Acute Activation of Maxi-K Channels (*hSlo*) by Estradiol Binding to the β Subunit

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Maxi-K channels consist of a pore-forming α subunit and a regulatory β subunit, which confers the channel with a higher Ca^{2+} sensitivity. Estradiol bound to the β subunit and activated the Maxi-K channel (*hSlo*) only when both α and β subunits were present. This activation was independent of the generation of intracellular signals and could be triggered by estradiol conjugated to a membrane-impenetrable carrier protein. This study documents the direct interaction of a hormone with a voltage-gated channel subunit and provides the molecular mechanism for the modulation of vascular smooth muscle Maxi-K channels by estrogens.

Women are less susceptible to cardiovascular disease before the onset of menopause when cessation of ovarian hormone production is accompanied by an increased incidence of coronary heart disease (*1*). In addition to the genomic actions of estrogens in the vasculature (*2*), 17β-estradiol acutely restores impaired coronary blood flow in postmenopausal women (*3*). Acute (nongenomic) vascular relaxation induced by 17β-estradiol is predominantly endothelium-independent (*4*) and mediated by stimulation of the gating of Maxi-K channels (*5, 6*) or an inhibition of L-type Ca^{2+} channels (*6, 7*) or both. However, identification of the nongenomic pathways mediating acute modulation of membrane ion channels by estrogens (*8*) has remained elusive (*9*). As yet there is no evidence at the molecular level linking the rapid modulation of ion channels with membrane binding sites for estrogens (*10*) or estrogen-generated intracellular signals (*11*).

Maxi-K channels, key modulators of vascular smooth muscle tone (*12*), are formed by two subunits: the pore-forming α subunit (*13*) and the regulatory β subunit, which increases the channel sensitivity to Ca^{2+} (*14, 15*). We studied the modulation by 17β-estradiol of both native and heterologously expressed Maxi-K channels and found that estradiol

activates the Maxi-K channel through its binding to the β subunit.

Xenopus laevis oocytes were injected with mRNA encoding the α subunit either alone or in combination with mRNA encoding the β subunit (*16*) and Maxi-K currents were recorded (*17*). Maxi-K currents recorded in patches expressing α subunits exhibited faster kinetics (Fig. 1A) than in patches expressing α and β subunits (Fig. 1C) (*15*). The presence of 17β-estradiol in the pipette solution elicited an increase in the currents recorded in patches expressing α and β subunits (Fig. 1D) but not in those expressing only the α subunit (Fig. 1B). 17β-Estradiol also accelerated the current kinetics (Fig. 1D) and shifted the open probability-voltage (P_o-V) curves obtained from the oocyte expressing α and β subunits (Fig. 1F), but not from that expressing only the α subunit (Fig. 1E). Figure 2A shows that half-activation voltage ($V_{1/2}$) of the P_o-V curves shifted with time of patch exposure to 17β-estradiol toward more negative potentials in those patches expressing α and β subunits, whereas $V_{1/2}$ obtained in α subunit–expressing patches showed no alteration. The estrogen-induced shift in $V_{1/2}$, when compared with the control taken immediately after 17β-estradiol addition (time 0) to patches expressing α and β subunits, was more pronounced at $[Ca^{2+}] < 10^{-7}$ M, tending to vanish as $[Ca^{2+}]$ was increased (Fig. 2B). This observation suggested that the calcium-dependent and estrogen-dependent effects of the β subunit on the pore-forming α subunit are mediated by different mechanisms. Estrogen increased the ionic current induced by the α-β complex in the nanomolar range of internal Ca^{2+}, unlike the Ca^{2+}-dependent increase in current associated with the expression of the β subunit, which started

developing at $[Ca^{2+}] > 300$ nM and was fully attained at 3 μM Ca^{2+}.

To obtain a recording condition in which Maxi-K currents could be monitored before and after the addition of 17β-estradiol, we used the cut-open oocyte voltage clamp technique (*18*) (Fig. 2C). A dose-response curve for the activation of Maxi-K currents by 17β-estradiol yielded a half-maximal concentration of 2.6 μM (Fig. 2D), which parallels the median inhibitory concentration (IC_{50}) (2.2 μM) of 17β-estradiol inhibition of vascular smooth muscle contraction in different mammalian species (*19*).

