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Effect of 17β-Estradiol on mRNA Expression of Large-Conductance, Voltage-Dependent, and Calcium-Activated Potassium Channel α and β Subunits in Guinea Pig

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Large-conductance, voltage- and calcium-activated potassium (MaxiK) channels play a key role in cell excitability. MaxiK channels are composed of a pore-forming α-subunit and a regulatory β-subunit, of which four (β1–4) genes have been identified. Previous findings suggested that MaxiK channel activity is regulated by estradiol. However, the underlying mechanisms have remained incompletely documented. Therefore, we used reverse transcriptase polymerase chain reaction to clone four cDNA fragments that were specific to the guinea pig α, β1, β2, and β4 genes. Using a sensitive ribonuclease protection assay, we found that the α and β4 mRNAs were the most abundant mRNAs in the brain and pituitary, whereas in the aorta, the α-subunit was coexpressed with the β1-subunit. Moreover, there was a significant upregulation of the α- but not the β1-subunit mRNA and the α-subunit protein in the aorta of the estrogen- vs oil-treated ovariectomized animals. In specific brain areas including preoptic area, ventral hypothalamus, hippocampus, and amygdala, and in the pituitary, neither the α- nor β4-subunit mRNAs were affected by estrogen. These findings suggest that estrogen may not affect the mRNA expression of MaxiK channels in the brain and pituitary. However, estrogen causes increased expression of MaxiK α in the aorta, which may explain some of the cardioprotective effects of estrogen in women.

Key Words: MaxiK channel subunits; mRNA expression; estradiol effects; brain; aorta.

Introduction

Large-conductance, voltage- and calcium-activated potassium (MaxiK) channels, also referred to as big K (BK) or slow-poke (Slo) channels, play a pivotal role in cell excitability. MaxiK channels are thought to modulate neuronal firing, hormone secretion, and smooth muscle tone by fine-tuning cell excitability *(1–5)*. Structurally, MaxiK channels are composed of a pore-forming α-subunit and a regulatory, α-binding β-subunit *(6,7)*. By binding to the α-subunit, the β-subunit may influence its calcium, voltage, and toxin sensitivities *(8–11)*. One gene (*slo*) encoding for an α-subunit has been identified, and its corresponding mRNA has been detected in both brain and peripheral tissues including the pituitary *(12–15)*. Four other genes encoding for 1–4β-subunits have also been identified. The expression of the respective transcripts displays a highly tissue-dependent specificity. For example, the β1, β2, β3, and β4 mRNAs are more enriched in smooth muscle, ovary, testis, and brain, respectively *(9,16)*.

Numerous findings suggest that MaxiK channels might constitute an important cellular target for 17β-estradiol. Indeed, this sex steroid hormone can affect the gating properties of the α/β complexes *(8,17)* by binding to the β-subunit *(18)*. In the brain, estradiol has been found to inhibit neurons in the area postrema by activating MaxiK channels *(19)*. In addition, estradiol has been shown to have cardioprotective effects through a number of mechanisms *(20)*. For example, during coronary hypoperfusion in dogs, estradiol increases coronary blood flow by increasing nitric oxide (NO) release and the opening of MaxiK channels *(21)*. In terms of transcriptional regulation of MaxiK channels, it has been reported that the expression levels of the α-subunit (mRNA and/or protein) are downregulated in the uteri of pregnant vs nonpregnant rats, suggesting a possible role for estradiol and/or progesterone hormones *(22,23)*.

Based on these and other observations, we predicted that estrogen treatment would alter the mRNA expression of the α- and/or β-subunit in the hypothalamus and other steroid-sensitive regions such as the hippocampus, amygdala, and pituitary. Therefore, we sought to document the subunit com-

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Table 1
Sequence Homology Between
Quinea Pig MaxiK Channel Subunit PCR Fragments
and Their Respective Mammalian Counterparts[a]

Guinea pig	Human (%)	Mouse (%)	Rat (%)	Rabbit (%)
α	92	93	91	90
β1	90	82	88	89
β2	94	87	NA	NA
β4	96	NA	92	NA

[a]Guinea pig MaxiK α, β1, β2, and β4 subunit PCR fragments were sequenced, and then each sequence was compared to the respective human, mouse, rat, and rabbit subunit sequences that were found in the GenBank database. The percentage of homology is indicated for each pair of sequences. NA, not available.

position of MaxiK channels in the guinea pig brain and peripheral tissues, and the effects of estrogen on the expression of MaxiK channel mRNAs.

