Estradiol Binding to Maxi-K Channels Induces Their Down-regulation via Proteasomal Degradation*

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Estrogens exert their biological action via both genomic and non-genomic mechanisms. Proteins different from classical estradiol receptors are believed to mediate the latter effects. Here we demonstrate that the maxi-K channel functions as an estrogen-binding protein in transfected HEK293 cells. Whole-cell maxi-K channel currents and protein expression were attenuated by exposure to either 17α- or 17β-estradiol. This effect was dose-dependent for 17β-estradiol at concentrations ranging from 10 nM to 1 μM, while 17α-estradiol inhibited channel expression only at 1 μM. These effects were mediated by direct low affinity binding of estradiol to the maxi-K channel but not to its accessory β1-subunit, as revealed by cell membrane estradiol binding assays. However, specific binding of estradiol to the channel was facilitated by the presence of the β1-subunit. Addition of MG-132, a blocker of proteasomal degradation, stabilized channel expression. These data suggest that channel down-regulation is mediated by estrogen-induced proteasomal degradation, similar to the pathway used for estrogen receptor degradation. Membrane expression of endogenous maxi-K channels in cultured vascular smooth muscle cells was also attenuated by prolonged exposure to 17α- and 17β-estradiol. Thus our studies demonstrate that estradiol binds to maxi-K channels and may directly regulate channel expression and function. These results will have important implications in understanding estradiol-induced effects in multiple tissues including vascular smooth muscle.

Existing epidemiological data suggest a beneficial role for estrogens in maintaining cardiovascular protection in pre-menopausal women. However, other data show that estrogen-containing preparations can negatively affect the health of pre-menopausal as well as post-menopausal women (1–6). These results indicate the existence of complex estradiol-signaling mechanisms, possibly mediated by proteins other than classical intracellular estradiol receptors (7).

One protein that has been hypothesized to be a potential effector of the action of estrogen is the large conductance Ca2+- and voltage-activated K+ channel (maxi-K channel). This channel provides a buffering repolarizing current in response to various physiological stimuli (8–11). The mechanism by which estrogen interacts with this channel is unknown. However, both 17β-estradiol and tamoxifen, a partial agonist of nuclear estrogen receptors, have been shown to activate maxi-K channels in both heterologous expression systems and smooth muscle cells, resulting in greater repolarizing currents (12, 13). Because acute incubation with either 17β-estradiol or membrane-impermeable estrogen derivatives activate maxi-K channels, signal transduction pathways other than those mediated by classical nuclear estradiol receptors α (ERα) or β (ERβ) are likely to be involved. Valverde et al. (14) showed that activation of maxi-K channels by 17β-estradiol occurs when the channel is associated with its accessory β1-subunit. This effect was not observed in the presence of 17α-estradiol or when the maxi-K channel was expressed alone. This observation led to the hypothesis that the β1-subunit directly binds estradiol and enhances channel activity by an unidentified mechanism (14). However, other studies have demonstrated that β1-subunit-independent decreases in maxi-K channel unitary conductance occur in response to (xeno)estrogens, implicating that either subunit can be a target for estrogen (15). Both studies emphasized the cell membrane-limited action of estrogen on the maxi-K channel, thus excluding a role for classical nuclear estradiol receptors. Other data indicate that ERα or ERβ may participate in membrane-associated signaling pathways when they are routed to the cell membrane by differential phosphorylation or binding to caveolins. Novel membrane-associated estradiol receptors are also a possible explanation for short term estrogen action (16–18).

Short term exposure of maxi-K channels to 17β-estradiol or to partial agonists of estradiol receptors is thought to activate the repolarizing current. Here, we report that exposure to either 17α- or 17β-estradiol induces both a decrease in maxi-K channel current and a down-regulation of channel protein expression in heterologous systems. Although 17α- and 17β-estradiol produce similar results, higher concentrations and longer exposure times are required for 17α-estradiol to mediate these effects. The presence of the β1-subunit did not alter channel expression, and its expression was inhibited by both estradiols. Further investigation into the mechanism underlying this response implicates estradiol-induced proteasomal degradation of the channel, which occurs after direct binding of estrogen to the maxi-K channel. Studies conducted in coronary artery smooth muscle cells suggest that 48 h of exposure to estrogens reduces maxi-K channel protein expression. The estrogen-induced loss of repolarizing current may have poten-

