Dual Effect of Tamoxifen on Arterial KCa Channels Does Not Depend on the Presence of the β1 Subunit*

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Tamoxifen has been reported to directly activate large conductance calcium-activated potassium (KCa) channels through the KCa β1 subunit, suggesting a cardio-protective role of this compound. The present study using knock-out (KO) mice for the KCa channel β1 subunit was aimed at understanding the molecular mechanisms of the effects of tamoxifen on arterial smooth muscle KCa channels. Single channel studies were conducted in excised patches from cerebral artery myocytes from both wild-type and KO animals. The present data demonstrated that tamoxifen can inhibit arterial KCa channels due to a major decrease in channel open probability (P_o), a mechanism different from the reduction in single channel amplitude reported previously and also observed in the present work. A tamoxifen-induced decrease in P_o was present in arterial KCa channels from both wild-type and β1 KO animals. This inhibition was concentration-dependent and partially reversible with a half-maximal concentration constant IC50 of 2.6 μM. The effect of tamoxifen was actually dual in nature. Single channel kinetic analysis showed that tamoxifen shortens both mean closed time and mean open time; the latter is probably due to an intermediate duration voltage-independent blocking mechanism. Thus, tamoxifen block would predominate when KCa channel P_o is >0.1–0.2, limiting the maximum P_o, whereas a leftward shift in voltage or Ca2+ activation curves can be observed for P_o values lower than those values. This dual effect of tamoxifen appears to be independent of the β1 subunit. The molecular specificity of tamoxifen, or eventually other xenoestrogen derivatives, for the KCa channel β1 subunit is uncertain.

KCa1 channels play a fundamental role in regulating and maintaining arterial tone (1). Extensive work on the molecular biology of these channels has revealed that KCa channels exist in most tissues as heterodimers formed by two different subunits termed α and β. The α subunit contains the pore-forming region, the voltage sensor, and the Ca2+-binding site, whereas the β subunit (first discovered in smooth muscle, Ref. 2) has regulatory functions (3, 4). Previous works on expressed KCa channels have shown that the smooth muscle-specific β1 subunit subtype increases the apparent Ca2+ sensitivity of the channel, slows the activation and deactivation kinetics, and increases the sensitivity of the agonist dehydrosoyasaponin-I (5–7). The KCa channel β1 subunit has also been shown to have a crucial role as a molecular tuner for vasoregulation. Indeed, animals with gene-targeted disruption of the β1 subunit showed an impaired KCa channel function that leads to an increased arterial tone associated with symptoms of hypertension (8, 9). More recently, down-regulation of the KCa channel β1 subunit was shown to have a role in a model of acquired hypertension (10) as well as in a model of genetic hypertension (11). Conversely, a recently discovered polymorphism of the β1 subunit that promotes a gain of function of the KCa channel is associated with a low prevalence of diastolic hypertension in humans (12). Given the crucial role of the KCa channel β1 subunit in arterial smooth muscle, together with its highly smooth muscle-specific expression, the search for a specific KCa channel β1 subunit pharmacology has recently increased. Tamoxifen, a nonsteroidal triphenylethylene derivative, is predominantly known as a competitive antagonist at the estrogen receptor and is used as an effective prescribed drug in the treatment of hormone-responsive breast cancer (13). Tamoxifen and other xenoestrogen and estrogenic compounds have also been reported as direct activators of KCa channels in the micromolar range, suggesting a potential cardio-protective role of these compounds. This nongenomic effect of estrogen-related compounds has been attributed to the presence of the β1 subunit of the KCa channel (14–18). Tamoxifen, however, has also been shown to have inhibitory effects on several potassium conductances in the cardiovascular system, such as IK1, Ito, Isus, and HERG (19, 20). In KCa channels from canine colonic smooth muscle cells, tamoxifen and other derivatives were also reported to decrease KCa macroscopic currents, mainly due to a reduction in single channel conductance (15, 17). The present study shows that tamoxifen can activate or inhibit native arterial KCa channels, depending on the level of channel activity. This study also demonstrates, with the use of animals with a targeted deletion of the KCa channel β1 subunit gene, that the dual effect of tamoxifen on arterial KCa channels is not conferred by the β1 subunit in native cerebral artery myocytes.

MATERIALS AND METHODS

Cell Isolation—Murine arterial smooth muscle cells were isolated as previously described (8). Briefly, wild-type C57Bl/6 (Black 6) mice and β1 subunit KO mice were used in the experiments. Adult mice (25–35 g; 3–8 months old) of either sex were euthanized by peritoneal injection of pentobarbital solution (150 mg/kg). The genotype of the mice was confirmed by PCR analysis of DNA obtained from mouse tails or blood samples following euthanization of the animals. Cerebral arteries were carefully dissected on ice and then digested with papain (0.3 mg/ml papain and 1 mg/ml dithioerythritol for 8 min at 37 °C) and collagenase (1 mg/ml collagenase type F and type H in a 70%-30% mixture, respectively, incubated for 7 min at 37 °C). The digested tissue was triturated with a fire polished glass Pasteur pipette.
pipette to yield single smooth muscle cells. Cells were kept on ice until use.