Results

Reverse Transcriptase Polymerase Chain Reaction Cloning and Sequencing

To evaluate the mRNA expression of MaxiK channel subunits in the guinea pig brain and periphery, we cloned guinea pig MaxiK α-, β1-, β2-, and β4-subunit cDNA fragments that were 357, 453, 381, and 432 bp, respectively. These fragments showed a high percentage of homology to their respective human MaxiK channel subunits. Indeed, the guinea pig α, β1, β2, and β4 cDNA fragments were found to be, respectively, 92, 90, 94, and 96% homologous to their human counterparts (Table 1). In addition, the guinea pig α, β1, and β4 fragments were found to be highly homologous to other mammalian MaxiK channel subunits (Table 1). For example, the guinea pig α PCR clone was found to be 93, 91, and 90% homologous to, respectively, mouse, rat, and rabbit MaxiK α sequences. The guinea pig β1 fragment was found to be 89, 88, and 82% homologous to the rabbit, rat, and mouse MaxiK β1 sequences, respectively. The guinea pig β4 fragment was found to be 92% homologous to the rat MaxiK β4 sequence. Moreover, during the preparation of this article, a mouse MaxiK β2 sequence was published (AY062429). We found that this sequence shares 87% homology with our guinea pig β2 sequence.

Expression Profile of MaxiK Channel Subunits as Demonstrated by Ribonuclease Protection Assay

We used a sensitive ribonuclease protection assay (RPA) to measure the expression of the MaxiK α, β1, β2, and β4 mRNAs in discrete brain regions and in other tissues of the guinea pig. Initially, we used male guinea pigs, because of the limited supply of females, to study the subunit composition and distribution in the brain and periphery. Known concentrations of sense RNA (62.5–8000 fg) were used as standard to quantify the mRNA expression (Figs. 1–3). Linear regression analyses of the respective MaxiK subunit standard curves revealed an r value of 0.992–0.993 (Figs. 1–3). The antisense [^{32}P]rUTP-labeled riboprobes of the MaxiK α-, β1-, β2-, and β4-subunits each protected a single RNA band of the appropriate nucleotide size (Figs. 1–3). The MaxiK α mRNA was broadly distributed throughout the brain (Fig. 1, Table 2; n = 2–4). Indeed, MaxiK α mRNA was highly detectable in 5-μg samples of total RNA obtained from a number of brain areas (Fig. 1, Table 2). The levels of MaxiK α mRNA expression ranged from a high of 377.3 ± 119.4 fg/μg in the prefrontal cortex to a low of 55.3 ± 24.0 fg/μg in the pons. The rank order of MaxiK α mRNA concentrations was as follows: prefrontal cortex ≥ sensorimotor cortex ≥ thalamus ≥ cerebellum ≥ hippocampus ≥ dorsal hypothalamus (dHTH) ≥ septum ≥ striatum ≥ preoptic area (POA) ≥ ventral tegmental area/substantia nigra ≥ ventral hypothalamus (vHTH) > pons.