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1 The abbreviations used are: ER, estradiol receptor; FBS, fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium; eGFP, enhanced green fluorescent protein.
FIG. 1. Dose-dependent inhibition of the maxi-K channel current by estrogens. Whole-cell recordings were elicited from −80 mV holding potential in 20 mV steps from −100 mV to +160 mV in HEK293 cells stably (n = 14) and transiently (n = 3) expressing maxi-K channels. Whole-cell currents were measured in the absence (A and B, control) and presence of 1 μM 17α- (A, 17α) and 17β-estradiol (B, 17β). Currents were significantly attenuated in the presence of 1 μM 17α-estradiol (C; n = 6) and 1 μM 17β-estradiol (D; n = 8) compared with control recordings. Asterisks indicate significant difference (p < 0.05) at a given membrane potential. Similar effects were seen in transiently expressed maxi-K channels current (data not shown, n = 3). Whole-cell current expression measured in the presence of 10 nM 17α-estradiol (E, n = 4) or 10 nM 17β-estradiol (F, n = 3) did not differ from control recordings.
Clonetics (San Diego, CA). The QuiKChange site-directed mutagenesis kit and GeneJammer transfection reagent were purchased from Stratagene (La Jolla, CA). The nuclear extract kit was obtained from Active Motif®(Carlsbad, CA). Proteasome inhibitor MG-132 and Anopore tissue culture inserts were purchased from Fisher Scientific. [6,7-3H]17β-Estradiol was obtained from Amersham Biosciences.

**Heterologous Expression of the Maxi-K Channel**—An adenoviral construct of the maxi-K channel (GenBank™ accession number U11058), encoding the epitope (GenBank™ accession number AF349445), was generated. HEK293F cells, grown to 60–70% confluence on 0.02-μm Anopore tissue culture inserts in phenol red-free DMEM:F-12 medium supplemented with 10% hormone-free FBS, were infected with 10 μl of purified construct (1–1.2 × 10^12 particles/ml). Heterologous expression of the maxi-K channel with its accessory β1-subunit was performed using 10 μl of an adenoviral construct of the human maxi-K channel β1-subunit (GenBank™ accession number NM 004157) at a concentration 10^10 particles/ml. Infection efficiency was monitored by eGFP reporter gene expression driven by a separate promoter. Cells cultured in estradiol-free medium as well as cells infected with the adenoviral vector alone were used as controls. One μl or 10 nl 17α- or 17β-estradiol was added to cultures 24 h post-infection and incubation continued for 30 min or 24 h. Proteasome-dependent proteolytic degradation was inhibited by incubating cell cultures with 1 μM MG-132 and either 1 μl 17α-estradiol for 24 h or 17β-estradiol for 30 min. Incubation with MG-132 alone was used as a control.

**Generation of the Maxi-K Channel Mutant**—The maxi-K channel putative PEST motif mutant was generated by substitution of the -SPKK- amino acid sequence for -AAAA- (SPKK642) using the QuiKChange site-directed mutagenesis kit. The mutant was fused at the C terminus with eGFP tags and expressed in HEK293F cells. Channel expression and intracellular trafficking in response to 17β-estradiol were examined after 48 h. Cell culture dishes were mounted on a Zeiss 510 confocal scanning microscope (Zeiss, Germany) at 37 °C. Cell cultures were subsequently incubated for 45 min in medium containing 1 μM 17β-estradiol and 15 μM HEPES. Images were taken in 1-min intervals.

**Human Coronary Artery Smooth Muscle Cell Culture**—Cell cultures at passages 3–5 were grown on coverslips to 80–90% confluency in DMEM:F-12 (1:1) medium supplemented with fibroblast growth factor basic (0.5 ng/ml), epidermal growth factor (2 ng/ml), insulin (5 mg/ml),

**Experimental Procedures**

Materials and Reagents—HEK293F cells, fetal bovine serum (FBS), gentamicin, and fungizone were from Invitrogen. Human coronary artery smooth muscle cells were purchased from Cell Applications (San Diego, CA). Diethylstilbestrol, 17α-estradiol, and 17β-estradiol, anti-α-smooth muscle actin-fluorescein isothiocyanate antibody, iiberitoxin, and polyethyleneimine were purchased from Sigma. Mouse hybridoma supernatant against a conserved region of the maxi-K channel was generated in our laboratory using a synthetic peptide for amino acids 913–926 of the channel. A sheep polyclonal antibody was generated by using a glutathione S-transferase fusion protein encoding the first structural motif of the maxi-K channel (GenBank™ accession number U11058), containing preparations.

