Tamoxifen Inhibits Inward Rectifier K⁺ 2.x Family of Inward Rectifier Channels by Interfering with Phosphatidylinositol 4,5-Bisphosphate-Channel Interactions

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ABSTRACT

Tamoxifen, an estrogen receptor antagonist used in the treatment of breast cancer, inhibits the inward rectifier potassium current (I_k) in cardiac myocytes by an unknown mechanism. We characterized the inhibitory effects of tamoxifen on Kir2.1, Kir2.2, and Kir2.3 potassium channels that underlie cardiac I_k. We also studied the effects of 4-hydroxytamoxifen and raloxifene. All three drugs inhibited inward rectifier K⁺ 2.x (Kir2.x) family members. The order of inhibition for all three drugs was Kir2.3 > Kir2.1 ~ Kir2.2. The onset of inhibition of Kir2.x current by these compounds was slow (T_{1/2} ~ 6 min) and only partially recovered after washout (~30%). Kir2.x inhibition was concentration-dependent but voltage-independent. The time course and degree of inhibition was independent of external or internal drug application. We tested the hypothesis that tamoxifen interferes with the interaction between the channel and the membrane-delimited channel activator, phosphatidylinositol 4,5-bisphosphate (PIP_2). Inhibition of Kir2.3 currents was significantly reduced by a single point mutation of I213L, which enhances Kir2.3 interaction with membrane PIP_2. Pretreatment with PIP_2 significantly decreased the inhibition induced by tamoxifen, 4-hydroxytamoxifen, and raloxifene on Kir2.3 channels. Pretreatment with spermine (100 μM) decreased the inhibitory effect of tamoxifen on Kir2.1, probably by strengthening the channel's interaction with PIP_2. In cat atrial and ventricular myocytes, 3 μM tamoxifen inhibited I_k, but the effect was greater in the former than the latter. The data strongly suggest that tamoxifen, its metabolite, and the estrogen receptor inhibitor raloxifene inhibit Kir2.x channels indirectly by interfering with the interaction between the channel and PIP_2.

Tamoxifen is a drug used in the treatment of hormone-responsive breast cancer and prevention of breast cancer in high-risk women (Fisher et al., 1998). The cardiovascular effects of tamoxifen include a reduction in myocardial infarction rates (McDonald and Stewart, 1991) and decreased mortality attributable to vascular disease (Early Breast Cancer Trialists’ Collaborative Group, 1992). However, increasing evidence suggests that tamoxifen may also have untoward cardiovascular effects. Several cases of QT prolongation and ventricular arrhythmias, in particular torsades de pointes, have been reported with tamoxifen (Pollack et al., 1997).

Tamoxifen has been shown to inhibit a number of ion channels in cardiac and noncardiac tissues (Zhang et al., 1994; Allen et al., 1998; Hardy et al., 1998; Liu et al., 1998; He et al., 2003). In rat ventricular myocytes, tamoxifen increases the ventricular action potential duration and decreases the sustained outward delayed rectifier K⁺ current, the transient outward K⁺ current, the inward rectifier K⁺ current (I_{K1}), and the Na⁺ current (He et al., 2003). In rabbit ventricular myocytes, tamoxifen inhibited rapidly activating delayed rectifier K⁺ current (I_{K1}) and the inward L-type calcium current (Liu et al., 1998). Inhibition of I_{K1} and I_{K1} may be an explanation for the arrhythmogenic effect of tamoxifen; however, the mechanisms by which tamoxifen interacts with ion channels and other proteins have not been well determined.