When compared with the expression of MaxiK α mRNA, the expression of MaxiK β1 mRNA in the brain was quite low, with values ranging from 59.2 fg/μg in the thalamus to 5.8 ± 2.1 fg/μg in the cerebellum (Fig. 2, Table 2; n = 2–4). The rank order of β1 mRNA concentrations in the guinea pig brain was as follows: thalamus ≥ POA ≥ VHTH ≥ pons ≥ hippocampus ≥ septum ≥ striatum ≥ sensorimotor cortex ≥ cerebellum (Fig. 2D, Table 2). In contrast to findings in the brain, we found high levels of β1 mRNA expression in peripheral smooth muscle–derived tissues such as the aorta, bladder, and colon, with values ranging from 1875.0 ± 475.8 fg/μg in the aorta to 440.2 ± 125 fg/μg in the colon (Fig. 2). A high level of β1 mRNA expression was also measured in the uterus (560.5 fg/μg; n = 2), which is consistent with its critical role in smooth muscle cell excitability *(24)*. By comparison, much lower levels of β1 mRNA expression were found in the skeletal muscle (92.0 fg/μg; n = 1) and heart (22.6 ± 11.3 fg/μg; n = 3).

Expression of the MaxiK β2 mRNA, similar to β1, was quite low in the brain, with values ranging from 29.1 fg/μg in the vHTH to 4.5 fg/μg in the cerebellum (Table 2). In addition, expression of the β2 mRNA subtype was also low in peripheral tissues such as the heart, colon, aorta, and bladder, with values ranging from 67.1 fg/μg in the heart to 8.1 fg/μg in the bladder. In the uterus, β2 mRNA expression was barely above background levels.

In contrast to the MaxiK β1 and β2 mRNAs, the concentrations of β4 mRNA in the brain were relatively high (Fig. 3, Table 2). The highest level of MaxiK β4 mRNA was found in the prefrontal cortex (424.8 ± 148.2 fg/μg; n = 4) and the lowest level was detected in the pons (34.3 fg/μg; n = 2) (Table 2). The rank order of concentrations of MaxiK β4 mRNA in the guinea pig brain was as follows: prefrontal cortex ≥ sensorimotor cortex ≥ septum ≥ striatum ≥ POA ≥ ventral tegmental area/substantia nigra ≥ thalamus