**Estradiol Effects on Maxi-K Channels**  

Estradiol was obtained from Amersham Biosciences. Mouse monoclonal anti-V5 antibody was obtained from Invitrogen, and rabbit polyclonal anti-β1-subunit antibody was from Affinity Bioreagents (Golden, CO). Biotin-conjugated IgG and streptavidin-conjugated fluorophores were purchased from Jackson ImmunoResearch (West Grove, PA). Human recombinant fibroblast growth factor basic and human epidermal growth factor were obtained from Insulin (5 mg/ml),

**FIG. 2.** Estrogens down-regulate membrane expression of transiently expressed maxi-K channels. Maxi-K channels in HEK293F cells were examined by immunocytochemistry. HEK293F cells express maxi-K channels in an estrogen-free environment on the cell membrane (A). 1 μM 17β-estradiol was added and protein expression examined 30 min later (B). The effect of 1 μM 17α-estradiol on maxi-K channel expression was examined following a 24-h incubation (C). Similarly, 10 nM 17β-estradiol (D) or 17α-estradiol (E) were introduced into the cell cultures and maxi-K channel expression examined 30 min later or 24 h later, respectively. Statistical significance of differences in expression levels (F) was assessed between estradiol-free controls (−/−E2) and 1 μM 17β-estradiol (1 μM, 17β) or 1 μM 17α-estradiol (1 μM, 17α). Effects of 10 nM 17β-estradiol (10 nM, 17β) or 10 nM 17α-estradiol (10 nM, 17α) were compared with controls (−/−E2). Significant differences (p < 0.05) from estrogen-free levels are indicated by asterisks. n = 9; scale bars = 10 μm (A, B, D, E) or 20 nM (C).

**FIG. 3.** Maxi-K channels are down-regulated in response to 17β-estradiol (live cell observation). The maxi-K channels fused at their C termini with eGFP tags were transiently expressed in HEK293F cells. The effect of 1 μM 17β-estradiol on channel expression was observed after applying the hormone at time 0 min (A) and 10 min later (B). Cells in hormone-free medium at 0 min (C) and 10 min (D) were used as controls. n = 3; scale bar = 10 μm.
Estradiol Effects on Maxi-K Channels

Fig. 4. Co-expression of the maxi-K channel with its accessory 
β1-subunit does not prevent estrogen-induced down-regulation. 
The maxi-K channels (maxi-K, A) expressed with their accessory β1-
subunits (β1, B) were incubated in either hormone-free medium or in 
medium supplemented with 1 μM 17β-estradiol for 30 min (C, D) or 
17α-estradiol for 24 h (E, F). Differences in expression levels of the 
maxi-K channels (G) was assessed between estradiol-free controls 
(−E2) and 17β- (17β) or 17α-estradiol (17α) incubated cells. Signif-
icant differences (p < 0.05) are indicated by asterisks. Expression levels 
of the β1-subunit resembled that of the maxi-K channels (not shown in 
a graph). n = 6; scale bars = 10 nm.

gentamicin (50 mg/ml), fungizone (250 μg/ml), and 5% FBS. Subconflu-
ent cultures were brought into a quiescent state by incubating in 
DMEM:F-12 (1:1) medium supplemented with 0.5% hormone-free FBS 
for 10–14 days and then incubated in the presence of 1 μM 17α-estradiol 
or 17β-estradiol for 48 h. Cultures incubated in medium devoid of 17α-
or 17β-estradiol were used as controls.