I_{K1} is the major determinant of the resting membrane potential in the heart, and it plays a major role in the formation of the plateau phase of the cardiac action potential. Kir2.1, Kir2.2, and Kir2.3 potassium channels are believed to underlie the cardiac inwardly rectifying current I_{K1} in various species, as well as in different regions of the heart...
(Dhamoon et al., 2004). However, several lines of evidence suggest that Kir2.1 is the predominant subtype that underlies $I_{K1}$ in human ventricle (Wang et al., 1998). Kir2.1, 2.2, and 2.3 channels are distinctly regulated by factors like pH, protein kinase C, and epidermal growth factor (Du et al., 2004). This modulation is stronger for channels that weakly interact with phosphatidylinositol 4,5-bisphosphate (PIP$_2$), e.g., Kir2.3 (Du et al., 2004). In a recent article (Rodriguez-Menchaca et al., 2008), we reported that the cationic amphiphilic antimalarial drug chloroquine (pK$_a$ of 10.8 and 8.4), which at pH 7.2 to 7.4 is ~99.9% in its cationic form, blocks Kir2.1 in a voltage-dependent manner. Chloroquine interacts with acidic residues (mainly Glu224 and Glu299) lining the cytoplasmic pore of the channel.

The working hypothesis of the present study was that the cationic amphiphilic drug tamoxifen (pK$_a$ of ~8.7; log D ~4.3), which readily inserts into lipid bilayers, may bind to phosphoinositides (Allan and Michell, 1975; Friedman, 1993) and interfere with the interaction between PIP$_2$ and inward rectifier K$^+$ (Kir2.x) channels. Thus, we studied the effects of tamoxifen on Kir2.1, Kir2.2, and Kir2.3 channels, expressed in human embryonic kidney (HEK)-293 cells, and on $I_{K1}$ of cat atrial and ventricular myocytes. It has previously been shown that inhibition of Kir2.3 currents by different modulators that decrease the interaction of the channel with PIP$_2$ was significantly reduced by a single point mutation of Kir2.3 (I213L), which enhances its interaction with membrane PIP$_2$ (Du et al., 2004). We compared the effect of tamoxifen on Kir2.3 wild type (WT) and the mutant I213L. In addition, we have compared the effect of tamoxifen alone and the effect of tamoxifen together with PIP$_2$.

We found that, in contrast to the results with chloroquine, tamoxifen inhibited all five channel currents in a voltage-independent manner. Moreover, our results indicate that the effects of tamoxifen are greater in cat atrial than in ventricular myocytes.

**Materials and Methods**

**Molecular Biology and Cell Transfection.** Kir2.1 cDNA (a kind gift from C. Vandenberg) and Kir2.2 and 2.3 (kind gifts from D. Logothetis) were subcloned into pcDNA3.1(+) plasmid (Invitrogen, Carlsbad, CA). Mutations were made by using the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). All mutations were confirmed by direct DNA sequencing. Kir2.1 constructs were expressed in HEK-293 cells as described. Cells were transfected with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions.

**Current Recordings in HEK-293 Cells.** Macroscopic currents were recorded in the whole-cell and inside-out configurations of the patch-clamp technique (Hamill et al., 1981), using an Axopatch-200B amplifier (Molecular Devices). Data acquisition and command potentials were controlled by pClamp 9.0 software (Molecular Devices). Patch pipettes with a resistance of 1 to 2 MΩ were made from borosilicate capillary glass (WPI). The external solution used to record membrane currents in isolated myocytes had the following composition: 140 mM NaCl, 5.4 mM Na$_2$HPO$_4$, 2.5 mM KCl, 1.8 mM CaCl$_2$, 0.1 mM MgCl$_2$, 11 mM glucose, and 10 mM taurine. The solution was equilibrated with 95% O$_2$ + 5% CO$_2$, pH 7.4. Nominally, calcium-free solution was prepared by omitting CaCl$_2$ from the Tyrode’s solution. The high-potassium, low-chloride solution (KB medium) had the following composition: 80 mM potassium glutamate, 40 mM KCl, 20 mM taurine, 3 mM KH$_2$PO$_4$, 10 mM glucose, 10 mM HEPES, and 0.2 mM EGTA. The pH was adjusted to 7.4 with KOH.