**Single Channel Recordings**—Single channel currents were recorded from inside-out membrane patches of isolated arterial myocytes in symmetrical solutions containing 140 mM KCl, 10 mM HEPES (pH 7.2), 1 mM MgCl₂, 5 mM EGTA or HEDTA, and CaCl₂ free concentration of 10 μM. For Ca²⁺—activation curves, bath solutions contained 140 mM KCl, 1 mM EGTA (or HEDTA; [Ca²⁺] > 6), 10 mM HEPES, and 5 mM Tris base. Free Ca²⁺ concentration was adjusted with different amounts of CaCl₂ calculated with MaxChelator software (C. Patton, Stanford University). Final free Ca²⁺ concentration was measured with a Ca²⁺—sensitive electrode calibrated according to the manufacturer's instructions (World Precision Instruments, Sarasota, FL). Single channel activity was recorded at steady potentials indicated in the text with an Axopatch 200B amplifier (Axon Instruments, Union City, CA). Currents were filtered at 2–5 kHz and digitized at 20 kHz.

**Solution Exchange**—Fast solution exchange was performed with a multibarreled application pipette positioned in front of the patch pipette and switched with a remote-controlled piezoelectric device. Sub-second exchange time is normally achieved using this system (SF-77B Perfusion Fast Step; Warner Instruments, Hamden, CT).

**Chemicals**—All chemicals were obtained from Sigma and Calbiochem. All experiments were conducted at room temperature (20–22 °C). Tamoxifen was dissolved in MeSO. Control solutions contained this vehicle (1:10,000 MeSO).

**Data Analysis and Statistics**—Single channel data analysis was performed with Clampfit 9.0 (Axon Instruments). Curve fitting and linear regression analyses were performed with Origin 6.1 software (OriginLab Corp., Northampton, MA). Concentration-response relationship for tamoxifen inhibition was fitted to a Hill equation of the following form: Inhibiton \( \text{Hill} = \frac{\text{IC}_{50}}{1 + \left(\frac{[\text{Tamoxifen}]}{\text{IC}_{50}}\right)^{\text{Hill}}}, \) where Inhibition is 1 — Pₘₐₓ tamoxifen/Pₘₐₓ control. Pₘₐₓ is the open probability of the channel in control conditions or in the presence of tamoxifen as indicated, Inhibition max is the maximal inhibition obtained, IC₅₀ is the concentration of tamoxifen needed to obtain half-maximal inhibition, and Tamoxifen is the tamoxifen concentration used. Calcium activation curves were also fitted to a Hill equation of the following form: \( P = P_{\text{max}} \left(1 + \left(\frac{[\text{Ca}^{2+}]}{[\text{IC}_{50}\text{Ca}^{2+}]}\right)^{\text{Hill}}\right), \) where P is the open probability of the channel, Pₘₐₓ max is the maximal Pₘₐₓ, IC₅₀ is the concentration of Ca²⁺ needed to obtain half-maximal Pₘₐₓ, and [Ca²⁺] is the Ca²⁺ concentration used. N_Hill is the Hill coefficient and represents the slope factor of the Hill equation. Voltage activation curves were fitted with a Boltzmann equation of following form: \( P = \frac{P_{\text{max}}}{1 + \exp\left(-\frac{V-V_{1/2}}{\text{Slope}}\right)}; \) where Pₘₐₓ is the voltage for half-maximal activation, and Vₐ₅₀ represents the slope factor. Unless indicated, data are presented by mean ± S.E. from n number of cells. Statistical analyses were made with paired and unpaired Student’s t test, as appropriate. The threshold for statistical significance was set at p < 0.05. Statistical tests were run in Origin 6.1 and SigmaStat 2.03 (Systat Software Inc., Point Richmond, CA).

**RESULTS**

**Tamoxifen Inhibits Arterial Smooth Muscle KCa Channels in the Presence or Absence of the β₁ Subunit**—Fig. 1 shows KCa single channel activity recorded in excised patches at 10 μM free Ca²⁺ concentration. Application of 5 μM tamoxifen to the bath promoted two types of changes in KCa channels from WT animals that are illustrated in Fig. 1A: a) a pronounced decrease in channel activity where NPₘₐₓ changes from 0.75 to 0.3; and b) a moderate reduction in single channel amplitude (from 10 to 9.3 pA) that was previously reported (15, 17). Corresponding point histograms on the right illustrate both the change in activity and the small change in channel amplitude. These types of changes can also be observed in the absence of the β₁ subunit (KO). Fig. 1B shows KCa channel activity from KO animals recorded, in this case, at +40 mV. Tamoxifen (5 μM) induced a decline in NPₘₐₓ (from 1.8 to 0.32) with a concomitant slight reduction in single channel amplitude (from 9.25 to 8.8 pA) that can be also observed in the corresponding point histogram on the right. Fig. 1C summarizes the inhibitory effect of 5 μM tamoxifen on arterial KCa channel activity from both WT and β₁ KO animals. Tamoxifen promoted a significant decrease in NPₘₐₓ (Fig. 1C, left panel) with respect to control conditions in WT KCa channels (46 ± 13% reduction, p < 0.05, n = 5, paired Student’s t test) and also in β₁ KO KCa channels (49 ± 8% reduction, p < 0.05, n = 9, paired Student’s t test). Tamoxifen also induced a small reduction in the absolute single channel amplitude (Fig. 1C, right panel) from 9.97 ± 0.26 to 8.81 ± 0.26 pA in WT (control versus tamoxifen, respectively, p < 0.05, n = 5, paired Student’s t test) and from 9.86 ± 0.22 to 8.61 ± 0.34 pA in KO (control versus tamoxifen, respectively, p < 0.05, n = 6, paired Student’s t test). These findings showed that, besides a small reduction in channel amplitude, tamoxifen inhibition of KCa channel activity is the main inhibitory mechanism and that this inhibition is independent of the presence of the β₁ subunit.

