(Xeno)estrogen Sensitivity of Smooth Muscle BK Channels Conferred by the Regulatory β1 Subunit

A STUDY OF β1 KNOCKOUT MICE*

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Estrogen and xenoestrogens (i.e. agents that are not steroids but possess estrogenic activity) increase the open probability (P_o) of large conductance Ca^{2+}-activated K⁺ (BK) channels in smooth muscle. The mechanism of action may involve the regulatory β1 subunit. We used β1 subunit knockout (β1−/−) mice to test the hypothesis that the regulatory β1 subunit is essential for the activation of BK channels by tamoxifen, 4-OH tamoxifen (a major biologically active metabolite), and 17β-estradiol in native myocytes. Patch clamp recordings demonstrate BK channels from β1−/− mice were similar to wild type with the exception of markedly reduced Ca^{2+}/voltage sensitivity and faster activation kinetics. In wild type myocytes, (xeno)estrogens increase NP_o (P_o × the number of channels, N), shifted the voltage of half-activation (V_{1/2}) to more negative potentials, and decreased unitary conductance. These effects were non-genomic and direct, because they were rapid, reversible, and observed in cell-free patches. None of the (xeno)estrogens increased NP_o of BK channels from β1−/− mice, but all three agents decreased single channel conductance. Thus, (xeno)estrogens increase BK NP_o through a mechanism involving the β1 subunit. The decrease in conductance did not require the β1 subunit and probably reflects an interaction with the pore-forming α subunit. We demonstrate regulation of smooth muscle BK channels by physiological (steroid hormones) and pharmacological (chemotherapeutic) agents and reveal the critical role of the β1 subunit in these responses in native myocytes.

BK channels are large conductance Ca^{2+}-sensitive K⁺ channels (1, 2). These channels are members of the voltage-gated K⁺ channel superfamily (3) and products of a nearly ubiquitously, alternatively spliced gene (Slk, 4). Whereas the pore-forming α subunit has a wide tissue distribution, BK channel function varies greatly among cell types because of the addition of specific regulatory β subunits (for example, see Ref. 5). In smooth muscle, biochemical purification and reconstitution have shown that α subunits combine with regulatory β subunits in a 1:1 ratio (6–8). This combination of α and β1 subunits in smooth muscle is manifested functionally. Specifically, the β1 subunit imparts greater Ca^{2+}/voltage sensitivity (9, 10), making BK channels important modulators of smooth muscle excitability (11–14). The β1 subunit also functions in responses to various pharmacological agents, including charybdotoxin (15) and dehydroepiandrosterone I (9). Additionally, the β1 subunit may bind estrogens and xenoestrogens (i.e. agents that are not steroids but possess estrogenic activity). Valverde et al. (16) have shown that smooth muscle BK channels are activated by 17β-estradiol and proposed that this action is because of binding on the β1 subunit. We have recently shown that tamoxifen (TX) also activates BK channels (17). We demonstrated in a heterologous expression system (human embryonic kidney 293 cells) that the presence of the β1 subunit is required for the effects of tamoxifen and 17β-estradiol on recombinant BK channels (17). The importance of the β1 subunit in the response of native smooth muscle cells to (xeno)estrogens is unknown.

Recently, genetically engineered mice in which the β1 subunit has been deleted (β1−/−) have been produced (14), and these animals provide the opportunity to assess the role of the β1 subunit in the actions of (xeno)estrogens in native myocytes. β1−/− mice are hypertensive and demonstrate altered vascular reactivity (13, 14), illustrating the physiological importance of the β1 subunit. Pharmacological manipulation of the regulatory β1 subunit may become a novel approach to treating conditions such as hypertension, because the expression of the β1 subunit is thought to be limited to smooth muscle (18, 19).