Immunocytochemistry—HEK293F cells were fixed in 2% paraform-
aldehyde and 0.1% Triton X-100 and then blocked in 5% heat-inacti-
vated FBS. The maxi-K channel was detected by subsequent incubations 
with a sheep polyclonal antibody directed against the first 
intracellular loop (1:100; 30 min at 37°C), biotin-conjugated donkey 
anti-sheep IgG (1:1000; 15 min at 37°C), and 1 μg/ml of streptavidin-
Cy5 (15 min at room temperature). β1-Subunit co-expressed with 
maxi-K channel was detected by rabbit polyclonal anti-β1 antibody 
(1:500) and donkey anti-rabbit-Cy3 (1:1600). Similarly, the maxi-K 
channels in human coronary artery smooth muscle cells were detected 
with maxi-K channel mouse hybridoma supernatant (1:50) and donkey 
anti-mouse-Cy3. α-Smooth muscle actin was then identified by direct 
immunofluorescence with anti-α-smooth muscle actin-fluorescein isothio-
cyanate antibody (1:500). Signals were visualized by Zeiss 510 confocal 
scanning microscope (Zeiss, Germany). The average signal intensity of 
each image was calculated using ImageJ software (National Institutes 
of Health).

Electrophysiology—All electrophysiological experiments were per-
formed at room temperature. HEK293 cells expressing the maxi-K 
channel stably (under G418 selection) or transiently were grown to 
confluence in 35-mm dishes in phenol red-free DMEM supplemented 
with 10% hormone-free FBS. Once confluent, cells were trypsinized and 
resuspended in bath solution. Whole-cell recording was performed and 
analyzed as described previously (19, 20) using a holding potential of 
−80 mV, prepulsed to −100 mV, and currents elicited at step potentials 
from −80 to +160 in 20-mV intervals. Iberiotoxin (200 nm) was used to 
confirm the presence of maxi-K channel current. 17α- and 17β-estradiol 
were added directly to the bath at a final concentration of either 10 nm 
or 1 μM.

Estrogen Binding Assay—HEK293F cells were transfected with ei-
ther the maxi-K channels or the maxi-K channel accessory β1-subunits 
or both constructs. Cells expressing the adenosivlar vector alone and 

Fig. 5. Inhibition of a proteasome-dependent proteolytic 
degradation pathway prevents estradiol-induced down-regulation 
of maxi-K channel membrane expression. HEK293F cells expressing 
the maxi-K channels were incubated in medium supplemented with 1 μM MG-132 (A) or 1 μM MG-132 + 1 μM 17β-estradiol for 30 min (B). 
Other cultures were incubated with MG-132 (C) or MG-132 + 1 μM 
17α-estradiol for 24 h (D). Statistical differences in expression levels (E) 
were assessed between 30 min control MG-132 and MG-132 + 17β or a 
24-h control MG-132 and MG-132 + 17α. Statistically significant dif-
ferences were not found. n = 5; scale bar = 10 nm.

MCF7 cells expressing endogenous nuclear ERα were used as controls. 
All cultured cells were grown in serum-free, phenol red-free medium 
24 h prior to harvesting. Preparation of cellular membranes was de-
scribed previously (21). Nuclear extracts of MCF7 cells were isolated 
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Estradiol Effects on Maxi-K Channels

Fig. 6. Mutation of the PEST motif in the maxi-K channel prevents estradiol-induced proteolytic degradation of the channel. The maxi-K channel containing the mutated PEST motif SPKK642–645AAAA was expressed in HEK293F cells. The mutant expression levels in estrogen-free medium (A) and medium supplemented with 1 μM 17β-estradiol for 30 min (B) were determined. No statistically significant difference in expression levels was detected (C). n = 4; scale bar = 10 nm.

5 ml of binding buffer using vacuum filtration and placed in 5 ml of scintillation mixture (RPI, Mount Pleasant, IL) overnight. Total and nonspecific binding was subsequently quantitated in a Beckman liquid scintillation spectrometer. Specific binding was calculated by subtracting nonspecific binding (competed) from total binding (non-competed).

Statistical Analysis—Data were expressed as mean ± S.E. Statistical significance of difference between control and experimental groups was calculated by paired t test. p value <0.05 indicated significant difference between groups.