**Tamoxifen Inhibition of KCa Channels Is Concentration-dependent and Partially Reversible**—Fig. 2A illustrates single channel activity at 10 μM Ca²⁺ and -40 mV holding potential from an inside-out patch containing three active channels from cerebral artery isolated myocytes from WT animals. KCa channel activity decreased with increasing concentrations of tamoxifen applied to the bath. Pₘₐₓ goes from 0.89 in control to 0.87, 0.71, 0.56, 0.40, 0.20, and 0.17 with increasing tamoxifen concentrations of 0.1, 1, 3, 5, 10, and 20 μM, respectively. Upon washout, the Pₘₐₓ is partially restored from 0.17 at 20 μM tamoxifen to 0.47 in washout. This reversibility was observed in three more independent experiments. Fig. 2B shows the concentration-dependent inhibition curve of WT KCa channels obtained from eight different patches at −40 mV and 10 μM Ca²⁺. Patches included in this curve lasted long enough to be assayed at least for three different concentrations of tamoxifen. The experimental points were fitted to a Hill function (continuous line) with an N_Hill of 1.47, suggesting more than one site for interaction between tamoxifen and the KCa channel protein. The half-maximal concentration constant (IC₅₀) obtained from the fit was 2.6 μM. Interestingly, analysis of the inhibition of single channel amplitude in the traces presented in Fig. 2A also shows a concentration-dependent behavior. The decay in single channel amplitude can be well fitted by a Hill function (data not shown). The values obtained for N_Hill and IC₅₀ were 1.26 and 2.5 μM, respectively, very similar to the values obtained for the inhibition of the Pₘₐₓ (1.47 and 2.6 μM, respectively, Fig. 2B). However, the maximum inhibition can only reach 20% compared with the 76% obtained for the Pₘₐₓ (Fig. 2B). These data indicate that tamoxifen inhibition of arterial KCa channels is largely due to a novel mechanism that is concentration-dependent and reversible and decreases the activity of the channel. In addition, a concentration-dependent small reduction of single channel amplitude can also be observed.

**Tamoxifen Can Have a Dual Effect, Depending on the Level of KCa Channel Activity**—To confront the present observations that tamoxifen reversibly inhibits KCa channels in a concentration-dependent manner with its well-documented activator effect on KCa channels (14, 15, 17), a series of experiments varying either voltage or free Ca²⁺ concentration were performed using arterial myocytes from WT animals. Fig. 3B shows for WT membrane patches the KCa channel voltage activation curve obtained from 12 different experiments in the presence or absence of 5 μM tamoxifen at a free Ca²⁺ concentration of 10 μM. The solid lines represent the fitting of the data to a Boltzmann equation (see “Materials and Methods”). Under these high Ca²⁺—concentration conditions, the maximum open probability (Pₘₐₓ max) is 0.82 ± 0.01, half activation voltage (V₁/₂) is −39 ± 2 mV, and the slope factor (dV) of the fitted curve is 23 ± 2 mV. Under these conditions, tamoxifen did not significantly change the V₁/₂ (−38 ± 4 mV, p > 0.7, unpaired Student’s t test) or the slope factor of the curve (25 ± 4 mV, p > 0.5, unpaired Student’s t test), but it did significantly reduce the Pₘₐₓ max of the WT KCa channels from 0.82 ± 0.01 to 0.49 ± 0.02.
Tamoxifen inhibits single KCa channel activity in cerebral artery myocytes from WT and β1 KO animals. A, left panels, single channel traces from an inside-out patch obtained before (Control) and after application of 5 μM tamoxifen to the bath solution. NPₒ of the patch was reduced from 0.75 to 0.3. Channels were held at -40 mV in symmetrical 140 KCl solutions at 10 μM free Ca²⁺. A, right panels, corresponding all point histograms illustrating the change in activity after tamoxifen inhibition. Single channel conductance was also reduced by tamoxifen from 10 pA in the control to 9.3 pA. c and o indicate the open and closed level of the channel, respectively. B, left panels, tamoxifen inhibition of single KCa channel activity in cerebral artery myocytes from β1 KO animals. c indicates the closed state of the channels, and o₁ and o₂ indicate open levels 1 and 2, respectively. Single channel traces from an outside-out patch containing two active channels, obtained in control conditions and after application of 5 μM tamoxifen to the bath solution. NPₒ of the patch was reduced from 1.8 to 0.32. Membrane patch was held at +40 mV in symmetrical 140 KCl solutions at 10 μM free Ca²⁺. B, right panels, corresponding all point histograms illustrating the change in activity after tamoxifen inhibition. Single channel conductance was also reduced by tamoxifen from 9.25 to 8.8 pA. C, summary data for the inhibitory effect of 5 μM tamoxifen on arterial KCa channel activity from both WT and β1 KO animals. Left panel, tamoxifen decreases NPₒ in WT KCa channels (46 ± 13% reduction, p < 0.05, n = 5, paired Student’s t test) and in β1 KO KCa channels (49 ± 8% reduction, p < 0.05, n = 9, paired Student’s t test). Right panel, tamoxifen reduces single channel amplitude from 9.97 ± 0.26 to 8.81 ± 0.26 pA in WT (control versus tamoxifen, respectively, p < 0.05, n = 5, paired Student’s t test) and from 9.86 ± 0.22 to 8.61 ± 0.34 pA in β1 KO (control versus tamoxifen, respectively, p < 0.05, n = 6, paired Student’s t test). Solid bars represent mean control values (black, WT; gray, KO). Diagonal filled bars represent mean values obtained in the presence of tamoxifen (white diagonals, WT; black diagonals, KO).