In this study, we have sought to determine whether tamoxifen, 4-OH tamoxifen, and 17β-estradiol activate BK channels in native smooth muscle cells. 4-OH tamoxifen is a very potent anti-estrogen and may be the biologically active product in some tissues (20). However, aside from binding the nuclear estrogen receptor, very little is known about its mechanism(s) of action, particularly non-genomic mechanisms. In this study, we have used β1−/− mice to test the hypothesis that the regulatory β1 subunit is essential for the response of BK channels to (xeno)estrogens, including tamoxifen and its P450 metabolite, 4-OH tamoxifen.

MATERIALS AND METHODS

Murine colonic myocytes were isolated by enzymatic dispersion as described previously (21). The genotype and phenotype of the β1−/− mice have been characterized previously (14) and corroborated in a similar model by others (13). Controls for this study were wild type C57BL (“Black 6”) mice. This is the same strain from which the β1−/− mice were derived (14). Mice were anesthetized with chloroform and killed by cervical dislocation. The proximal colon was placed in Krebs solution and rinsed free of contents. Krebs contained (in mM) 125 NaCl, 5.9 KCl, 2.5 CaCl₂, 1.2 MgCl₂, 15.5 NaHCO₃, 1.2 NaH₂PO₄, 11.5 glucose, pH 7.4, with 95% O₂ and 5% CO₂. Salts for Krebs and other solutions were purchased from Sigma and Fisher Scientific. In Ca^{2+}-free Hanks’ solution, the smooth muscle layer was dissected free of...
muco and submuco. Ca2⁺-free Hank's solution contained (in mM) 125 NaCl, 5.36 KCl, 15.5 NaHCO3, 0.336 Na2HPO4, 0.44 KH2PO4, 10 glucose, 2.9 sucrose, 11 HEPES, pH 7.4 with NaOH. The muscle layers were treated with collagenase (345 units/ml, Worthington) in Ca2⁺-free Hank's solution at 37 °C to produce suspensions of single cells. Dispersed myocytes were placed in a recording chamber on an inverted microscope for electrophysiological studies.

After adhering to the glass-bottomed recording chamber, myocytes were suffused with a solution containing (in mM) 140 KCl, 10 HEPES, and 5 Tris, pH 7.1. Ca2⁺ was added to the bath solution buffered with 1 mM EGTA or N-2-hydroxyethyl)EDTA to achieve the desired free Ca2⁺ concentration (MaxChelator, Pacific Grove, CA) (22). Myocytes were approached with heat-polished pipettes having tip resistances of 2–10 megohm when filled with bath solution. BK channel currents were recorded from inside-out patches in symmetrical K⁺ or with an asymmetrical gradient (5 mM K⁺ in the pipette with a Na⁺ balance). An Axopatch 1D amplifier with a CV-4 headstage was used for data acquisition (Axon Instruments, Foster City, CA). Data were acquired in pCLAMP 5.5.1 (Clampex and Fetchex, Axon) by an IBM-compatible computer interfaced via a TL-1 analog-digital converter. The digitization rate was 4 times greater than the low pass cut-off frequency for filtration (1 kHz). Data were analyzed using pCLAMP 6 (Clampfit; Axon) and the Analysis of Single Channel Data program (University of Leuven, Leuven, Belgium). NP, and conductance were calculated from all-points amplitude histograms. Data are expressed as the means ± S.E. of n cells. Statistical analyses were made with the Mann-Whitney rank sum test on medians or Student’s t test on means as appropriate. The threshold for statistical significance was set as p < 0.05.