RESULTS

HEK293 cells, which do not express endogenous estrogen receptors, were utilized to study the effects of 17α- and 17β-estradiol on maxi-K channels. Whole-cell recordings were performed in both HEK293 cell lines expressing the maxi-K channels either transiently or stably. In stable cell lines, both 1 μM 17α- and 17β-estradiol attenuated whole-cell maxi-K channel currents (Fig. 1, A and B). Maxi-K current was decreased ~45% by 17α-estradiol at +160 mV, although current was significantly inhibited at lower depolarizing voltages (Fig. 1C). A similar effect was observed with 17β-estradiol with an attenuation of maxi-K channel current by ~38% at +160 mV (Fig. 1D). Similar levels of current inhibition were seen in transiently transfected cells. Current inhibition was not detected within 10 min after addition of 10 nM 17α- and 17β-estradiol to the bath solution (Fig. 1, E and F). Currents were recorded for up to 15 min until seal integrity was compromised. Due to reports of endogenous K+ current in HEK293 cells (23), iberiotoxin, a maxi-K channel inhibitor, was added to the bath solution to determine the contribution of the maxi-K channel to the estrogen-sensitive whole-cell K+ current. The maxi-K channel comprised ~77% of the total K+ current in HEK293F cells and ~100% of the estrogen-sensitive component (data not shown).

The effects of estradiol on protein expression of transiently expressed maxi-K channels was studied immunocytochemically. A thirty minute incubation with 1 μM 17β-estradiol induced a profound (~80%) attenuation of channel expression as compared with estradiol-free controls (Fig. 2, A, B, and F). At a concentration of 1 μM, 17α-estradiol demonstrated a similar level of inhibition, but only after 24 h exposure (Fig. 2, C and F). Although down-regulation of maxi-K channels has also been observed following a 30 min incubation with 10 nM 17β-estradiol (~45%, Fig. 2, D and F), 17a-estradiol at the same concentration did not demonstrate an inhibition of channel expression after either a 30 min or 24 h incubation (Fig. 2, E and F). At a concentration of 100 nM, 17β-estradiol inhibited channel expression by ~60%, while 17α-estradiol was again ineffective at this concentration (data not shown). Preincubation of cells with 17β-estradiol (10 nM or 1 μM) for 24 h prior to infection inhibited maxi-K channels by ~80%. 17α-Estradiol produced a similar effect but only at 1 μM (data not shown). Neither estrogen affected infection efficiency as assessed by GFP reporter gene expression. Additionally, an inhibitory effect of 1 μM 17β-estradiol was observed in live HEK293F cells expressing maxi-K channels fused C-terminally with an eGFP tag. The response was detected as early as 10 min after addition of 17β-estradiol, although some cells demonstrated a delayed reaction detected after 40 min (Fig. 3, A and B). The difference in timing of the response may be related to the presence of the large C-terminal tag affecting the estrogen sensitivity of the channel. This response was clearly estrogen-dependent, since cells incubated in estradiol-free medium did not show a similar decrease in signal intensity (Fig. 3, C and D).

The activation of the maxi-K channels by estradiol has been reported to require the presence of ancillary β1-subunits (14, 15). We speculated that the accessory β1-subunit may be a stabilizing factor for the maxi-K channel in an estradiol-enriched environment. Endogenous β1-subunit transcript was not detected in HEK293F cells by reverse transcriptase-PCR (data not shown). Estradiol-induced down-regulation of maxi-K channels was observed in cells co-expressing the maxi-K channel with its accessory β1-subunit (Fig. 4, A and B) after a 30-min incubation with 17β-estradiol (Fig. 4C) or a 24-h incubation with 17α estradiol (Fig. 4E). In addition, β1-subunit expression was attenuated in a similar manner (Fig. 4, D and F). Thus the presence of the β1-subunit yielded the same degree of channel suppression observed when maxi-K channels were expressed alone (Fig. 4G).
Estradiol-mediated down-regulation of maxi-K channel membrane expression may indicate that proteasome-dependent proteolytic degradation may be responsible, since this mechanism is involved in down-regulation of the ligand-activated ERα (24). According to this hypothesis, inhibition of the proteasome complex would stabilize the maxi-K channel protein on the cell membrane. We examined the effects of the proteasome inhibitor, MG-132, on either a 17β-estradiol- (Fig. 5, A and B) or a 17α-estradiol-induced (Fig. 5, C and D) down-regulation of the maxi-K channel. The data demonstrate that incubation in the presence of MG-132 was sufficient to prevent down-regulation of maxi-K channels by estradiola (Fig. 5E). A similar effect was detected using 10 µM lactacystin, another proteasome inhibitor (data not shown). Analysis of the secondary structure of maxi-K channel using several Web-based databases (ExPASy, PESTFind) revealed the presence of a putative PEST motif -RLEDEQPSTLSPKK- in its intracellular C terminus, which may be mediating recognition and degradation of the maxi-K channel by the proteasome (25, 26). The functional significance of this motif was determined by substituting the -SPKK- amino acids with -AAAA- (SPKK642–645AAAA) and assessing the response of the mutant to 17β-estradiol (Fig. 6, A and B). Resistance to estrogen-induced proteolytic degradation and a tendency toward increased channel expression was observed following a 30-min incubation with 1 µM 17β-estradiol (Fig. 6, A–C). These data indicate that the proteasome mediates channel degradation in response to estrogens.

