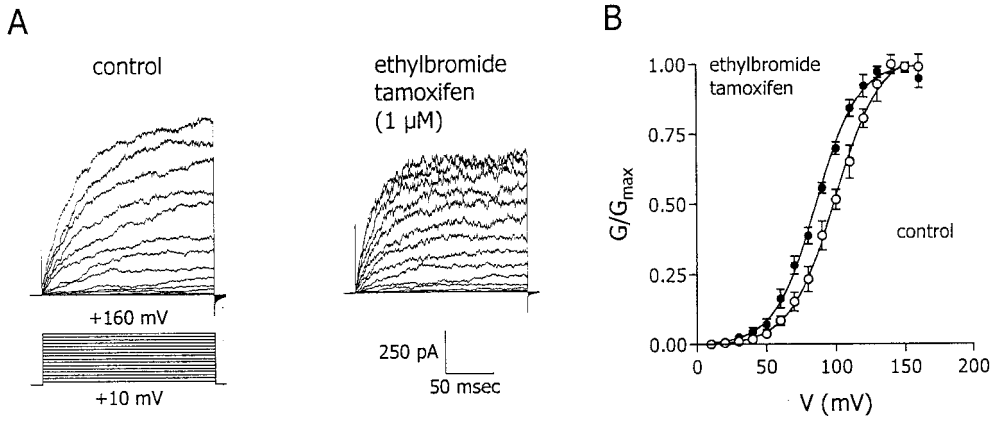


Fig. 3. Shift of BK channel activation curve by ethylbromide tamoxifen in outside-out patches. BK channel currents were measured in outside-out patches in symmetrical (140 mM) K^+ with 100 nM free Ca^{2+}. A, average current traces from five trials on the same patch before and after the addition of 1 μM ethylbromide tamoxifen. The patch was held at 0 mV and stepped from +10 to +160 mV in 10-mV increments. Ethylbromide tamoxifen (1 μM) decreased current. B, group data for the effect of 1 μM ethylbromide tamoxifen on BK channel activation ($n = 4$). Conductance was calculated from current and voltage, normalized to the maximum, and plotted. Ethylbromide tamoxifen (1 μM) shifted the curve significantly ($p < 0.05$ between +60 and +130 mV; two-way repeated measures ANOVA with Bonferroni post hoc analysis).