Macroscopic currents were recorded in the whole-cell configurations of the patch-clamp technique (Hamill et al., 1981), using an Axopatch-200B amplifier (Molecular Devices). Data acquisition and command potentials were controlled by pClamp 9.0 software (Molecular Devices). Patch pipettes with a resistance of 1 to 2 MΩ were made from borosilicate capillary glass (WPI). The external solution used to record membrane currents in isolated myocytes had the following composition: 140 mM NaCl, 5.4 mM KCl, 0.1 mM CaCl$_2$, 0.4 mM MgCl$_2$, 1.0 mM KF, 10 mM HEPES, and 11 mM glucose, pH adjusted to 7.4 with NaOH. The pipette solution had the following composition: 90 mM potassium aspartate, 54 mM KCl, 10 mM KH$_2$PO$_4$, 5 mM HEPES, EGTA 0.1 mM, pH 7.3 with KOH.

**Drugs.** Tamoxifen, 4-hydroxytamoxifen, and raloxifene (Sigma-Aldrich) were dissolved directly in the external solution at the desired concentration. HEK-293 cells and myocytes were exposed to drug solutions until steady-state effects were achieved. The effects of the drugs were measured 15 to 20 min after bath application in whole-cell experiments and inside-out patches. To determine the concentration-effect relationships, a single cell was exposed to only one concentration of the drug.

**Data Analysis.** Patch-clamp data were processed by using Clampfit 9.0 (Molecular Devices) and then analyzed in Origin 7 (OriginLab Corp., Northampton, MA). Data are presented as mean ± S.E.M. (n = number of cells). For whole-cell experiments, currents were normalized to the current recorded at ~120 mV under control conditions; for inside-out experiments, currents were normalized to the current recorded at ~80 mV under control conditions. The fractional block of current (f) was plotted as a function of drug concentration ([D]), and the data were fit with a Hill equation, f = 1/(1 + ([D]/IC$_{50}$))$^n$, to determine the IC$_{50}$ and the Hill coefficient, n$_H$.

Statistical analyses were performed using the Statistica (Tulsa, OK) software package, version 10.0. Concentration-response data were tested for significance by Student’s t test applying Bonferroni’s correction.
correction for multiple comparisons. Student’s t test was applied to compare individual data sets. A two-tailed probability value of less than 0.05 was considered statistically significant.

**Results**

**Tamoxifen Reduces Current through Kir2.x Channels.** The effects of tamoxifen on Kir2.1, 2.2, and 2.3 channels were first studied in the whole-cell configuration in transfected HEK-293 cells. Voltage ramps of 3 s were applied from −120 to −20 mV every 20 s for Kir2.1, Kir2.2, and Kir2.3. Under control conditions all three channels showed large inward currents but small outward currents, which is typical of the strong inward rectifier channels (Fig. 1, Aa–Ac). In all three channels, 1 μM tamoxifen significantly decreased current amplitude throughout the whole range of voltages studied. However, the effect on Kir2.3 was stronger. Concentration-response curves for the inhibitory effects of tamoxifen on current measured at −120 mV in all three channels are shown in Fig. 1B. The IC\textsubscript{50} of tamoxifen on Kir2.1 (0.93 ± 0.07 μM; n = 5 cells) and Kir2.2 (0.87 ± 0.02 μM; n = 5 cells) were not significantly different, but IC\textsubscript{50} on Kir2.3 (0.31 ± 0.06 μM; n = 5 cells) was significantly different (P < 0.05, from both Kir2.1 and Kir2.2).

**The Inhibitory Effect of Tamoxifen on Kir2.x Currents Was Slow and Voltage-Independent.** The temporal course of tamoxifen inhibition of Kir2.x currents measured at −120 mV is shown in Fig. 2A. The temporal course tamoxifen inhibition (3 μM on Kir2.1 and 2.2; 1 μM on Kir2.3) was slow with a T\textsubscript{1/2} of 378 ± 62 s for Kir2.1 (n = 5), 339 ± 31 s for Kir2.2 (n = 5), and 345 ± 22 s for Kir2.3 (n = 3) (Fig. 2B). Washout of the effects of 3 μM tamoxifen on Kir 2.1 and Kir2.3 channels was slow and incomplete with a current amplitude recovering of 31 ± 6% (Fig. 2C). The inhibitory effects of tamoxifen on Kir2.1 and Kir2.3 currents were independent of test voltage (Fig. 2D).