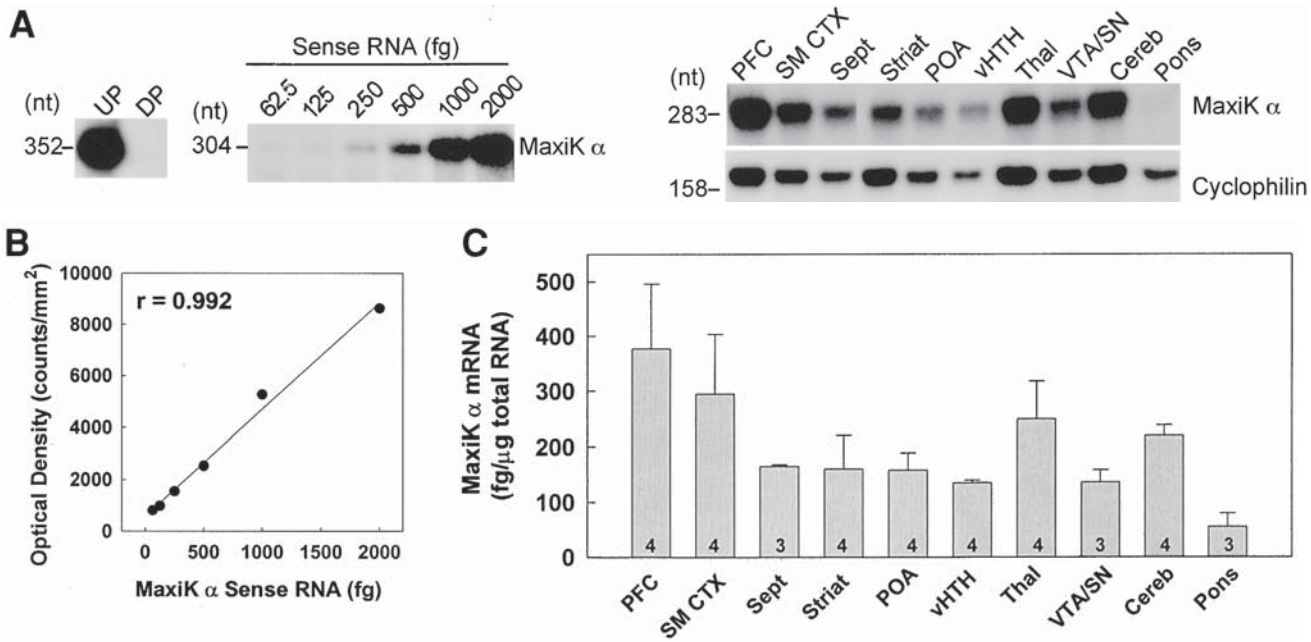

Fig. 1. Quantitative distribution of MaxiK α-subunit mRNA in guinea pig brain. (**A**) Representative film image of RPAs illustrating ^{32}P-labeled undigested (UP) and digested (DP) MaxiK α riboprobe, its respective sense RNA standard curve (62.5–2000 fg), and specific MaxiK α signal (exposure time of ~16 h; cyclophilin exposure time of ~7 h) obtained from ~5 μg of total RNA in each brain region. (**B**) Linear regression analysis of the MaxiK α sense standard curve using a phosphorimager revealed $r = 0.992$. (**C**) Distribution and quantitative analysis of MaxiK α mRNA in discretely dissected brain regions of guinea pig. Each MaxiK α band was normalized to its corresponding cyclophilin band and quantified from each sense mRNA standard curve. Each bar represents a mean ± SEM of three to four measurements. The number of animals is indicated at the bottom of each bar. PFC, prefrontal cortex; SM CTX, sensorimotor cortex; Sept, septum; Striat, striatum; POA, preoptic area; vHTH, ventral hypothalamus; Thal, thalamus; VTA/SN, ventral tegmental area/substantia nigra; Cereb, cerebellum; nt, nucleotide.

≥ cerebellum ≥ VHTH ≥ dHTH ≥ hippocampus ≥ pons. In the periphery, however, expression of MaxiK β4 mRNA in smooth muscle–derived tissues such as the aorta and the uterus (8.6 and 8.4 fg/μg, respectively; $n = 2$ each) was much lower than MaxiK β1 mRNA. The mRNA expression of β4 mRNA was also low in the heart (24.1 fg/μg; $n = 2$).

Distribution in Female and Effect of 17β-Estradiol on Expression of MaxiK Channel Subunits in Brain and Pituitary

Analysis of MaxiK channel subunit distribution as outlined earlier indicated that expression of the α/β4 mRNAs predominated in the brain, whereas that of the α/β1 mRNAs predominated in the smooth muscle–enriched tissues. Since a number of studies suggested that estrogen may regulate MaxiK channels, we used a sensitive and quantitative