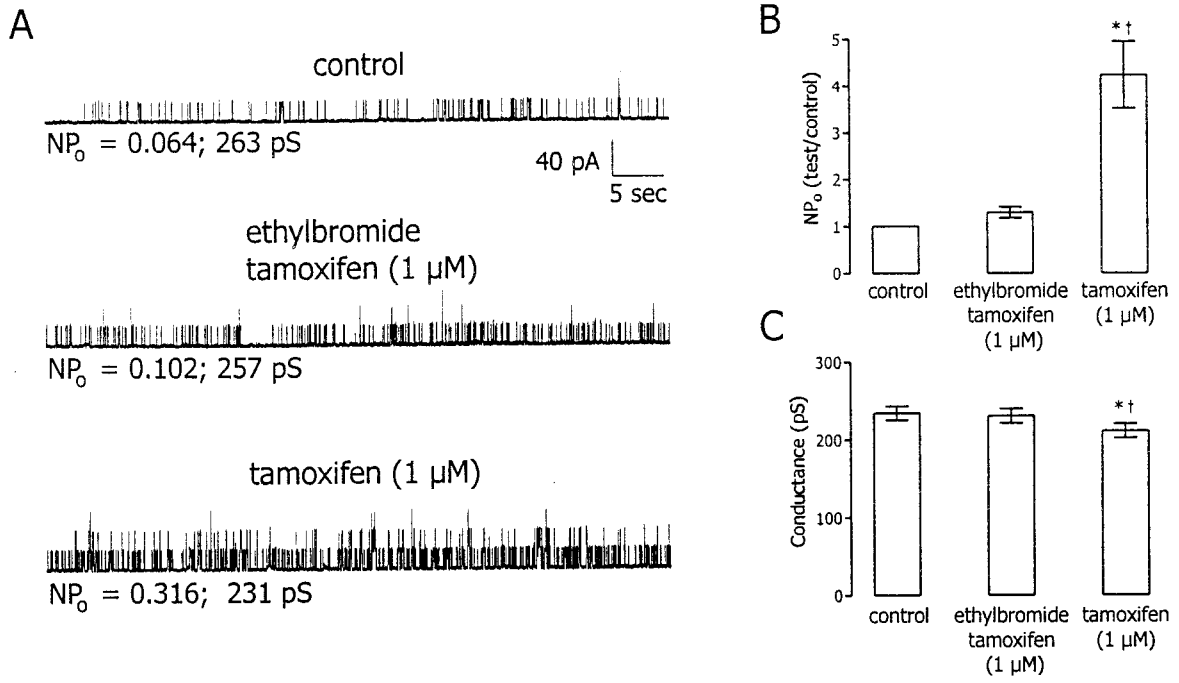


Fig. 4. Effect of ethylbromide tamoxifen on BK channels in cell-attached patches. Single-channel BK currents were recorded in cell-attached patches from canine colonic myocytes. The pipette and bath contained 140 mM K^+ and 100 nM free Ca^{2+}. NP_o and conductance were measured under control conditions, after the addition of 1 μM ethylbromide tamoxifen, and after the addition of 1 μM tamoxifen. A, representative 30-s traces of current. The patch potential was +80 mV. NP_o was slightly increased, whereas conductance was slightly decreased, by 1 μM ethylbromide tamoxifen. The subsequent addition of 1 μM tamoxifen, when ethylbromide tamoxifen was washed out, greatly increased NP_o and decreased conductance significantly. B, different effects of ethylbromide tamoxifen and tamoxifen on channel activity (n = 9). NP_o was normalized to that observed under control conditions. *, significant difference between tamoxifen and control; †, significant difference between ethylbromide tamoxifen and tamoxifen (one-way repeated measures ANOVA with Bonferroni post hoc analysis). C, effects of ethylbromide tamoxifen and tamoxifen on single-channel conductance. *, significant difference between tamoxifen versus control; †, significant difference between ethylbromide tamoxifen and tamoxifen (one-way repeated measures ANOVA with Bonferroni post hoc analysis).