**The Inhibitory Effects of Tamoxifen on Kir2.x Currents of Internal and External Membrane Application Were Similar.** In excised, inside-out patches expressing Kir2.3 channels, using symmetrical high [K\textsuperscript{+}], 1 μM tamoxifen was applied to the internal side of the membrane; current was elicited by a 3-s voltage ramp from −80 to +80 mV.
The magnitudes of the inhibitory effect and the voltage independence of tamoxifen inhibition were similar to those of externally applied tamoxifen, in the presence of $[K^+]_o = 4$ mM. The inhibition time course was faster in the inside-out than in the whole-cell configuration. For inside-out patches, $T_{1/2} = 236 \pm 29$ s ($n = 4$) was significantly different from the $T_{1/2} = 345 \pm 22$ s observed in the whole cell configuration. Thus, the slow onset of inhibition from either membrane surface suggests that tamoxifen must incorporate into the lipid membrane to exert its inhibitory effect.

The Inhibitory Effects of Tamoxifen on Kir2.x Channels Are Not Due to Pore Block. The effects of tamoxifen (1 to 10 $\mu$M) on Kir2.1 WT and Kir2.1 (E224G/E299S) mutant channel were similar in magnitude, and the same inhibitory effect was observed at negative and positive membrane potentials, suggesting that the effect of tamoxifen is not due to pore blockade by interaction with the two acidic residues at the cytoplasmic pore of the channel (E224G/E299S). The IC$_{50}$ of tamoxifen on the Kir2.1 (E224G/E299S) mutant was $1.2 \pm 0.26$ $\mu$M, $n = 6$ (Fig. 4B). This value was not significantly different from the IC$_{50}$ of Kir2.1 WT ($0.93 \pm 0.07$ $\mu$M). All of the tamoxifen results, including the lack of effect of the above mutations on potency, slow time course of inhibition, voltage and $[K^+]_o$ independence, and the similar effects of applying the drug from either side of the membrane, suggest that the effects were not due to pore blockade.

Evidence That Tamoxifen Interferes with Kir2.x Channels-PIP$_2$ Interaction. In Fig. 5A, the effect of tamoxifen on Kir2.3 (I213L) is shown. The potency of inhibition by tamoxifen was reduced 4-fold by the point mutation. The IC$_{50}$ of tamoxifen on the Kir2.3 (I213L) mutant was $1.19 \pm 0.04$ $\mu$M, $n = 5$ cells (Fig. 5B). To further characterize a possible relationship between tamoxifen and PIP$_2$, exogenous PIP$_2$ was added in Fig. 6A. The effect of 1 $\mu$M tamoxifen on Kir2.3 current was studied by applying tamoxifen together with 30 $\mu$M PIP$_2$, or applying PIP$_2$ at the same concentration, 2 min before the application of tamoxifen (Fig. 6, A and B). Both experimental maneuvers significantly decreased the inhibitory effects of tamoxifen on Kir2.3. Similar effects were obtained with 3 $\mu$M tamoxifen on Kir2.1 (data not shown).

We determined whether the long polyamine spermine was able to modulate the inhibitory effect of tamoxifen on Kir2.1 channels. Because outward current through Kir2.1 channels is very sensitive to contamination by residual polyamines and other cations, we used the inward current at $-80$ mV to index channel activity. As shown in Fig. 7A, 3 $\mu$M tamoxifen...
caused 65 ± 10% (n = 5) inhibition in the absence of polyamines. However, in the presence of spermine (100 μM), the same concentration of tamoxifen caused only 40 ± 2.4% (n = 4) inhibition (Fig. 7B). As illustrated in Fig. 7C, the IC50 of tamoxifen on Kir2.1 in the absence of polyamines was 1.39 ± 0.16 μM, n = 5; in the presence of 100 μM spermine, it was 4.14 ± 0.44 μM (p < 0.05).