RPA to explore MaxiK channel subunit distribution in ovariectomized (OVX), oil- and estrogen-treated female guinea pigs, focusing on steroid-sensitive tissues in the brain, pituitary, and aorta. Using radioimmunoassay (RIA) for estrogen, we confirmed that 24 h after a single injection of 17β-estradiol benzoate (EB) (25 μg), the circulating levels of 17β-estradiol were highly elevated in the EB-injected group (202 ± 20.6 pg/mL; $n = 6$) compared with the oil-injected group (16.6 ± 3.6 pg/mL; $n = 6$).

In the oil-treated, OVX guinea pigs ($n = 4$–6), MaxiK α mRNA was heterogeneously expressed in the POA, vHTH, hippocampus, and amygdala, with values ranging from 217.3 ± 51.1 fg/μg in the hippocampus to 86.5 ± 9.17 fg/μg in the POA (Fig. 4). Analysis of variance (ANOVA) indicated that there were significant differences ($p < 0.002$) in mRNA expression within the different brain regions (Fig. 4). Post hoc analysis demonstrated that MaxiK α mRNA was significantly higher in the hippocampus and amygdala compared to the levels found in the POA and vHTH (Fig. 4; $p < 0.05$). MaxiK α mRNA was also detected in the pituitary, but the expression was significantly less (24.4 ± 1.4 fg/μg) than that found in most brain regions (Fig.4; $p < 0.05$). Similar to MaxiK α mRNA, MaxiK β4 mRNA was also heterogeneously expressed in the POA, vHTH, hippocampus, and amygdala, with the highest levels measured in the amygdala (245.2 ± 26.5 fg/μg) and the lowest levels in the vHTH (30.0 ± 7.5 fg/μg; Fig. 5). ANOVA indicated that there were significant differences ($p < 0.001$) in mRNA expression within the different brain regions (Fig. 5). Post hoc analysis demonstrated that MaxiK β4 mRNA was significantly higher in the hippocampus and amygdala compared to the levels found in the POA and vHTH (Fig. 5; $p < 0.01$). In addition, similar to MaxiK α, we measured a low expression of MaxiK β4 mRNA in the pituitary (24.4 ± 1.4 fg/μg; Fig. 5). How-