(control versus tamoxifen, respectively, p < 0.001, unpaired Student’s t test). If external Ca²⁺ was reduced to 100 nm, KCa channel activity decreased dramatically, and the number of events to determine the Pₒ of the channel with accuracy at hyperpolarized voltages was very low. Nonetheless, under these conditions, the tamoxifen activator effect can be detected.
at a holding potential of +80 mV, which is the maximum depolarized voltage tested without compromising the stability of the membrane patch. Under these conditions, the $P_\alpha$ of the KCa channel increases from 0.038 ± 0.01 to 0.11 ± 0.3 (control versus tamoxifen, respectively, $n = 7$, $p < 0.05$, paired Student’s $t$ test). The dual effect of tamoxifen was illustrated for comparison at +80 mV in the paired experiment shown in Fig. 3A, in which KCa channel activity was inhibited by 5 μM tamoxifen at 10 μM Ca$^{2+}$ concentration (from 0.6 to 0.43, control versus tamoxifen, respectively; 10 μM Ca$^{2+}$) and activated by 5 μM tamoxifen at 100 nM Ca$^{2+}$ concentration (from 0.016 to 0.075, control versus tamoxifen, respectively; 100 nM Ca$^{2+}$). Single channel amplitude also diminished in the presence of tamoxifen (from 17.3 to 15.5 pA, control versus tamoxifen, respectively; 10 μM Ca$^{2+}$). Similarly, at low Ca$^{2+}$ concentrations, single channel amplitude was also diminished by the presence of tamoxifen (from 20.5 to 17.3 pA, control versus tamoxifen, respectively; 100 nM Ca$^{2+}$).

The dual effect of tamoxifen on WT KCa channels was also studied in an experimental series involving inside-out patches held at a single membrane potential (+40 mV) and tested at different intracellular Ca$^{2+}$ concentrations. Rapid solution exchange (see “Materials and Methods”) was used to expose the cytosolic side of the channels to several Ca$^{2+}$ concentrations in the presence and absence of 5 μM tamoxifen. Recordings at concentrations of 100 and 300 nM Ca$^{2+}$ showed an increase in KCa channel activity with the increase in Ca$^{2+}$ concentration (2.1E-5 and 0.009, 100, and 300 nM Ca$^{2+}$, respectively). Addition of 5 μM tamoxifen increased $P_\alpha$ (0.0061 and 0.041) at both Ca$^{2+}$ concentrations (100 and 300 nM Ca$^{2+}$, respectively; 5 μM tamoxifen). The traces also show that tamoxifen was interacting with the channels present in the patch based on the reduction observed in single channel amplitude (from −10.4 to −8.4 pA, control versus tamoxifen, measured at 300 nM Ca$^{2+}$). When the Ca$^{2+}$ concentration was increased to 1 μM, channel activity still increased in control conditions with respect to the previous traces (at Ca$^{2+}$ concentrations of 100 and 300 nM), but tamoxifen promoted a decrease in channel activity (from 0.51 to 0.28, control versus tamoxifen, respectively; 1 μM Ca$^{2+}$). Fig. 4B shows Ca$^{2+}$ activation curves obtained for group data from four similar experiments in the presence and absence of 5 μM tamoxifen. Experimental points were fitted to a Hill equation represented by the solid lines. A crossover of the fitted Hill curves can be observed, indicating that the overall effect of tamoxifen is indeed dependent on the level of KCa channel activity. Tamoxifen diminished the $P_\alpha$ max from 0.75 ± 0.06 to 0.24 ± 0.02. (control versus tamoxifen, respectively, $p < 0.001$, unpaired Student’s $t$ test). Tamoxifen also changed the half-maximal activation constant from 700 to 270 nM intracellular Ca$^{2+}$ ($p < 0.001$, unpaired Student’s $t$ test). The $N_{max}$ for Ca$^{2+}$, however, remained −3 (2.7 versus 3, control versus tamoxifen, respectively, $p > 0.9$, unpaired Student’s $t$ test). The inset in Fig. 4B shows the same plot in a log-log scale to expand the changes observed at low Ca$^{2+}$ and low P$\alpha$ values. These data indicated that tamoxifen reduces the maximum $P_\alpha$ of the KCa channels, probably due to a channel block. In addition, tamoxifen also promoted a leftward shift in the Ca$^{2+}$ activation curve of the channels.