**Effects of 4-Hydroxytamoxifen and Raloxifene on Kir2.x Channels.** The effects of the active tamoxifen metabolite 4-hydroxytamoxifen and the estrogen receptor modulator drug raloxifene on Kir2.1 and Kir2.3 channels are shown in Figs. 8 and 9, respectively. For Kir2.1 WT and mutant E224G/E299S channels, currents were measured at -120 mV. The IC50 of tamoxifen for the mutant E224G/E299S was 1.2 ± 0.26 μM (n = 6) and 0.93 ± 0.07 μM (n = 5) for Kir2.1 WT. Difference between both groups was not significantly different (p > 0.05).
Tamoxifen Inhibits IK1 in Cat Atrial and Ventricular Myocytes.

The effects of tamoxifen on cat atrial and ventricular myocytes were studied in the whole-cell configuration. Voltage ramps of 3 s were applied from −120 to −20 mV every 20 s. Under control conditions, atrial and ventricular currents showed large inward currents but small outward currents, which is typical of the strong inward rectifier channels (Fig. 10, A and B).

**Fig. 5.** Effect of tamoxifen on the mutant channel Kir2.3 (I213L). A, current traces recorded from the mutant Kir2.3 (I213L) in whole-cell configuration ([K+]o = 4 mM). Currents were elicted by 3-s ramps from −120 to −20 mV (holding potential, −80 mV) under control conditions (trace C) and in the presence of 1 µM tamoxifen (trace T). B, concentration-response curves for the effects of tamoxifen on Kir2.3 WT and mutant I213L channels. Currents were measured at −120 mV. The IC50 of tamoxifen for the mutant I213L channel 1.19 ± 0.04 µM (n = 6) and for Kir2.3 WT 0.31 ± 0.06 µM (n = 5) were not significantly different (p > 0.05).

**Fig. 6.** Effect of tamoxifen in the presence of PIP2. A, temporal course of the Kir2.3 current inhibition induced by 1 µM tamoxifen alone (left panel), after preincubation with 30 µM PIP2 (middle panel), and in the continuous presence of 30 µM PIP2 (right panel) in different excised, inside-out macropatches. B, summary of the inhibition induced by 1 µM tamoxifen alone (n = 5), after preincubation with PIP2 (n = 5), and in the continuous presence of PIP2 (n = 5), ∗, p < 0.05.

was significantly greater for 4-hydroxytamoxifen (p < 0.05) on Kir2.3 (IC50 = 0.97 ± 0.33 µM) than on Kir2.1 (IC50 = 7.8 ± 1.3 µM) (Fig. 8B); in addition, the potency of block was significantly greater for raloxifene on Kir2.3 (IC50 = 0.53 ± 0.13 µM) than on Kir2.1 (IC50 = 7.56 ± 0.13 µM) (Fig. 9B).

**Tamoxifen Inhibits IK1 in Cat Atrial and Ventricular Myocytes.** The effects of tamoxifen on cat atrial and ventricular myocytes were studied in the whole-cell configuration. Voltage ramps of 3 s were applied from −120 to −20 mV every 20 s. Under control conditions, atrial and ventricular currents showed large inward currents but small outward currents, which is typical of the strong inward rectifier channels (Fig. 10, A and B). In both atrial and ventricular myocytes, 3 µM tamoxifen significantly de-
Fig. 7. Effect of tamoxifen in the presence of long polyamine spermine. A, superimposed Kir2.1 current traces obtained under control conditions (trace C) and in the presence of 3 μM tamoxifen (trace T). Kir2.1 current was recorded in the inside-out configuration, evoked by depolarizing pulses to +50 mV, followed by a hyperpolarizing pulse to −80 mV. B, superimposed Kir2.1 current traces obtained in the continuous presence of 100 μM spermine before tamoxifen (trace C) and in the presence of 3 μM tamoxifen (trace T). C, concentration-response curve constructed from current measured at −80 mV. The IC_{50} of tamoxifen on Kir2.1 was 1.39 ± 0.16 μM (n = 5) and 4.14 ± 0.44 μM (n = 5) in the continuous presence of 100 μM spermine (p < 0.05).