17β-Estradiol coupled to bovine serum albumin (BSA)–activated Maxi-K channels only when added externally (Fig. 3A). This result provided evidence for an extracellular site of estradiol action. We screened effects of the stereoisomer 17α-estradiol. When added externally, 17α-estradiol shifted the P_o-V curve to the left along the voltage axis (Fig. 3B), although it was much less potent than 17β-estradiol. At 0 mV, 17β-estradiol induced an ~50-fold increase in P_o, whereas the increase in P_o evoked by 17α-estradiol was only ~3-fold above the control. Similar partial agonist effects have been described for 17α-estra-
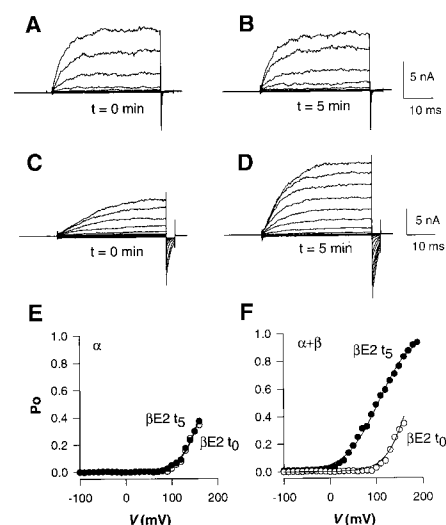
**Fig. 1.** Differential effect of 17β-estradiol (βE2) on Maxi-K currents recorded in oocytes expressing α or α and β channel subunits. Currents were recorded from inside-out macropatches held at 0 mV and pulsed from −100 to +150 mV in 10-mV voltage steps of 40-ms duration. Tail currents were recorded at −60 mV. Internal $[Ca^{2+}]$ was 56 nM. The pipette solution contained 5 μM 17β-estradiol. Currents from macropatches expressing α (**A** and **B**) and α and β (**C** and **D**) subunits immediately after seal formation [(A and C); $t = 0$ min] and 5 min later [(B and D); $t = 5$ min]. Open probability versus voltage (P_o-V) curves were obtained from tail current measurements from the experiments shown in (A) and (B) (**E**), and (C) and (D) (**F**). Boltzmann fitting to the experimental data is indicated by solid lines.

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Fig. 2. (A) Kinetics of the estrogen-induced effect on Maxi-K channels. The half-activation voltage ($V_{1/2}$) was calculated from a Boltzmann's fit to P_o-V curves measured at different times (0 to 5 min) after patch excision into a solution containing 56 nM Ca^{2+}. The pipette solution contained 5 μM 17β-estradiol. Values are from patches expressing α (open circles; $n \geq$ 3) or α and β (solid circles, $n \geq 3$) subunits and are presented as mean ± SD. Changes in $V_{1/2}$ measured in oocytes expressing α and β subunits were different from those obtained in oocytes expressing α subunits ($P = 0.024$ at 2 min and $P = 0.035$ at 5 min, t test). **(B)** The Ca^{2+} dependence of the estrogen-induced activation of Maxi-K channel. Values of $V_{1/2}$ (mean ± SD) were plotted against internal $[Ca^{2+}]$ in oocytes expressing α and β subunits in the presence (solid circles; $n \geq 3$) or in the absence of (open circles; $n = 3$ to 62) 5 μM 17β-estradiol. **(C)** Maxi-K currents recorded with the oocyte cut-open voltage-clamp technique in an oocyte expressing α and β subunits. Current traces were obtained in response to a +100-mV step from 0 mV in the absence (control) or in the presence of 5 μM 17β-estradiol (βE2). **(D)** Dose-response curve of estrogen-induced activation of Maxi-K. The ordinate shows the quotient between maximum steady-state currents for a +100-mV step in the presence ($I_{βE2}$) and in the absence ($I_{control}$) of 17β-estradiol. The solid line is a fit to the data (closed circles; $n = 3$ to 4) determined with an equation of the form $I_{βE2}/I_{control} = (1 + I_{βE2max}/I_{control})(1 + (K/([βE2]))^n)$ where $I_{βE2max}$ is the current obtained at the highest $[βE2]$, K is the apparent dissociation constant, and n is the Hill coefficient. The best fit determined with a nonlinear least squares fitting procedure gave $n = 1.4$, $K = 2.6$ μM, and $I_{βE2max} = 4.25$.
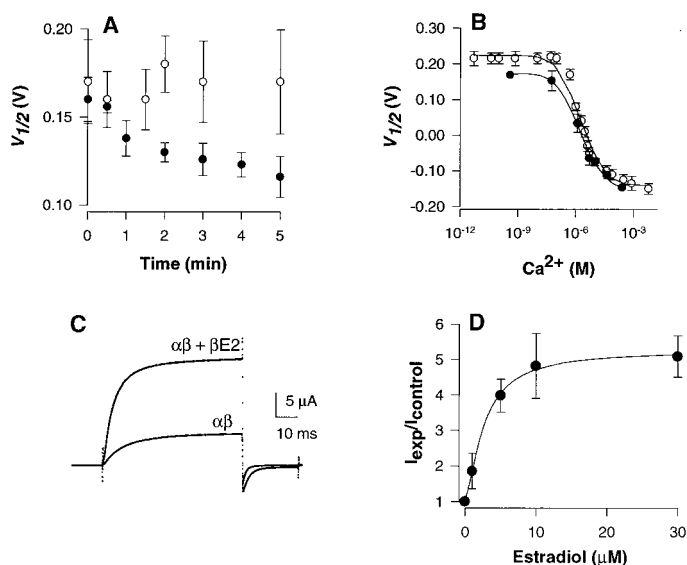
Fig. 3. (A) Activation of Maxi-K channels by membrane-impermeable estrogens. A pipette solution containing 1 μM 17β-estradiol 6-(O-carboxymethyl)oxime-BSA was used to elicit currents from a macropatch of an oocyte expressing α and β subunits. Current traces were obtained by pulsing from 0 to 100 mV, immediately (BSA-βE2 t_0) and 5 min after excision into a solution containing 56 nM Ca^{2+} (BSA-βE2 t_5). **(B)** Stereospecificity of estradiol effect. P_o-V curves were obtained from Maxi-K tail current measurement in response to 17α-estradiol measured at $t = 0$ min (solid squares, αE2 t_0) and $t = 5$ min (open circles, αE2 t_5) or 17β-estradiol (solid circles, $t = 5$ min); $n = 3$ for both 17α- and 17β-estradiol. G, conductance. **(C)** Binding of [³H]estradiol to oocyte membranes. The quantity of [³H]estradiol bound is expressed in counts per minute per milligram of protein. The amount bound to membranes from control oocytes was subtracted from the values obtained with membranes from oocytes injected with α, β, or α and β subunits of the *hSlo* channel. Data were obtained from five different membrane preparations. The number of binding experiments in each condition was 10. Error bars are standard deviation from the mean. A Kruskal-Wallis multiple comparison z-value test showed that medians for the percentage of binding