Single Channel Analysis Reveals That Tamoxifen Abbreviated Both Mean Closed Time ($\tau_c$) and Mean Open Time ($\tau_o$) in WT KCa Channels—Kinetic analysis of the effect of tamoxifen on WT KCa channels was performed in a subset of five experiments from Fig. 3B in which only one active channel was present in the membrane patches. Patches were studied at a Ca$^{2+}$ concentration of 10 μM at several holding potentials (from −80 to +80 mV). Fig. 5A (top panel) shows that in control conditions, single channel activity was evident at all the voltages studied. The addition of 5 μM tamoxifen to the bath solution promoted a decrease in single channel amplitude together with a decrease in single channel activity at voltages more positive than −40 mV. At more negative potentials (i.e. −60 and −80 mV), single channel openings appear to be more frequent. This change in activity is summarized in Fig. 5B (top right panel). Experimental points obtained in these five experiments were fitted to a Boltzmann function (solid lines). The plot shows that tamoxifen promotes a reduction in $P_\alpha$ max (from 0.84 ± 0.02 to 0.53 ± 0.01, control versus tamoxifen, respectively, $p < 0.001$, unpaired Student’s $t$ test) but does not significantly change $V_{1/2}$ (−40 ± 2 versus −30.5 ± 8 mV, control versus tamoxifen, respectively, $p > 0.2$, unpaired Student’s $t$ test). However, a decrease in the slope of the Boltzmann function could be detected with tamoxifen in this subset of experiments. Slope factor changed from 17 ± 2 to 33 ± 7 mV (control
Fig. 3. The dual effect of tamoxifen depends on the KCa channel activity level in excised patches from WT animals. A, left panels, channel activity recorded under control conditions at 10 μM and 100 nM internal Ca\(^{2+}\). The patch pipette was then exposed to the same solutions with 5 μM tamoxifen added. Channel activity diminishes with respect to control conditions at 10 μM Ca\(^{2+}\) (top traces) but increases with respect to the control at 100 nM Ca\(^{2+}\) (bottom traces). Arrows indicate the zero current level. B, voltage dependence of KCa channels at high (10 μM) and low (100 nM) Ca\(^{2+}\), before and after the addition of 5 μM tamoxifen. Top panel, P\(_o\) versus voltage plot at 10 μM Ca\(^{2+}\) under control conditions (●) and after the addition of 5 μM tamoxifen (○). Experimental points (n = 12 patches) were fitted with a Boltzmann equation (solid lines). Tamoxifen did not significantly change V\(_{1/2}\), which remains around −40 mV (±39 ± 2 mV in control and −38 ± 4 mV with tamoxifen). Tamoxifen mainly produces a reduction of P\(_{\text{max}}\) (from 0.82 ± 0.01 to 0.49 ± 0.02, p < 0.01). Slope factor remains similar with 22 ± 3 versus 25 ± 4 mV, control versus tamoxifen, respectively. Bottom panel, at 100 nM Ca\(^{2+}\), tamoxifen promotes a leftward shift in the V\(_{1/2}\) from 153 ± 25 to 127 ± 5 mV (n = 5 patches) with no changes in the slope (22.8 ± 7 versus 22.9 ± 2 mV, control versus tamoxifen, respectively).