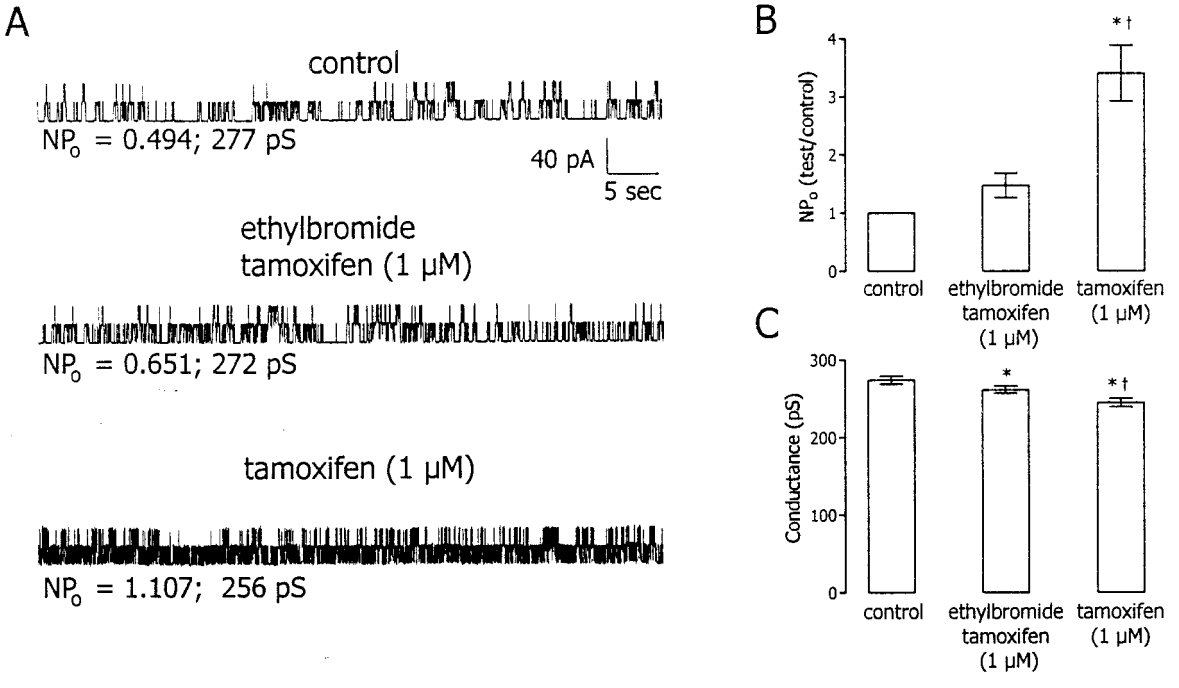


Fig. 5. Tamoxifen and ethylbromide tamoxifen have different effects on BK channels in inside-out patches. A, single-channel BK currents recorded from an inside-out patch at +80 mV in symmetrical 140 mM K^+ with 100 nM free Ca^{2+}. These representative current traces show 30 s of data. Ethylbromide tamoxifen (1 μM) increased NP_o; however, washout of ethylbromide tamoxifen and addition of 1 μM tamoxifen greatly increased BK channel NP_o. Both agents decreased BK channel unitary conductance, with tamoxifen having a greater effect. B, group data for the effect of 1 μM ethylbromide tamoxifen and 1 μM tamoxifen on the normalized NP_o of BK channels (n = 14). *, a difference between tamoxifen versus control; †, a difference between ethylbromide tamoxifen and tamoxifen (one-way repeated measures ANOVA with Bonferroni post hoc analysis). C, single-channel conductance was decreased slightly by ethylbromide tamoxifen and more greatly by tamoxifen. *, significant difference between both ethylbromide tamoxifen and tamoxifen versus control; †, significant difference between ethylbromide tamoxifen and tamoxifen (one-way repeated measures ANOVA with Bonferroni post hoc analysis).

way repeated measures ANOVA with Bonferroni post hoc analysis). The extracellular face of the membrane surrounding the channels is within the recording pipette; therefore, adding ethylbromide tamoxifen to the bath does not reach them, but tamoxifen does.

Inside-out patches were used to determine whether ethylbromide tamoxifen activated BK channels from the intracellular surface (Fig. 5). Inside-out patches were obtained from canine colonic myocytes by making a GigaOhm seal then pulling the patch from the cell. The solutions were 140 mM K^+ and 100 nM free Ca^{2+}. Negative voltages were applied to activate BK channels. Ethylbromide tamoxifen (1 μM) did not significantly increase NP_o (60 $\pm$ 22% increase; $p > 0.20$ by one-way repeated measures ANOVA with Bonferroni post hoc analysis; $n = 14$). Ethylbromide tamoxifen did, however, decrease single-channel conductance (5 $\pm$ 1%; $p < 0.001$ by one-way repeated measures ANOVA with Bonferroni post hoc analysis Fig. 5A). Adding 1 μM tamoxifen, after ethylbromide tamoxifen was washed out, increased NP_o and decreased single-channel conductance to a much greater extent (Fig. 5A). Tamoxifen increased BK channel NP_o 264 $\pm$ 42% (Fig. 5B; $p < 0.001$ by one-way repeated measures ANOVA with Bonferroni post hoc analysis; $n = 14$). Tamoxifen also decreased single-channel conductance to a greater extent than did ethylbromide tamoxifen (12 $\pm$ 2%; $p < 0.001$ by one-way repeated measures ANOVA with Bonferroni post hoc test). Thus, in inside-out patches, ethylbromide tamoxifen and tamoxifen have greatly different effects on BK channel NP_o and conductance.