Fig. 8. Effects of 4-hydroxytamoxifen on Kir2.1 and Kir2.3 channels. A, superimposed current traces induced by ramps, 3 s in duration, applied every 20 s from −120 to −20 mV (holding potential, −80 mV) in the whole-cell configuration, obtained under control conditions (trace C) and in the presence of 4-hydroxytamoxifen, 1 μM (trace T). Effects of 4-hydroxytamoxifen on Kir2.1 (left panel) and Kir2.3 (right panel) channels. B, concentration-response curves constructed from current measured at −120 mV. The IC_{50} of 4-hydroxytamoxifen on Kir2.1 was 7.8 ± 1.3 μM (n = 5) and 0.97 ± 0.33 μM (n = 5) for Kir2.3 (p < 0.05).
increased current amplitude throughout the whole range of voltages studied. The inhibitory effects of tamoxifen on current measured at −120 mV in both cell types are shown in Fig. 10C. The inhibitory effect of tamoxifen on atrial $I_{K1}$ was significantly stronger than on ventricular $I_{K1}$. At −120 mV, tamoxifen inhibited $I_{K1}$ in atrial myocytes ($n = 5$) by $58 \pm 5\%$ and inhibited $I_{K1}$ in ventricular myocytes by $33 \pm 6\%$ ($n = 5$; $p < 0.05$).

Fig. 9. Effects of raloxifene on Kir2.1 and Kir2.3. A, superimposed current traces induced by ramps, 3 s in duration, applied every 20 s from −120 to −20 mV (holding potential, −80 mV) in the whole-cell configuration, obtained under control conditions (trace C) and in the presence of 1 μM raloxifene (trace T). Effects of raloxifene on Kir2.1 (left panel) and Kir2.3 (right panel) channels. B, concentration-response curves constructed from current measured at −120 mV. The $IC_{50}$ of raloxifene on Kir2.1 was $7.56 \pm 0.13 \mu M$ ($n = 5$) and $0.53 \pm 0.13 \mu M$ ($n = 5$) for Kir2.3 ($p < 0.05$).

Fig. 10. Effects of tamoxifen on $I_{K1}$ of cat atrial and ventricular myocytes. A, effects of 3 μM tamoxifen (trace T) on $I_{K1}$ recorded from an atrial myocyte, in the whole-cell configuration. Currents were elicited by ramps of 3 s, applied from −120 to −20 mV. B, effects of 3 μM tamoxifen (trace T) on $I_{K1}$ recorded from a ventricular myocyte, in the whole-cell configuration. Currents were elicited by ramps of 3 s, applied from −120 to −20 mV. C, summary of the inhibition induced by 3 μM tamoxifen on atrial ($n = 5$) and ventricular ($n = 5$) myocytes. Difference between both groups was significant ($p < 0.05$).
Discussion

We demonstrate that tamoxifen, a synthetic nonsteroidal triphenylethylene derivative, which has estrogenic, antigestagenic effects, 4-hydroxytamoxifen, an active metabolite of tamoxifen, and raloxifene, the selective estrogen receptor modulator used to treat osteoporosis in postmenopausal women, inhibit the strong inward rectifier potassium channels Kir2.x. The order of inhibition for all three drugs was Kir2.3 > Kir2.1 ~ Kir2.2. The inhibition of Kir2.x current by tamoxifen, 4-hydroxytamoxifen, and raloxifene occurred slowly ($T_{1/2}$ ~ 6 min), and the currents only partially recovered after washout (~30%). Tamoxifen also inhibited $I_{K1}$ in cat atrial and ventricular myocytes, and the effects were greater in the former than the latter.

The inhibition induced by tamoxifen, 4-hydroxytamoxifen, and raloxifene was concentration-dependent but voltage-independent. The potency of tamoxifen to inhibit Kir2.1 channel was greater than 4-hydroxytamoxifen and raloxifene. The $IC_{50}$ of inhibition by tamoxifen on Kir2.1 was 0.93 μM, 7.8 μM for 4-hydroxytamoxifen, and 7.56 μM for raloxifene. The results were similar when the drugs were externally applied in the whole-cell configuration and when applied to the internal side of the membrane in the excised inside-out macropatch, suggesting that the effect on Kir2.x channels was membrane delimited. During breast cancer treatment, tamoxifen plasma levels yield 0.54 to 2.0 μM (Murphy et al., 1987). However, tamoxifen plasma concentrations have been reported to reach up to 1.56 to 7.0 μM during high-dose therapy (Berman et al., 1995). Therefore, the concentrations of tamoxifen found to significantly inhibit Kir2.x channels are clinically relevant.