Fig. 4. Ca\(^{2+}\) dependence of the WT KCa channel under control conditions and after addition of 5 μM tamoxifen at +40 mV. A, representative traces illustrate channel activity at several Ca\(^{2+}\) concentrations. Under control conditions (left panels), P\(_o\) values were as follows: 2.1E-5, 0.009, and 0.51 (100 nM, 300 nM, and 1 μM Ca\(^{2+}\), respectively). After addition of 5 μM tamoxifen, P\(_o\) changes to 0.0061, 0.041, and 0.28, (100 nM, 300 nM, and 1 μM Ca\(^{2+}\), respectively). A reduction in single channel amplitude is also observed (e.g. from −10.4 to −8.4 pA, control versus tamoxifen, measured at 300 nM Ca\(^{2+}\)). B, Ca\(^{2+}\) activation curves obtained for group data from four experiments in control (●) and in the presence of 5 μM tamoxifen (○). Experimental points were fitted to a Hill equation (continuous lines). Tamoxifen diminished the maximal activation from 0.7 to 0.24. Tamoxifen also changes the IC\(_{50}\) value from 700 to 270 nM Ca\(^{2+}\). The Hill coefficient remained ~3 (2.7 versus 3, control versus tamoxifen, respectively). The inset shows the same plot in a log-log scale.
versus tamoxifen, respectively, $p < 0.05$, unpaired Student’s $t$ test). This change most likely reflects the combined block and activation mechanisms of tamoxifen. Single channel amplitude was also reduced by tamoxifen (Fig. 5B, top left panel). Slope conductance changed from $227 \pm 0.001$ to $194 \pm 0.002$ picosiemens (15% reduction, control versus tamoxifen, respectively, $p < 0.001$, unpaired Student’s $t$ test). Under control conditions, both $\tau_c$ and $\tau_o$ were found to vary with voltage. Fig. 5B (bottom left panel) shows that $\tau_c$ can be well described by the exponential function of the following form: $\tau_c = \tau_c(0) \exp(-V/C)$ (solid lines), where $\tau_c(0)$ represents the mean closed time constant at 0 mV, and C is a slope factor (in mV). Under control conditions, $\tau_c$ decreased e-fold by 26 mV, whereas in the presence of tamoxifen, $\tau_c$ decreased e-fold by 40 mV. Analysis of the kinetic changes promoted by tamoxifen in this experimental series revealed that tamoxifen-induced reduction in $\tau_c$ becomes significant at $-80$ mV (from $29.6 \pm 5$ to $11.6 \pm 3$ ms, control versus tamoxifen, respectively, $p < 0.05$, unpaired Student’s $t$ test). Tamoxifen, however, did not change $\tau_o(0)$ (1.3 ± 0.4 versus 1.5 ± 0.7 ms; control versus tamoxifen, respectively, $p > 0.8$, unpaired Student’s $t$ test). On the other hand, $\tau_o$ increased linearly with depolarization from 1.9 ms at $-80$ mV to 4.3 ms at $+80$ mV, with a slope from the linear regression (solid line) of 0.015 ms/mV (regression coefficient $r = 0.99$, $p < 0.001$). Addition of tamoxifen almost abolished the voltage dependence of $\tau_c$ that remained around 1.6 ms at every potential tested, ranging from a minimum of 1.2 ms to a maximum of 1.9 ms (Fig. 5B, bottom right panel). The linear regression after the addition of tamoxifen (solid line, $r = 0.8$, $p < 0.02$) showed a 7-fold decrease in the slope from 0.015 to 0.002 ms/mV ($p < 0.05$, unpaired Student’s $t$ test) These data indicated that tamoxifen induces a voltage-independent block of the channel, together with a destabilization of long-lived closed states present at hyperpolarized voltages.

The Dual Effect of Tamoxifen Is Also Present in \( \beta1 \) KO KCa Channels—The absence of the \( \beta1 \) subunit in arterial KCa channels does not prevent tamoxifen inhibition of KCa channels as demonstrated in Fig. 1. The question remains whether the activator effect of tamoxifen can also be present in this preparation. To further explore the effect of tamoxifen, in the absence of the \( \beta1 \) subunit, a series of nine concentration-response experiments were conducted with excised membrane patches.

**Fig. 5.** Single channel analysis of the effect of tamoxifen in a single WT KCa channel at different voltages and 10 \( \mu \)M internal Ca\(^{2+} \). A, representative single channel activity obtained at different holding voltages (from $-80$ to $+80$ mV, as indicated) in control (left panels) and in the presence of 5 \( \mu \)M tamoxifen (right panels). Arrows indicate the closed state of the channel. B, grouped data obtained from five different experiments with only one active channel present in the patch. Top left panel, bar plot of single channel amplitudes in the absence and presence of 5 \( \mu \)M tamoxifen with voltage steps between +20 and +80 mV; control amplitude values (black bars) of 4.7 ± 0.1, 9.1 ± 0.2, 12.4 ± 0.4, and 14.8 ± 1 pA, respectively; tamoxifen amplitude values (diagonal filled bars) of 3.72 ± 0.2, 7.42 ± 0.2, 10.4 ± 0.3, and 13 ± 0.5 pA, respectively. Tamoxifen significantly reduced the slope conductance by 15% (from 227 ± 0.001 to 194 ± 0.002 picosiemens, control versus tamoxifen, respectively, $p < 0.001$). Top right panel, $P_o$ versus voltage plots in control (○) and in the presence of 5 \( \mu \)M tamoxifen (●). Experimental points were fitted to a Boltzmann function (solid lines). Tamoxifen reduces $P_{max}$ (from 0.9 ± 0.02 to 0.57 ± 0.01; control versus tamoxifen, respectively, $p < 0.001$). A change in the slope factor of the Boltzmann function was also observed with tamoxifen from 17 ± 2 to 33 ± 7 mV (control versus tamoxifen, respectively, $p < 0.05$). Bottom left panel, tamoxifen decreases mean closed time ($\tau_c$). $\tau_c$ values were fitted to a single exponential function (see text). Under control conditions, $\tau_c$ decreased e-fold by 26 mV, whereas in the presence of tamoxifen, $\tau_c$ decreased e-fold by 40 mV. Bottom right panel, mean open time ($\tau_o$) is also affected by tamoxifen. $\tau_o$ in control (○) increased linearly with depolarization (see text) from 1.9 ms at $-80$ mV to 4.3 ms at $+80$ mV, with a slope from the linear regression (solid line) of 0.015 ms/mV. In the presence of tamoxifen (●), $\tau_o$ voltage dependence is almost abolished, showing a 7-fold decrease in the slope from 0.015 to 0.002 ms/mV (control versus tamoxifen, respectively, $p < 0.03$).
The dual effect of tamoxifen can be detected in the absence of the β1 subunit of KCa channels. A, P0 versus tamoxifen concentration plot from β1 KCa channels held at +40 mV and 1 μM internal Ca2+. Tamoxifen response depended on the initial P0 of the channel. Black circles represent mean P0 values from four different patches with high KCa channel activity (P0 > 0.3). Black diamonds represent mean P0 values from five different patches with lower KCa channel activity (P0 < 0.3). For comparison, P0 values from WT channels from Fig. 2A are included (gray circles). B, P0 versus voltage relationship of β1 KO KCa channels at 1 μM internal Ca2+. Data represent the mean from five different experiments at increasing concentrations of tamoxifen. Data were fitted to a Boltzmann function (solid lines). Tamoxifen (1 μM) promotes both a leftward shift of 14 mV (V1/2 Control = 36 ± 2 mV versus V1/2 tamoxifen = 22 ± 3 mV, p < 0.005) and a reduction of P0 max (from 0.58 ± 0.02 to 0.39 ± 0.02, control versus tamoxifen, respectively). A change in the slope factor of the Boltzmann curve is also observed (from 18 ± 1 to 24 ± 2 mV, control versus tamoxifen, respectively, p < 0.05, unpaired Student’s t test). At 5 and 10 μM tamoxifen, P0 max drops to 0.29 and 0.18, respectively, but a leftward shift can no longer be detected.