For another comparison of the effects of ethylbromide tamoxifen and tamoxifen on BK channels in inside-out patches, we reversed the order of drug application. We added tamoxifen first, washed it out, and then replaced it with ethylbromide tamoxifen. Tamoxifen (1 μM) increased BK channel NP_o and decreased single-channel conductance (Fig. 6A). Replacement of tamoxifen with 1 μM ethylbromide tamoxifen returned NP_o and single-channel conductance toward the control value (Fig. 6A). Tamoxifen (1 μM) increased NP_o 228 $\pm$ 26% and decreased single-channel conductance 11 $\pm$ 2% ($n = 14$). The removal of tamoxifen and subsequent replacement with 1 μM ethylbromide tamoxifen returned NP_o to 145 $\pm$ 8% of control in 5 min ($n = 14$; Fig. 6B). With ethylbromide tamoxifen, single-channel conductance similarly returned to within 6 $\pm$ 1% of control ($n = 14$; Fig. 6C). The effect of tamoxifen on NP_o was significant ($p < 0.001$ by one-way repeated measures ANOVA with Bonferroni post hoc test) but that of ethylbromide tamoxifen was not ($p > 0.10$). The effects of both tamoxifen and ethylbromide tamoxifen on single-channel conductance were significant ($p < 0.001$ by one-way repeated measures ANOVA with Bonferroni post hoc test). These data, obtained with a reversed order of drug application, are very similar to those described above (Fig. 5).

Because ethylbromide tamoxifen decreased the current of outside-out macropatches (Fig. 3) and unitary conductance of BK channels (Fig. 2), we investigated a putative interaction of tamoxifen with the pore-forming α-subunit. This was accomplished by assessing the effect of tamoxifen on the TEA concentration-response curve (Fig. 7). TEA occludes the BK channel pore, reducing current. Outside-out patches of membrane were taken from canine colonic myocytes. The pipette