Ligand binding is required for the estradiol receptor to be targeted to, and degraded by, proteasomes (24, 27). It has also been reported that the estrogen sensitivity of the maxi-K channel is conferred by its accessory β1-subunit (14, 15). Therefore, we attempted to determine whether estrogen binding to the maxi-K channels, independent of the β1-subunit, induces proteolytic degradation of the channel protein. Specific binding was compared between sham-transfected controls (Fig. 7, sham) and each experimental group. Maxi-K channels (Fig. 7, maxi-K), but not β1-subunits (Fig. 7, β1), demonstrate specific binding when incubated with 10 nM [6,7-3H]17β-estradiol. However, accessory β1-subunits co-expressed with maxi-K channels (Fig. 7, maxi-K+β1) significantly increased specific binding of estradiol to the channel. MCF7 cells expressing endogenous estradiol receptors (Fig. 7, MCF7) were used as a positive control and also demonstrated specific binding. The presence of estradiol receptors in these cells was confirmed by immunoblotting (data not shown).

The physiological relevance of the estrogen effect on maxi-K channels was confirmed in cultured human coronary artery smooth muscle cells. Similar to what was detected in the heterologous system, prolonged exposure to 1 µM 17β-estradiol (Fig. 8, A and B) and 17α-estradiol (Fig. 8C) resulted in significant down-regulation of endogenous maxi-K channels. Down-regulation of the maxi-K channels occurred despite the presence of endogenous estrogen receptors in smooth muscle cells.

The data presented in this paper suggest that estrogens have an inhibitory effect on the expression of the maxi-K channel protein and on the current generated by this channel. Down-regulation of the channel appears to be induced by the binding of 17β- or 17α-estradiol to the channel, followed by subsequent proteasome-mediated proteolytic degradation. The ancillary β1-subunit did not prevent estrogen from down-regulating the maxi-K channel. Although the β1-subunit itself does not function as a binding site for 17β-estradiol, it appears to greatly facilitate estrogen binding to the maxi-K channels.

**DISCUSSION**

Controversy stirred by the findings of recent clinical trials on hormone replacement therapy has renewed interest in the physiological mechanisms underlying estrogenic protective effects in peripheral tissues that might explain cardiovascular and cognitive health benefits of estrogens in premenopausal women (1, 28). It is becoming apparent that estrogen per se is not sufficient for such protection, and the ultimate effects of this steroid may differ depending on unidentified signaling pathways. In the present study, we present one potential pathophysiological foundation for the increase in risk of various complications associated with continuous exposure to estradiol (as in prolonged use of estrogen-containing preparations, environmental pollution with estrogenic compounds, etc.). We provide evidence that exposure to estrogens may cause a significant down-regulation of the membrane expression of maxi-K channels. These channels generate significant repolarizing K⁺ current to buffer cell excitability and mediate a variety of functions in normal and diseased tissues including brain, smooth, and skeletal muscle. Consequences of such down-regulation would be tissue-specific and may be potentially negative for the function of normal tissue. In vascular smooth muscle, for example, these channels maintain the normal myogenic tone of the vascular wall. Loss of this channel may lead to an impaired vascular relaxation and response to contractile stimuli.