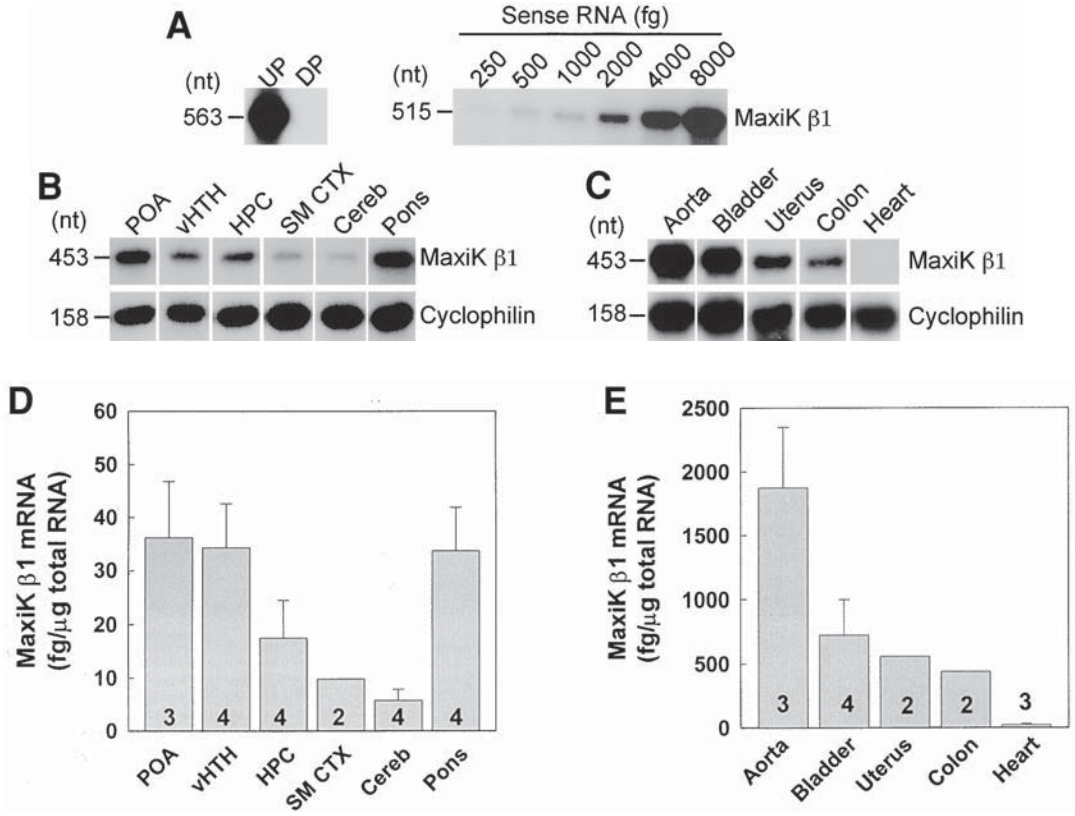

Fig. 2. Quantitative distribution of MaxiK β1-subunit mRNA in guinea pig brain and peripheral tissues. (**A**) Representative film image of RPAs illustrating ^{32}P-labeled undigested (UP) and digested (DP) MaxiK β1 riboprobe and its respective sense RNA standard curve (250–8000 fg). Linear regression analysis of the standard curve (not shown) revealed $r = 0.997$. (**B,C**) Specific MaxiK β1 signals (exposure time of 14–17 h; cyclophilin exposure time of ~5 h) measured in ~20 (B) or 5 μg (C) of total RNA obtained from brain and periphery, respectively. (**D,E**) Distribution and quantitative analysis of MaxiK β1 mRNA in discretely dissected brain regions (D) or peripheral tissues (E). Each MaxiK β1 band was normalized to its corresponding cyclophilin band and quantified from each sense mRNA standard curve using a phosphorimager. For each tissue the number of animals is given. HPC, hippocampus; see Fig. 1 for additional abbreviations.

ever, an estrogen treatment regimen, which inhibited pituitary luteinizing hormone (LH) secretion, had no significant effect on MaxiK α or MaxiK β4 mRNA expression in the brain and pituitary (Figs. 4 and 5).

Effects of 17β-Estradiol on Expression of MaxiK Channel Subunits in Aorta

In OVX, oil-treated animals, we measured high levels of MaxiK α and β1 mRNAs in the aorta (Fig. 6). Thus, the expression of α mRNA was 346.9 ± 56.3 fg/μg ($n = 6$) and that of β1 mRNA was 752.9 ± 151.3 fg/μg ($n = 4$). More important, measurements in the EB-treated group revealed that the mRNA expression of the MaxiK α subunit was significantly upregulated after EB treatment as compared with oil treatment (Fig. 6; $p < 0.02$). By contrast, mRNA expression of the β1-subunit was not different between the two groups of animals (Fig. 6). To elucidate the effects of EB treatment on MaxiK α-subunit protein, we conducted Western blot analysis of aorta membrane fractions using a selective antibody to the α-subunit (Alomone, Jerusalem, Israel). The antibody detected a single band at the expected size of ~125 kDa (Fig. 7), and this signal was eliminated when the antibody was preabsorbed with the antigenic peptide. Moreover, in support of our mRNA data, the protein levels were increased based on the immunoreactive MaxiK α-subunit density in the aorta from EB-treated animals (Fig. 7; $p < 0.02$, $n = 7$).

Discussion

Results of the present study provide insight into the quantitative expression of MaxiK channel subunit mRNAs and their regulation by estrogen. We found that the MaxiK α/β4 mRNA combination was highly expressed in the brain and that the α/β1 mRNA combination was expressed in the aorta. There was no significant change in the steady-state levels of hypothalamic or pituitary α/β4 mRNAs at the time of negative feedback of estradiol on the secretion of gonadotropin-releasing hormone (GnRH) and LH. However, we found, for the first time, an estradiol-mediated upregulation