of membranes containing β and α and β subunits of the *hSlo* channel is significantly different from control (z-value > 1.96). Binding of the estrogen to membranes containing β or α and β subunits was not statistically different (z-value > 0.84). Fluorescence labeling of HEK-293 cells stably expressing α subunit **(D)** or α and β subunits **(E)** and control untransfected cells **(F)**. Fluorescence pictures are at left with their corresponding relief-contrast images at right.
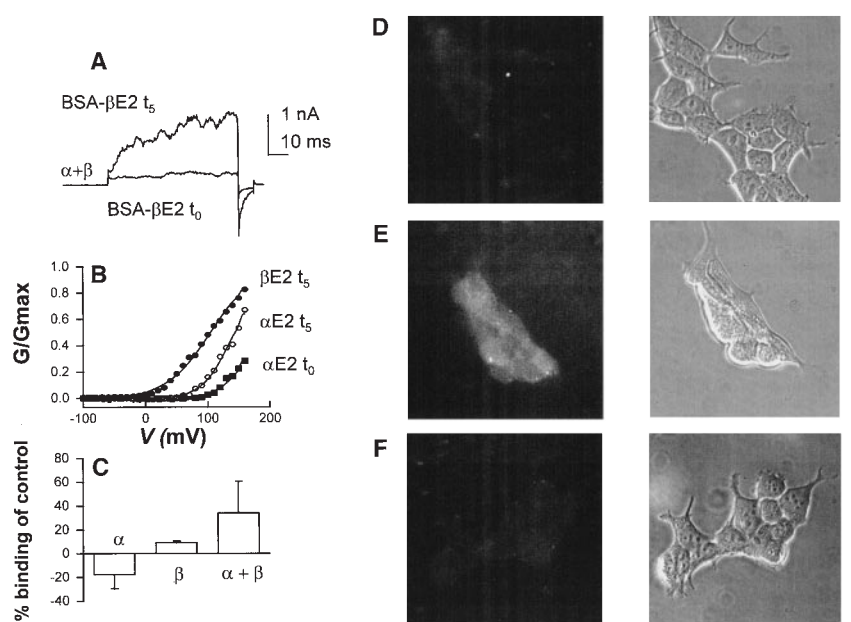
diol in arterial smooth muscle preparations (*19*). All these observations suggested a direct interaction of estrogens with the channel. We confirmed this idea by measuring binding of [³H]estradiol to membrane fractions obtained from control oocytes (water injected) or oocytes injected with mRNA coding for α, β, or α and β subunits (*20*). A significant increase in [³H]estradiol binding was accomplished only in membranes obtained from oocytes injected with mRNA coding for the β subunit or α and β subunits (Fig. 3C). We

applied 50 μM 17β-estradiol linked to fluorescein isothiocyanate (FITC)–labeled BSA to HEK-293 cells stably expressing α or α and β subunits (*21*). HEK-293 cells lack either a nuclear-cytoplasmic estrogen receptor (*22*) or an estrogen-binding site on their membrane (*23*), ruling out the possibility that expression of β subunits may increase the expression (or translocation to the membrane) of an estrogen receptor endogenous to these cells.