Although estrogen-induced degradation of the maxi-K channel protein has not been described, previous studies have reported variable effects of estrogens on the maxi-K channel current. Increases in repolarizing current and open-state probability, as well as decreases in unitary conductance, have been demonstrated (12, 15, 29). In our experiments the effects of 17β-estradiol on maxi-K channel expression were consistently inhibitory over a wide range of concentrations, whereas 17α-estradiol inhibited expression only at a high concentration. If
both estrogens use the same binding site(s) to inhibit maxi-K channels, 17α-estradiol may bind with lower affinity (24). Although the exact binding affinity of 17α- and 17β-estradiol for the maxi-K channel has not been evaluated, the dose response data for 17β-estradiol are consistent with a binding affinity lower than that reported for intracellular estrogen receptors. It is important to mention that maxi-K channels lack a region homologous to the ligand-binding domain of the estradiol receptor. Thus, other motifs may be recognized by estrogen as potential binding sites. Elucidating this site may provide new insights into estrogenic effects not mediated by nuclear estradiol receptors.

The data presented here do confirm a role for the maxi-K channel accessory β1-subunit in regulation of the maxi-K channels sensitivity to estrogen, although our results do not suggest direct binding of estradiol to this subunit. Analysis of our binding assays revealed that total estradiol binding in cells expressing the β1-subunits alone was significantly higher when compared with other experimental groups and controls, as reported previously (14). However, nonspecific binding was not statistically different from total binding. It is possible that the β1-subunits expressed alone may change the biophysical properties of the cell membrane to induce trapping rather than specific binding of estradiol. The enhanced level of specific binding observed in maxi-K channels co-expressed with their β1-subunits implies a role for these subunits in inducing conformational changes in the maxi-K channel protein favorable for specific estradiol binding. Two mechanisms potentially explain the observed increase: 1) conformational change increases the affinity of the binding site or 2) a conformational change involving more binding sites on the maxi-K channels available to estradiol. Further research is necessary to determine the mechanism responsible for this increase. It is also possible to speculate, although it was not specifically addressed here, that an increase in specific binding would allow for easier recognition of estrogen-bound maxi-K channels by the proteasome and, thus, a faster turnover rate.

It has been confirmed by numerous studies that endogenous estrogens exert effects beyond their reproductive actions. Our study, as well as previously published work (30), suggest that the multiplicity of peripheral actions of estrogen may be explained by its recognition by proteins in addition to the classical estrogen receptors, that are capable of low affinity binding. Estrogen-bound proteins are then directed along estradiol signaling pathways and degraded by the proteasome similar to classical nuclear estrogen receptors (27). Consequences of such down-regulation for cell or tissue function would depend on the particular protein involved and the degree of inhibition. The presence of nuclear estrogen receptors does not prevent low affinity binding to other proteins, as shown by the experiments on the effects of estrogens on the expression of endogenous maxi-K channels in smooth muscle cells. Although conditions necessary for estradiol binding to proteins besides the estradiol receptors have not been investigated, it is reasonable to speculate that saturation of "traditional" estradiol binding sites (i.e. estradiol receptors) by high concentrations of estrogen and continuous supply of free unbound estradiol would favor low affinity binding. Use of therapeutic or environmental estrogenic compounds may produce such conditions due to relatively high doses and a lack of diurnal variations characteristic of ovarian production of estrogen (31, 32).

In summary, estrogens down-regulate maxi-K channel cell surface expression and repolarizing current. Down-regulation involves specific binding of estrogen to the maxi-K channel and induction of a proteasome-dependent proteolytic degradation.

This action can be elicited independently of classical nuclear estrogen receptors or the accessory β1-subunit, but in the presence of β1-subunits specific binding of estradiol to maxi-K channels is increased significantly. A similar down-regulation has been reported in response to chronic exposure to glucocorticoids, although this study did not address the possibility that this response could be mediated by binding of this class of steroids to the channel (33). The results from this study demonstrate the importance of elucidating the mechanisms of non-reproductive action of estradiol given the widespread therapeutic and environmental use of agents with estrogenic activity.

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