To further explore the observation about biphasic response to tamoxifen, an experimental series at nonsaturating cytosolic Ca2+ (1 μM) was performed at varying voltages and tamoxifen concentrations to expand the range at which tamoxifen-induced changes in channel activity can be detected. Original β1 KO KCa channel traces shown in Fig. 6C demonstrate that the dual effect of tamoxifen can also be detected in the absence of the β1 subunit. Channel activity recorded at +20 mV show that 1 μM tamoxifen increases β1 KO KCa channel P0 from 0.02 to 0.12 (control versus tamoxifen, respectively).
5 and 10 μM tamoxifen, in which $P_o_{\text{max}}$ drops to 0.29 and 0.18, respectively, but a leftward shift can no longer be detected.

**DISCUSSION**

**Tamoxifen Inhibition of KCa Channels**—Despite recent reports suggesting the potential vasodilatory effect of tamoxifen and other xenoestrogen-related compounds acting through activation of KCa channels, the present findings revealed that tamoxifen instead can be an effective blocker of the KCa channels present in arterial smooth muscle. Tamoxifen inhibition of KCa channel was observed for other smooth muscle preparations and attributed to a reduction in single channel conductance produced by tamoxifen. In the present work, the change in unitary current observed in arterial KCa channels accounts only for a marginal reduction of the current (10–15%). However, a substantial decrease in channel $P_o$ is observed in the presence of tamoxifen. The analysis of single channel $P_o$ provided here allows a close discrimination between changes in unitary conductance and changes in channel activity. Tamoxifen inhibition of arterial KCa channel $P_o$ is concentration-dependent and also partially reversible. KCa channel $P_o$ can drop to 50% of the control values at a tamoxifen concentration of ~3 μM. This inhibition in channel activity is probably also responsible for the reduction in macroscopic KCa currents observed in other smooth muscle preparations (15, 17). However, this pronounced decrease in macroscopic currents was attributed to a decrease in channel conductance rather than a change in $P_o$.

The evidence provided here suggests that tamoxifen inhibition of KCa channels can be better described by a decrease in channel $P_o$ probably due to an intermediate block that was entirely overlooked in the past.