RESULTS

Currents were recorded from inside-out patches of membrane from murine colonic myocytes in symmetrical (140 mM) K⁺. NP, increased when the patch potential was made more positive or when Ca2⁺ concentration of the bath was increased (Fig. 1A). In myocytes from wild type mice, the slope conductance of BK channels in symmetrical K⁺ was 258 ± 5 pS (Fig. 1B, n = 6). The I-V relationship in asymmetrical K⁺ (5 mM pipette and 140 mM pipette) was outwardly rectifying, and conductance increased to 265 ± 6 pS at +80 mV (n = 3). The slope conductance of BK channels from β1−/− mice was 259 ± 6 pS in symmetrical K⁺ (Fig. 1B, n = 9). For β1−/− mice, BK channel conductance in asymmetrical K⁺ increased to 267 ± 13 pS at +80 mV (n = 3). These characteristics of BK channels in colonic myocytes from β1−/− mice were not significantly different from those of wild type mice. Furthermore, the characteristics of BK channels from β1−/− mice are similar to those attributed to the cloned mSlo α subunit (4) and of cerebral arterial myocytes from same animals (14).

Presence of the regulatory β subunit in smooth muscle BK channels confers greater Ca2⁺/voltage-sensitivity and slows gating kinetics, therefore, we tested the Ca2⁺ dependence of steady-state activation of BK channels from wild type and β1−/− mice as an index of the presence of the β1 subunit (23). Currents were measured from inside-out patches in symmetrical K⁺ with 100 mM free Ca2⁺ in the bath. Patches were held at 0 mV and depolarized in 10 mV increments until conductance was maximal (Fig. 1C). Currents activated with a different time course in myocytes from control and β1 knockout mice. The time constant of activation at +150 mV was 10 ± 2 ms in control myocytes (n = 6) and 4 ± 1 ms in myocytes from β1−/− mice (n = 10, p < 0.05). Conductance was normalized and plotted versus voltage (Fig. 1D). The V1/2 was 117 ± 3 mV in patches from control myocytes. In contrast, V1/2 was significantly greater in patches from β1−/− myocytes (151 ± 5 mV, p < 0.05). Thus, BK channels from the β1−/− mice demonstrated more rapid activation kinetics and reduced Ca2⁺/voltage-sensitivity.

Expression of canine Slo a and β1 subunits in human embryonic kidney 293 cells has revealed that tamoxifen activates cloned BK channels only in the presence of the β1 subunit (17). We tested whether tamoxifen activated BK channels in colonic...
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Tamoxifen shifted the voltage dependence of steady-state activation of BK channels to more negative potentials only in wild type mice (Fig. 3). Inside-out patches were studied in symmetrical 140 mM K+ and two Ca2⁺ concentrations (either 100 nM or 10 μM). The patch potential was held at 0 mV and stepped to different potentials to determine steady-state activation before and after the addition of 1 μM tamoxifen. Potential was made more positive until conductance was maximal, and then activation curves (i.e. normalized conductance versus voltage) were generated. In patches from control myocytes with 100 nM free Ca2⁺, the V1/2 was 118 ± 3 mV and shifted to 94 ± 2 mV by tamoxifen (n = 9). With 10 μM free Ca2⁺, the V1/2 was −52 ± 3 mV and shifted to −66 ± 3 mV by tamoxifen in control myocytes (n = 4). Increasing the free Ca2⁺ concentration, two orders of magnitude made V1/2 more negative, however, tamoxifen still caused a hyperpolarizing shift in V1/2. Tamoxifen decreased conductance by 29 ± 10 and 30 ± 12% in 100 nM and 10 μM free Ca2⁺, respectively (p < 0.05). In patches from β1−/− mice, tamoxifen did not change the voltage dependence of steady-state activation. With 100 nM free Ca2⁺, V1/2 was 152 ± 3 mV under control conditions and 153 ± 4 mV in the presence of tamoxifen (n = 12). With 10 μM free Ca2⁺ in patches from β1−/− mice, V1/2 was 34 ± 5 mV under control conditions and 31 ± 4 mV in the presence of tamoxifen (n = 8). However, tamoxifen did significantly decrease the conductance of channels from β1−/− mouse myocytes by 27 ± 4 and 28 ± 6% in 100 nM and 10 μM free Ca2⁺, respectively (p < 0.05, not different from patches from wild type mice).