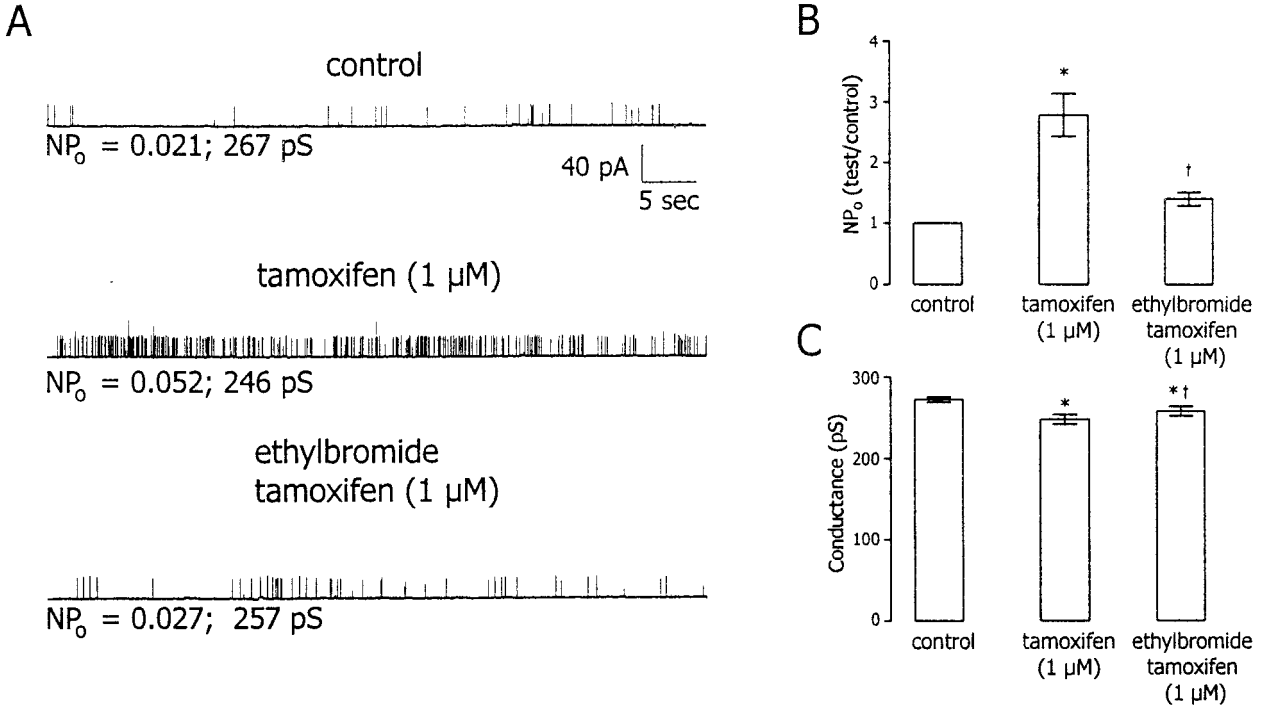


Fig. 6. Effect of sequential additions of tamoxifen and then ethylbromide tamoxifen to BK channels inside-out patches. A, single-channel BK currents recorded for 30 s from an inside-out patch at +80 mV in symmetrical 140 mM K^+ with 100 nM free Ca^{2+}. Tamoxifen (1 μM) increased NP_o; however, washout of tamoxifen and subsequent addition of ethylbromide with 1 μM tamoxifen reduced BK channel NP_o toward control. Both agents decreased BK channel unitary conductance, with tamoxifen having a greater effect. B, group data ($n = 14$) for the effect of 1 μM tamoxifen and 1 μM ethylbromide tamoxifen on BK channel normalized NP_o. *, difference between tamoxifen and control; †, difference between tamoxifen and ethylbromide tamoxifen (one-way repeated measures ANOVA with Bonferroni post hoc analysis). C, single-channel conductance was decreased by tamoxifen but less by ethylbromide tamoxifen. *, difference between tamoxifen and ethylbromide tamoxifen versus control; †, difference between tamoxifen and ethylbromide tamoxifen (one-way repeated measures ANOVA with Bonferroni post hoc analysis).

contained 140 mM K^+ with 10 μM free Ca^{2+}, whereas the bath solution contained 5 mM K^+ and was nominally Ca^{2+}-free. Patches of membrane were held at −80 mV and depolarized to +40 mV to activate current. Current was measured before and after the addition of TEA (0.01–10 mM). TEA inhibited current with an IC_{50} value of 0.14 ± 0.08 mM (n = 7; Fig. 7C). TEA was washed out and current returned to the control value. The TEA concentration-response curve was repeated in the presence of 1 μM tamoxifen, which decreased current itself. Tamoxifen shifted the TEA concentration response curve to the right (IC_{50} = 0.47 ± 0.05 mM; Fig. 7C). The IC_{50} values in the absence and presence of 1 μM tamoxifen were significantly different ($p < 0.001$ by Student's paired t test). We also tested the effect of tamoxifen on the block of BK channels by 10 nM charybdotoxin or iberiotoxin, specific peptide antagonists. These experiments were performed with bath solutions containing 0.01% fatty acid-free albumin to prevent nonspecific binding of the peptides. Tamoxifen reduced the degree of block produced by either 10 nM charybdotoxin or iberiotoxin (Fig. 7, D and E).

Discussion

Estrogen and xenoestrogens activate smooth muscle BK channels (Dick et al., 2001; Dick and Sanders, 2001; Valverde et al., 1999). The mechanism of action, as determined on recombinant BK channels in heterologous expression systems and in β1-subunit knockout mice, involves the regulatory β1-subunit. However, the exact site and mechanism of action are unknown. On the basis of a previous study using a membrane-impermeant conjugate of estrogen (Valverde et al., 1999), we hypothesized that tamoxifen activates BK channels through a site on the extracellular surface of the membrane. We used ethylbromide tamoxifen, which is a charged analog of tamoxifen and, therefore, membrane-impermeant, to test this hypothesis in whole-cell, outside-out, cell-attached, and inside-out patches from canine colonic smooth muscle cells. Ethylbromide tamoxifen increased whole-cell K^+ current at potentials positive to +40 mV but, unlike tamoxifen, did not inhibit delayed rectifier current. These data suggest that ethylbromide tamoxifen inhibits delayed rectifier K^+ channels from the intracellular surface; however, it is possible that delayed rectifier K^+ channels do not interact with ethylbromide tamoxifen in the same manner that they do with tamoxifen. Ethylbromide tamoxifen increased BK NP_o in outside-out patches, suggesting that there is an extracellular binding site. As we have demonstrated previously (Dick et al., 2001; Dick and Sanders, 2001), BK channel NP_o increased when 1 μM tamoxifen was added to inside-out patches; however, equimolar ethylbromide tamoxifen had no effect. Thus, the tamoxifen binding site of BK channels is likely to be on the extracellular side of the membrane, perhaps within the extracellular loop of the BK channel β1-subunit. As an alternative, the interaction may occur on an as-yet-unidentified mediator molecule that has an extracellular site.