**The Mechanisms of the Effect of Tamoxifen on KCa Channels**—The fact that tamoxifen concentration-dependent inhibition affinity ($IC_{50} = 2.6 \mu M$) is very close to the reported affinity for dose-dependent activation (~1 μM, Ref. 14) emphasizes the dual nature of tamoxifen action in the same concentration range and the need for a very detailed analysis to account for the possible mechanisms. The dual nature of the effects of tamoxifen produces, under certain conditions, a clear crossover of the voltage activation curves or the Ca$^{2+}$ activation curves (Figs. 4A and 6B). Single channel kinetics revealed that tamoxifen operates through a reduction in the mean open time, but also a through reduction in the mean close time (Fig. 5B). A simple explanation is that tamoxifen can be well described as an intermediate channel blocker of KCa channels (as opposed to a fast or flickery block). However, single channel kinetics simulations (data not shown) have thus far excluded the possibility of tamoxifen behaving as an open channel blocker or a closed channel blocker, at least for very simplified models of three to five states (closed-open-block, closed-blocked-open, or closed1-closed2-closed3-open-blocked). Thus, it appears that the activator effect of tamoxifen cannot be simply described as a release from blocked states. In addition, the Hill coefficient obtained in the present study for the blocking reaction ($N_{Hill} = 1.47 > 1$) also suggests that tamoxifen inhibition cannot be taken as a simple bimolecular reaction. Tamoxifen appears to act by destabilizing long-lived closed states while, at the same time, producing a block state of intermediate duration not previously described. Both processes appear to have a very similar tamoxifen affinity, which further complicates the analysis. It is not completely clear at this point whether the intermediate blocking site is physically separated from the activating site in the KCa channel protein. Moreover, it is not clear whether tamoxifen inhibition takes place as a true physical occlusion of the permeation pathway in the mouth of the channel, or whether tamoxifen is acting by shifting the equilibrium between normal gating states of KCa channels. Nonetheless, competition experiments have shown in the past that tamoxifen can reduce the inhibition produced by known KCa channel pore blockers such as tetraethylammonium, iberiotoxin, and charybdotoxin (17), favoring the hypothesis that tamoxifen inhibition can take place within the mouth of the channel. The presence of several channels in the patch pipette in the present work precludes any detailed analysis, which, in combination with a dwell time of the intermediate block in the range of the pre-existing dwell times in normal gating of the channel, further complicates the discrimination between a pore occlusion of the channel or a modification in the gating process of the channel. Competition experiments in outside-out configuration patches, a configuration difficult to obtain under the present recording conditions, will be needed to determine whether tetraethylammonium or iberiotoxin can effectively reduce the tamoxifen inhibition in $P_o$ described here. This will help to distinguish between an occlusion mechanism or a gating modifier mechanism.

**The Role of the $\beta 1$ Subunit**—Tamoxifen inhibition of KCa channels appears to reside only in the $\alpha$ subunit. As stated above, this inhibition is mainly due to an intermediate duration block that results in a dramatic reduction of $P_o$, which differs from the reduction in single channel conductance also observed here and elsewhere (15, 17). Dick et al. (17) demonstrated that ethylbromide tamoxifen (a membrane-impermeant tamoxifen analog) can reduce single channel amplitude from both the extracellular side and (to a minor extent) the intracellular side. They also showed that ethylbromide tamoxifen can reduce KCa macroscopic currents in outside-out patches. Although the present work does not address which side applies to the tamoxifen effect, it is conceivable that the tamoxifen intermediate blocking site can only be reached from the extracellular side of the channel protein. Regardless of its side, this intermediate blocking site is most likely located only in the $\alpha$ subunit, due to the fact that tamoxifen inhibition takes place in both WT and $\beta 1$ KO KCa channels with very similar affinities and partial reversibility. The present study also demonstrates that the $\beta 1$ subunit is not required to allow tamoxifen-mediated activation of arterial KCa channels. This observation suggests that tamoxifen binding sites are probably located in the $\alpha$ subunit, opening the possibility that both activation and inhibition are physically related, if not the same, sites. It is worth noting, however, that in the absence of the $\beta 1$ subunit, the activator effects of tamoxifen are less pronounced. The leftward shift shown in Fig. 6B for KO conditions is only 14 mV compared with the 24-mV shift reported for colonic smooth muscle under WT conditions (15), suggesting that perhaps the role of the $\beta 1$ subunit is to stabilize or facilitate tamoxifen-bound states rather than being the actual forming subunit of the tamoxifen receptor. The receptor for charybdotoxin (or iberiotoxin) (21, 22), Ca$^{2+}$ (23), dehydrosoyasaponin-I (7), or, in this case, tamoxifen (14) appears to be modulated directly or indirectly by the $\beta 1$ subunit, rather than the $\beta 1$ subunit being part of those receptors in the channel protein (5, 24, 25). The weaker effect for tamoxifen activation obtained in the absence of the $\beta 1$ subunit, in combination with the inhibitory properties of tamoxifen, can probably explain the differences between the present work and the previously reported lack of effect in KCa channels also from $\beta 1$ KO animals, although colonic smooth muscle cells were used in that study (15). Alternatively, another auxiliary $\beta$ subunit could have emerged in arterial smooth muscle as a compensatory mechanism in $\beta 1$ KO animals. In this case, this compensatory $\beta$ subunit should also
preserve similar pharmacological characteristics. To date, there is no information about the role of other KCa channel β subunits in tamoxifen-mediated KCa channel activation. In either case, the exclusiveness of the β1 subunit in tamoxifen activation becomes questionable, as does the use of tamoxifen as a pharmacological probe for the presence of β1 subunits in KCa channels (10, 11).

In conclusion, tamoxifen interaction with arterial KCa channels appears to be rather complex, promoting two types of blocks: a previously described fast flickery block, also present here, and an intermediate block, first characterized here, which accounts for a great deal of the inhibition observed in arterial KCa channels in the present work and probably in other studies elsewhere. These blocking effects co-exist with an activator effect through the destabilization of long-lived closed states. Together, these effects appear to reside exclusively on the α subunit or, at minimum, do not appear to depend on the presence of the β1 subunit of KCa channels.

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