The effects of tamoxifen were rapid and reversible, suggesting a non-genomic mechanism. Furthermore, the mechanism seems direct, as tamoxifen increased NPo in excised patches, free from cellular signaling mechanisms. The time course of activation by tamoxifen was studied in inside-out patches. The potential of patches from wild type and β1−/− mouse myocytes was held constant, and the bath-contained 100 nM free Ca2⁺. NPo and conductance were measured over 8-s intervals and plotted versus time (Fig. 4). In patches from wild type mice, tamoxifen increased NPo and decreased conductance in a reversible manner (Fig. 4A). NPo reached its maximum (3.5 ± 0.6-fold increase) in 83 ± 9 s (n = 3), and conductance simultaneously reached a minimum (12 ± 5% reduction). In contrast, in patches from β1−/− mice, tamoxifen decreased conductance (13 ± 3% reduction, n = 3) without affecting NPo (Fig. 4B). The effect on conductance was reversible. Washout commenced within 1 min of tamoxifen removal or approximately the time required to exchange the bath solution. In wild type mice after 5 min of washout, NPo and conductance returned to 94 ± 8% and 100 ± 1% of control, respectively (n = 3, p > 0.05).

In vivo metabolism of tamoxifen produces 4-OH tamoxifen, a very potent anti-estrogen, that may be the biologically active product in some tissues (20). However, aside from binding the nuclear estrogen receptor, very little is known about its mechanism(s) of action. To determine whether this hydroxylated P450 metabolite of tamoxifen interacts with the β1 subunit to increase BK channel NPo, currents were recorded before and after the addition of 1 μM 4-OH tamoxifen. In myocytes from wild type mice, 4-OH tamoxifen (1 μM) increased BK channel NPo (Fig. 5A) and decreased the single channel conductance 12 ± 3% (n = 9). In contrast, 4-OH tamoxifen had no significant effect on the NPo of BK channels from β1−/− mice (Fig. 5A). It did, however, decrease unitary conductance 13 ± 4% (n = 4). In vivo, most 4-OH tamoxifen is bound to proteins such as albumin, therefore, the amount of free 4-OH tamoxifen is reduced. To mimic this situation and determine whether binding to proteins might negate the effect of 4-OH tamoxifen, we added 20 mg/ml albumin to a solution containing 1 μM 4-OH tamox-
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Fig. 3. Tx shifts the voltage dependence of steady-state activation in the presence of the β1 subunit. A, current traces from a representative inside-out patch taken from a wild type mouse (symmetrical 140 mM K+ 10 μM free Ca2⁺). The patch was held at 0 mV and stepped from −120 to +30 mV under control conditions. The addition of 1 μM Tx decreased the current and shifted the V1/2 to more negative potentials. To measure steady-state activation in the presence of Tx, the patch was stepped from −140 to +10 mV. B, representative currents from an inside-out patch taken from a β1−/− mouse (conditions are the same as in A). Ca2⁺/ voltage sensitivity was reduced, therefore, the patch was stepped from −40 mV to +110 mV to measure activation. Tx (1 μM) decreased current but did not shift the V1/2. C, group data showing activation curves for wild type and β1−/− BK channels at 100 nM and 10 μM free Ca2⁺. Tx shifted the activation curves of wild type but not β1−/− BK channels.

Fig. 4. Rapid and reversible effect of Tx on NPo, depends on the presence of the β1 subunit. A, BK NPo and γ versus time in an inside-out patch taken from a control myocyte. The patch was held at +60 mV in a bath containing 100 nM free Ca2⁺ (symmetrical 140 mM K⁺). Application of 1 μM Tx increased NPo and decreased γ. These effects were reversible upon washout. B, BK NPo and γ versus time in an inside-out patch taken from a β1−/− mouse myocyte. Conditions were similar to those in A with the exception that the patch potential was +80 mV. Application of 1 μM Tx had no effect on NPo but decreased γ. This effect was reversible upon washout.