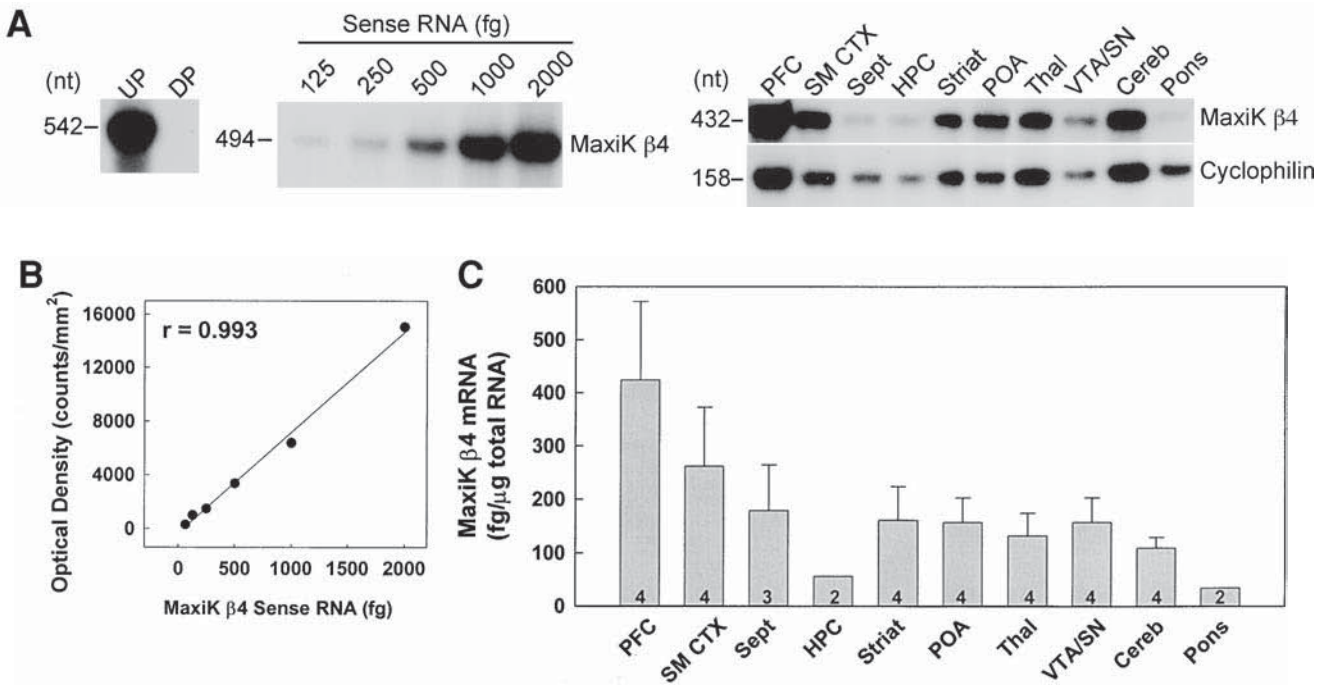

Fig. 3. Quantitative distribution of MaxiK channel β4-subunit mRNA in the guinea pig brain. (**A**) Representative RPA images illustrating ^{32}P-labeled undigested (UP) and digested (DP) MaxiK β4 riboprobe, its respective sense RNA standard curve (125–2000 fg), and specific MaxiK β4 signal (exposure time of ~16 h; cyclophilin exposure time of ~7 h) obtained from ~5 μg of total RNA in different brain regions. (**B**) Linear regression analysis of the MaxiK β4 sense standard curve using a phosphorimager revealed $r = 0.993$. (**C**) Distribution and quantitative analysis of MaxiK β4 mRNA in discretely dissected brain regions of the male guinea pig. Each bar represents an average of two measurements or a mean ± SEM of three to four measurements. The number of animals is given at the bottom of each bar. For abbreviations, see Figs. 1 and 2.

Table 2
Quantitative Distribution of MaxiK Subunit mRNAs (fg/μg total RNA) in Guinea Pig Brain[a]

Brain regions	MaxiK α	MaxiK β4	MaxiK β1	MaxiK β2
PFC	377 ± 119 (4)	424 ± 148 (4)	2 (1)	11 (1)
SM CTX	295 ± 109 (4)	262 ± 111 (4)	10 ± 1 (2)	—
Thalamus	250 ± 23 (4)	132 ± 42 (4)	59 ± 35 (2)	—
Septum	165 ± 4 (3)	180 ± 87 (3)	13 ± 10 (2)	—
Striatum	160 ± 60 (4)	161 ± 63 (4)	9 (1)	—
Hippocampus	204 ± 107 (2)	56 ± 20 (2)	17 ± 7 (4)	16 ± 8 (2)
POA	158 ± 32 (4)	158 ± 47 (4)	36 ± 11 (3)	—
dHTH	168 ± 30 (2)	82 ± 1 (2)	—	
vHTH	135 ± 5 (4)	86 ± 4 (3)	34 ± 8 (4)	29 (1)
VTA/SN	136 ± 23 (3)	157 ± 47 (4)	2 (1)	—
Pons	55 ± 24 (3)	34 ± 3 (2)	34 ± 8 (2)	15 (1)
Cerebellum	219 ± 19 (4)	110 ± 20 (4)	6 ± 2 (4)	4 (1)