Our findings also suggest that novel pharmacological targeting of the β1-subunit with xenoestrogens may be useful for treatment of diseases and conditions that involve smooth muscle, such as hypertension, myocardial ischemia, asthma, impotence, and constipation. This is because the β1-subunit is expressed highly in, and may be limited to, smooth muscle cells (Tseng-Crank et al., 1996; Jiang et al., 1999). Tamoxifen has effects on smooth muscle consistent with the activation of BK channels. Tamoxifen inhibits spontaneous and agonist-induced contractions of myometrium (Kostrzewska et al.,

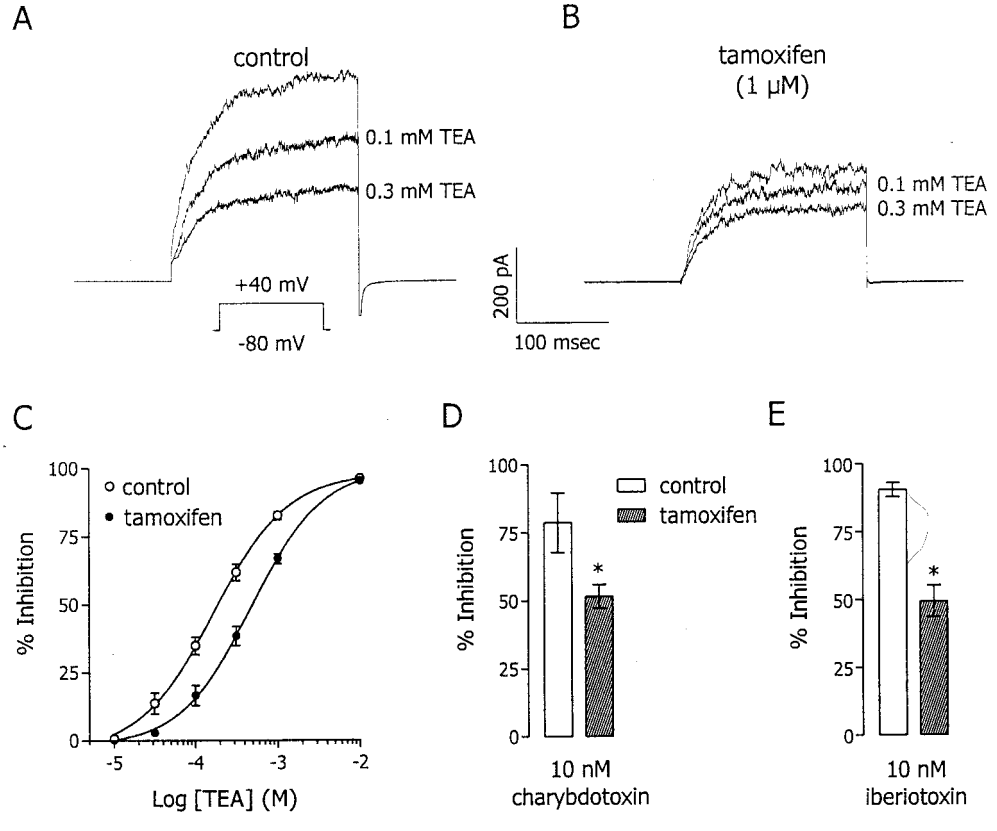
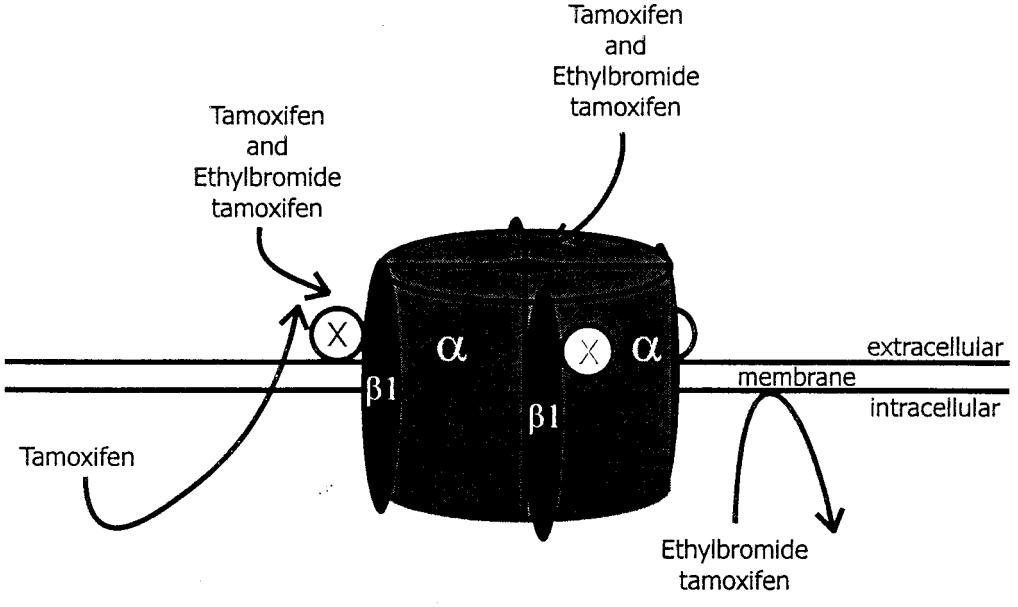