Fig. 5. Activation of BK channels by 4-OH tamoxifen requires the regulatory β1 subunit. A, group data showing that 4-OH tamoxifen (1 μM) increases the NPo of BK channels in myocytes from wild type (n = 9) but not β1−/− (n = 4) mice. Currents were recorded from inside-out patches of in symmetrical 140 mM K⁺ and 100 mM free Ca2⁺. B, the voltage dependence of steady-state activation is shifted to more negative potentials by 4-OH tamoxifen.

Tamoxifen and tested the effect on BK channel NPo. Albumin decreased the effectiveness of 4-OH tamoxifen, but NPo was still increased significantly (2.2 ± 0.6-fold increase, n = 4). 4-OH tamoxifen also shifted the activation curve of wild type BK channels to more negative potentials (Fig. 5B). In patches from wild type mice with 100 nM free Ca2⁺, the V1/2 was 109 ± 3 mV and shifted to 93 ± 5 mV by 1 μM 4-OH tamoxifen (n = 9). 4-OH tamoxifen decreased conductance by 30 ± 5% (p < 0.05). As a comparison to our previous results with tamoxifen (17), a parallel set of experiments was performed on myocytes from the canine colon. We constructed a complete concentration-response curve (5 concentrations between 100 nM and 10 μM) for 4-OH tamoxifen on BK channels in canine colonic myocytes. 4-OH tamoxifen increased BK channel NPo with an EC50 of 0.87 μM (n = 10), a value very similar to that observed previously with tamoxifen (0.65 μM, see Ref. 17).

Reconstitution of human and canine Slo α and β subunits in heterologous expression systems has indicated that 17β-estradiol activates BK channels only in the presence of the β1 subunit (16, 17). The regulatory β1 subunit may confer sensitivity to 17β-estradiol upon recombinant smooth muscle BK channels, however, this has not been determined in native myocytes. Thus, it is unknown what molecular components of the BK channel are necessary for activation by 17β-estradiol in native smooth muscle cells. We tested the effects of 17β-estradiol on native smooth muscle cells from wild type and β1−/− mice. 17β-estradiol (10 μM) activated BK channels from wild type mouse colonic myocytes (Fig. 6A). BK conductance was increased significantly (2.2 ± 0.69% p < 0.05, n = 7, Fig. 5B). In contrast to the effects observed in wild type myocytes, 17β-estradiol did not activate BK channels in colonic myocytes from β1−/− mice (Fig. 5A). Fig. 6B shows that although NPo was not changed by 17β-estradiol, BK conductance was decreased 3.34 ± 0.20% by 17β-estradiol (p < 0.05, n = 9). The activation of BK channels by 17β-estradiol, but not the reduction in conductance, depends on the presence of the regulatory β1 subunit.

DISCUSSION

We have shown that (xeno)estrogens activate smooth muscle BK channels, important regulators of smooth muscle tone
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Tamoxifen in vitro and in vivo are multifaceted and difficult to identify specifically.

The mechanism of action of tamoxifen and 17β-estradiol, determined on recombinant BK channels in heterologous expression systems, involves the regulatory β1 subunit (16, 17). However, such a mechanism had not been demonstrated in native smooth muscle cells. A wide variety of smooth muscle cells express the β1 subunit (18, 19), thus it has been difficult to test the importance of the β1 subunit in responses of native myocytes to (xeno)estrogens. Genetically engineered mice have provided the opportunity to test a β1 subunit-null smooth muscle cell type (13, 14). We tested the hypothesis that the regulatory β1 subunit was essential for the activation of native smooth muscle BK channels by tamoxifen, 4-OH tamoxifen, and 17β-estradiol. The data support the hypothesis and strengthen the idea that the regulatory β1 subunit functions as a membrane receptor for (xeno)estrogens that transduces the signal to effects on BK channels. The effects of (xeno)estrogens to increase BK channel NPo are non-genomic, as they were rapid, reversible, and occurred in cell-free patches that were removed from nuclear influences. These are well established characteristics of non-genomic mechanism of steroid hormone action (36). Whereas the β1 subunit of the BK channel is essential for conferring sensitivity to 17β-estradiol, tamoxifen, and 4-OH tamoxifen, the exact mechanism of action has yet to be elucidated, but several possibilities can be excluded from our studies and include changes in Ca2+-, nitric-oxide production, and changes in kinase activity.