Tamoxifen, 4-hydroxytamoxifen, and raloxifene are cat-ionic amphipathic drugs (CADs). Previous reports have shown that tamoxifen, 4-hydroxytamoxifen, and raloxifene affect different ion channels in cardiac and noncardiac tissues; however, the underlying mechanisms have been poorly understood (Zhang et al., 1994; Allen et al., 1998; Hardy et al., 1998; Liu et al., 1998; He et al., 2003). Even though the ultimate pharmacological effects may depend on drug-protein interactions, drug molecules must cross lipid membrane barriers before reaching the target site. Therefore, the interactions of drugs with phospholipids and phospholipid-containing membranes play critical roles in drug disposition and drug action. It has been shown that CADs bind to the negatively charged phosphoinositides (Allan and Michell, 1975). The cationic group of CADs is normally placed between the polar head groups of phospholipids, and the hydrophobic portion is directed toward the hydrophobic interior of the membrane; thus, the drug molecule intercalates between lipid molecules (Conrad and Singer, 1981). It has long been suspected that CADs could regulate ion channels by adsorbing to the host lipid bilayer and thereby altering the bilayer's physical properties and that such a mechanism could be involved in many nonspecific effects (Andersen and Koepppe, 2007). Tamoxifen and 4-hydroxytamoxifen readily insert into lipid bilayers (Jordan, 1984; Custódio et al., 1993a,b). It has previously been suggested that tamoxifen and 4-hydroxytamoxifen interact with membrane phospholipids, alter lipid structure or lipid-protein interactions, and consequently may exert their effects on ion channels or other proteins indirectly. However, the mechanism by which both drugs inter- fere with ion channel function has not been determined (Song et al., 1996; Verrecchia and Hervé, 1997).

PIP$_2$ is an essential signaling phospholipid that is needed for many plasma membrane processes, including the function of ion channels and transporters, the control of endocytosis, and the nucleation of cytoskeletal networks (Suh and Hille, 2005). PIP$_2$ is a major phosphoinositide of the plasma membrane that comprises approximately 1% of plasma membrane phospholipids. Each PIP$_2$ molecule consists of an inositol head group and fatty acid side chains inserted into the membrane. The negatively charged PIP$_2$ head groups are anchored just below the plane of the membrane by the lipid tails of the molecule. Activation by PIP$_2$ is a common feature of all Kir channels; the putative location of the PIP$_2$ activation gate in the Kir channels has been studied by several groups (Xiao et al., 2003; Pegan et al., 2005). It has been proposed that the channel opens when the negative head charges of PIP$_2$ interact with positively charged residues in the cytoplasmic domain, near the plasma membrane (Logothetis et al., 2007). It has been suggested that PIP$_2$ binding causes conformational changes at bundle crossing formed by M2 helix (Xiao et al., 2003) or the G-loop (girdle) structure near the junction between membrane pore and cytoplasmic pore domain (Pegan et al., 2005), which in turn promotes the open state, and the channel closes when the interaction with PIP$_2$ is lost. Characteristics of PIP$_2$-Kir channel interactions determine the sensitivity of Kir to many regulatory factors like pH, protein kinase C, and membrane receptors such as M1 (type 1 muscarinic) and epidermal growth factor receptors; a Kir channel like Kir2.3 or Kir3.1/3.4 that has a weak interaction with PIP$_2$ is sensitive, whereas channels like Kir2.1 that have strong interactions with PIP$_2$ are less sensitive to these regulators (Du et al., 2004).

The effects of the cationic pore blocker chloroquine on Kir2.1 channels were strongly attenuated by neutralization of two acidic residues within the cytoplasmic pore of the channel (E224G/E299S) (Rodríguez-Menchaca et al., 2008). The effects of tamoxifen (1–10 μM) on Kir2.1 WT and Kir2.1 (E224G/E299S) mutant channel were similar in magnitude, and the same inhibitory effect was observed at negative and positive membrane potentials, suggesting that the effect of tamoxifen is not due to pore blockade by interaction with the two acidic residues at the cytoplasmic pore of the channel (E224G/E299S).