Fluorescence labeling was absent in native (Fig. 3F) or α subunit–expressing

HEK-293 cells (Fig. 3D), whereas cells stably expressing α and β subunits were clearly labeled (Fig. 3E). These results imply that the Maxi-K channel, through its β subunit, may act as a low-affinity membrane estrogen receptor.

We also studied the effect of estradiol on Maxi-K channels in native channels, obtained from rat skeletal muscle (Fig. 4A) and bovine aortic smooth muscle (Fig. 4B), that had been incorporated into artificial lipid bilayers (*24*). Although the single-channel properties of both preparations are
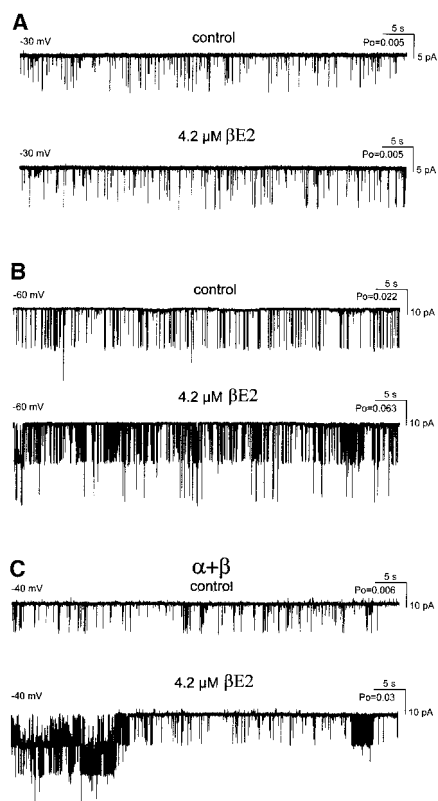
Fig. 4. Effect of 17β-estradiol on Maxi-K channels reconstituted in lipid bilayers. Channels obtained from skeletal muscle (**A**), smooth muscle (**B**), or *Xenopus* oocytes expressing α and β subunits (**C**) were recorded at −30 mV, −60 mV, and −40 mV, respectively, before and after the addition of 4.2 μM 17β-estradiol to the external side of the membrane.

identical (*25*), due to the presence of a highly preserved α subunit, skeletal muscle is almost devoid of β subunit (*26*). 17β-Estradiol only activated Maxi-K channels from aortic smooth muscle preparations (Fig. 4B). The P_o increased from 0.022 to 0.063 upon addition of 4.2 μM 17β-estradiol. Similarly, the P_o of Maxi-K channels reconstituted from membrane fractions of oocytes expressing α and β subunits (Fig. 4C) increased from 0.006 to 0.03. In contrast, the P_o of the channel from skeletal muscle (Fig. 4A) did not change significantly with the addition of the steroid ($P_o = 0.005$). The mean increase in aortic smooth muscle Maxi-K channel activity in response to 17β-estradiol ($P_{oβE2}/P_{ocontrol}$) at concentrations of 0.42 and 4.2 μM was 3.6 ± 1.3 ($n = 4$) and 5.9 ± 1.9 ($n = 4$), respectively. The bilayer experiments also demonstrated that no intracellular signaling is required for 17β-estradiol activation of

the Maxi-K channel from either aortic smooth muscle cells (Fig. 4B) or oocytes expressing α and β subunits (Fig. 4C).

Our electrophysiological and binding studies demonstrate that direct interaction of estradiol with an external binding site available in the presence of β subunit is sufficient to mediate the effects of estrogen on Maxi-K channels. These results provide insight into the significance of the so-called regulatory subunit of the Maxi-K channel and may explain the functional diversity of estrogen action on these channels in terms of the coassembly of α and β subunits in different tissues.

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solution. Free [Ca^{2+}] was measured with a Ca^{2+} electrode. The current signal was digitized to a frequency equal to five times the filter cut-off frequency. The external solution used when the cut-open oocyte voltage clamp was used consisted of 110 mM KMES, 10 mM Hepes, 2 mM $CaCl_2$, pH 7. The acquisition and basic analysis of the data were performed with pClamp 6.0 software.
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21. Human embryonic kidney cells (HEK-293) were cultured in phenol red–free Dulbecco's modified Eagle's medium containing 10% fetal calf serum in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. HEK-293 cells stably expressing α or α and β Maxi-K channel subunits [A. G. Clark, S. K. Hall, M. J. Shipston, *J. Physiol. London* **516**, 45 (1999)] were cultured for 24 hours in 13-mm cover slips, then the cells were incubated in 10 μM 17β-estradiol 6-(O-carboxymethil)oxime-BSA-FITC conjugate for 1 hour at 37°C. Cells were washed twice with phosphate-buffered saline (PBS) and viewed with a Leica DMIL inverted microscope. Fluorescence studies are representative of three independent experiments.
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