Cellular signaling mechanisms are known to influence BK channel activity. Nitric-oxide activates BK channels directly (37) and indirectly via protein kinase G (38). Protein kinase A has also been shown to regulate the NPo of BK channels (39, 40). In this study, excised patch recordings were performed in solutions with clamped Ca2+ and in the nominal absence of substrates for cellular regulatory mechanisms that affect BK channel NPo (e.g., nitric-oxide production and phosphorylation via cGMP- and cAMP-dependent kinases). Therefore, (xeno)estrogens appear to directly activate BK channels. Our studies, however, do not rule out the possibility that these other mechanisms contribute to the effects of (xeno)estrogens in intact myocytes. If some of these other cellular signaling mechanisms contribute to the increase in NPo in intact myocytes, then our experiments have underestimated the full effect of (xeno)estrogens to increase BK NPo, as the total cellular response would be a summation of direct and indirect effects. Another critical reason why phosphorylation by protein kinase G is an improbable mechanism underlying BK activation by tamoxifen, 4-OH tamoxifen, and 17β-estradiol in the excised patches in our study is that a stimulatory protein kinase G phosphorylation site exists on the pore-forming α subunit (41), but our study shows that (xeno)estrogens have no effects on NPo in β1−/− mice. Additionally, the effects of tamoxifen are also not attributed to periodic fluctuations of NPo or “wanderlust” kinetics (24), because the responses to tamoxifen are concentration-dependent (17) and, as we have shown here, completely reversible. Regardless of any other potential influence or signaling mechanism, the β1 subunit is crucial for the effects of tamoxifen, 4-OH tamoxifen, and 17β-estradiol on BK NPo.

Tamoxifen, its 4-hydroxy metabolite, and 17β-estradiol produce effects on BK channels directly at the cell membrane, probably by binding the regulatory β1 subunit itself, however, this is yet to be confirmed. A previous study (16) has indicated that a membrane-impermeant conjugate of 17β-estradiol acts only from the extracellular surface, giving a sidedness to the putative binding site. We have obtained preliminary results...
indicating that a membrane-impermeant form of tamoxifen, i.e. ethylbromide tamoxifen, activates BK channels only from the extracellular surface.\textsuperscript{2} It is unknown whether the 17β-estradiol and xen estrogen binding sites are the same, however, pilot data from our lab indicates that they are the same as 17α-estradiol, an isomer without estrogenic properties, antagonizes the effects of both tamoxifen and 17β-estradiol.\textsuperscript{2} Furthermore, it is unknown whether the binding site is actually on the β1 subunit or on another unidentified effector molecule that acts through the β1 subunit. Neither this study, our previous work (17), nor the investigation of Valverde et al. (16) has determined the location of the binding site (xeno)estrogens. Regardless, the effects of 17β-estradiol, tamoxifen, and 4-OH tamoxifen to increase BK NP\textsubscript{a} represent a non-genomic mechanism of ion channel activation involving a regulatory subunit. Furthermore, our data suggest that wild type and β1\textsuperscript{−/-} mice could be used to test the effects of (xeno)estrogens on smooth muscle function in vivo and in vitro in the presence or absence of the BK channel regulatory β1 subunit. Thus, the contribution of BK channels to the effects of tamoxifen on smooth muscle reported previously (25–28) might now be determined and separated from the effects on other ion channels (29–32).

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