The slow inhibition time course (resembling the run-down phenomenon) independent of internal or external drug and the voltage independence of the inhibition suggest that tamoxifen, 4-hydroxytamoxifen, and raloxifene probably insert into the lipid membrane (Allan and Michell, 1975; Jordan, 1984; Custódio et al., 1993a,b) and might interfere with PIP$_2$-channel interactions. Inhibition of Kir2.3 currents was significantly reduced by a single point mutation of (I213L), which enhances channel interaction with membrane PIP$_2$. More recently, it was found that long polyamines were capable of strengthening the PIP$_2$-channel interaction (Xie et al., 2005). In the present work, pretreatment with spermine (100 μM) decreased the inhibitory effect of tamoxifen on Kir2.1, perhaps by strengthening the channel interaction with PIP$_2$. (Xie et al., 2005). In support of this hypothesis, pretreatment or continuous application of PIP$_2$ markedly decreased the inhibition induced by tamoxifen, 4-hydroxy-tamoxifen (data not shown), and raloxifene (data not shown). Thus, our re-
sults suggest that membrane PIP$_2$ may likely be involved in the Kir current inhibition by tamoxifen, 4-hydroxytamoxifen, and raloxifene.

Members of the Kir2 subfamily (Kir2.1, 2.2, and 2.3) contribute to macroscopic IK$_{\text{C}}$ current in cardiac muscle. Protein isoform distribution is tissue- and species-dependent; Kir2.3 message was found to be nearly 10-fold more concentrated in the atria than ventricle in humans (Wang et al., 1998), dogs (Melnik et al., 2002), and sheep (Dhamoon et al., 2004). We have found that Kir2.3 channels are more sensitive than Kir2.1 and 2.2 to tamoxifen, 4-hydroxytamoxifen, and raloxifene. These results suggest that atrial IK$_{\text{C}}$ in those species could be more sensitive to inhibition by those drugs, which may have importance in the development of new antiarrhythmic approaches. For example, recent studies have demonstrated a major role for IK$_{\text{C}}$ in controlling the stability and frequency of ventricular fibrillation. They have shown that BaCl$_2$, which at low concentrations (1–50 $\mu$M) selectively blocks IK$_{\text{C}}$, effectively terminates ventricular fibrillation in the hearts of both guinea pigs (Warren et al., 2003; Grzedza et al., 2009) and transgenic mice overexpressing IK$_{\text{C}}$ (Noujaim et al., 2007). Because inward rectifier currents are also thought to be important in controlling atrial fibrillation (Ehrlich, 2008), it is tempting to surmise that tamoxifen and its analogs might be effective in terminating atrial fibrillation, particularly because in humans Kir2.3 is the dominant strong inward rectifier isomorph. In the present work, we found that tamoxifen inhibits IK$_{\text{C}}$ in cat atrial myocytes more potently than in ventricular myocytes, suggesting that in atrial myocytes the Kir2.3 isomorph may be participating more in the composition of the IK$_{\text{C}}$ channels than in ventricular myocytes. It is unfortunate that there is not information about the relative expression of Kir2.x isoforms in cat atrial and ventricular myocytes. Our experiments performed in channels expressed in a heterologous system (HEK-293 cells), although far from being physiological, yielded results that pointed in the right direction.

Although it has been suspected that lipophilic drugs may modulate membrane proteins by altering physical properties of the lipid bilayer, evidence for this interaction is lacking (Lundbaek, 2008). A serious problem with lipid theories is the fact that it was rarely, if ever, explained how the proposed perturbation in the lipid bilayer would then result in a dysfunctional membrane protein (Franks, 2006). In the present work, we provide evidence for a novel mechanism of Kir channel inhibition by drugs. Our data suggest that lipophilic compounds such as tamoxifen may insert in the lipid membrane bilayer and interfere with the interaction between Kir channels and PIP$_2$. However, more direct evidence on such interference should be provided.

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References


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