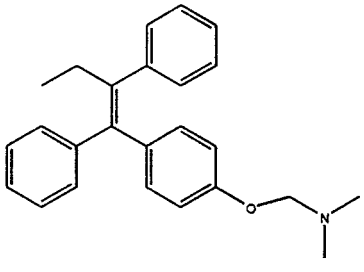
Fig. 7. Interaction of tamoxifen with the pore-forming α-subunit. A and B, effect of TEA on BK channel current before and after the addition of 1 μM tamoxifen. Both sets of traces are from the same outside-out patch dialyzed with 140 mM K^+ and 10 μM free Ca^{2+} and suffused with physiological saline containing 5 mM K^+. Under control conditions (A), TEA inhibited current in a concentration-dependent manner and was fully reversible upon washout. Tamoxifen (1 μM) decreased current by itself. The TEA concentration-response curve was repeated in the presence of 1 μM tamoxifen (B). C, group data (n = 7) for the effect of TEA before and after the addition of 1 μM tamoxifen, which shifted the curve significantly to the right (see text for details). D and E, group data (n = 3–5) for the effect of 10 nM charybdotoxin or iberiotoxin in the presence or absence of 1 μM tamoxifen. Tamoxifen decreased the effect of both peptide antagonists (*, $p < 0.05$ by Student's unpaired t test).

1997). Tamoxifen relaxes myometrial arteries (Marshall and Senior, 1987), increases uterine blood flow (Marshall and Senior, 1987), and hyperpolarizes and relaxes cerebral arteries (Nelson et al., 1997). Elevated extracellular K^+, which limits the degree to which openings of BK channels can hyperpolarize smooth muscle, inhibits the relaxing effect of tamoxifen on myometrial smooth muscle (Kostrzewska et al., 1997). However, it is important to realize that tamoxifen also affects other ion channels, including volume-sensitive Cl^-, L-type Ca^{2+}, and delayed rectifier K^+ currents in canine colonic smooth muscle (Dick et al., 1999). Tamoxifen also inhibits voltage-gated Na^+ (Hardy et al., 1998), L-type Ca^{2+} (Doughty et al., 1998; Dick et al., 1999), and nonselective cation channels (Allen et al., 1998; Welsh et al., 2000). Ethylbromide tamoxifen appears to be somewhat more specific, because it is known to inhibit only nonselective cation channels (5-HT_3 receptor) (Allen et al., 1998) without effect on delayed rectifier K^+ channels or volume-sensitive Cl^- channels (Sahebgharani et al., 2001). Thus, charged [xeno]estrogens may be potentially useful therapeutic agents that alter cellular excitability without genomic side effects.

Tamoxifen effects on ion channels may be responsible for some therapeutic side effects (e.g., for example Q-T interval prolongation and neurological symptoms) (Trump et al., 1992). The prolongation of the Q-T interval may be due to inhibition of cardiac delayed rectifier K^+ channels (Liu et al., 1998). Tamoxifen chemotherapy commonly causes facial flushing, reflecting a loss of vasomotor tone (Love et al., 1991). Such mechanism could be explained, in part, by the activation of vascular smooth muscle BK channels. This is similar to the effect of other xenoestrogens to reduce coronary vascular tone by inhibiting L-type Ca^{2+} channels and activating BK channels (Ruehlmann et al., 1998). It is possible, considering our results, that ethylbromide tamoxifen could be used as a more selective tool for targeting the BK channel β1-subunit in smooth muscle. For example, ethylbromide tamoxifen could be used to activate BK channels (and possibly hyperpolarize and relax smooth muscle) without interacting with nuclear estrogen receptors or inhibiting delayed rectifier K^+ channels.

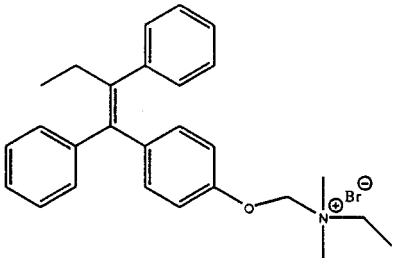
Although it is clear that tamoxifen and ethylbromide tamoxifen have effects on ion channels, the mechanisms of action are not yet clearly delineated. Only the integral role of the β1-subunit and a sidedness have been determined. Ethylbromide





Tamoxifen

[Z]-1-[p-dimethylaminoethoxy-phenyl]-1,2-diphenyl-1-butene



Ethylbromide tamoxifen

[Z]-1-[p-dimethylammoniumbromide ethoxy-phenyl]-1,2-diphenyl-1-butene

Fig. 8. Schematic of ethylbromide tamoxifen effects. Tamoxifen activates BK channels from the extracellular side. A cartoon schematic of a BK channel with α- and β1-subunits is shown. In addition, the possibility that an unidentified molecule (X) serves as the extracellular receptor is included. Tamoxifen and ethylbromide tamoxifen activate BK channels from the outside. However, tamoxifen, but not ethylbromide tamoxifen, activates BK channels when applied to the intracellular surface. Tamoxifen can freely cross the membrane. Both agents decrease single-channel conductance, an interaction with the pore. The structures of tamoxifen and its charged analog, ethylbromide tamoxifen, are shown.

tamoxifen has two effects on BK channels. First, ethylbromide tamoxifen activates BK channels. This is likely to be dependent on the presence of the β1-subunit (as the effect of tamoxifen is) and may be mediated directly by that subunit. Second, ethylbromide tamoxifen decreases the unitary conductance of BK channels, as does tamoxifen (Dick et al., 2001; Dick and Sanders, 2001). This probably represents a direct interaction with the pore-forming α-subunit, because we have demonstrated previously that tamoxifen attenuates single-channel conductance of α-subunits expressed in isolation of β1-subunits (Dick et al., 2001; Dick and Sanders, 2001). Furthermore, tamoxifen seems to interact with the pore, because it decreased the blocking effect of TEA, iberiotoxin, and charybdotoxin. Figure 8 shows a schematic of the effects of ethylbromide tamoxifen and tamoxifen on BK channels. Our findings indicate that the binding site for xenoestrogens is probably external and these compounds do not activate BK channels from the internal surface of the membranes. These findings give insight into BK channel structure and function, and define an extracellular (i.e., nongenomic) [xeno]estrogen receptor that regulates the properties of BK channels through the β1-subunit.

Acknowledgments

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