[a]RPA was used to measure the expression levels of MaxiK α, β1, β2, and β4 mRNAs in different areas of the guinea pig brain. The number of animals is given in parentheses. PFC, prefrontal cortex; SM CTX, sensorimotor cortex; POA, preoptic area; dHTH, dorsal hypothalamus; vHTH, ventral hypothalamus; VTA/SN, ventral tegmental area/substantia nigra. —, Not measured.

of the MaxiK α, but not β1, mRNA and MaxiK α protein in the aorta. Therefore, these observations suggest that estrogen's negative feedback on the hypothalamo-pituitary-gonadal axis may not involve the regulation of MaxiK α/β4 mRNAs. However, circulating estrogen may be involved in the regulation of the MaxiK α/β1 complex in major blood vessels such as the aorta.

The guinea pig α, β1, β2, and β4 gene fragments that we cloned were used as templates to generate the respective riboprobes that were utilized in a quantitative and highly

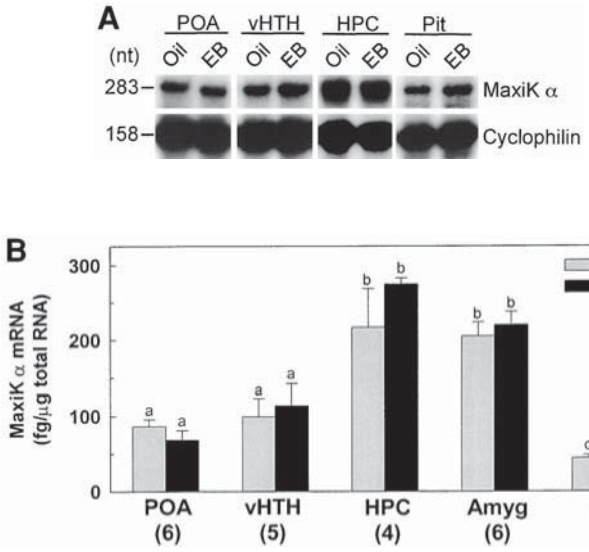

Fig. 4. Quantification of MaxiK α mRNA in female guinea pig brain. (**A**) Representative film images of RPAs of total RNA (~5 μg/lane) from oil- and EB (25 μg)-treated female guinea pigs illustrating mRNA expression of MaxiK α (exposure time of 16 h) in POA, vHTH, HPC, amygdala (Amyg), and pituitary (Pit). (**B**) Distribution and quantitative analysis of MaxiK α mRNA in discretely dissected brain regions and Pit of female guinea pig. Each MaxiK α band was normalized to its corresponding cyclophilin band and quantified from each sense mRNA standard curve using a phosphorimager. For each area, the number of animal pairs is given in parentheses. Bars with the same letter are not significantly different; those with different letters represent significantly different populations. See Figs. 1 and 2 for abbreviations.

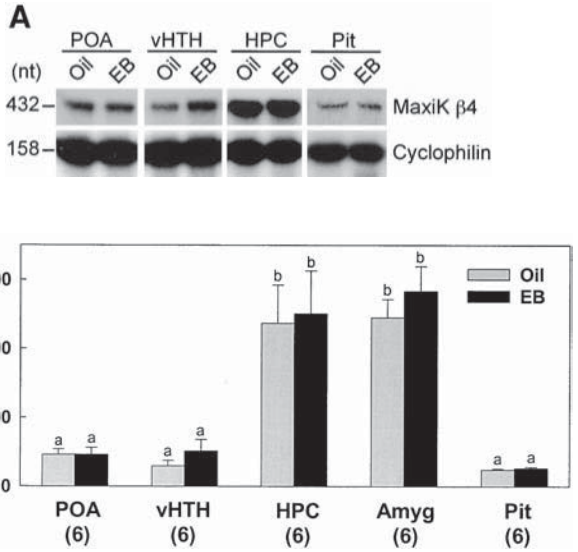

Fig. 5. Quantification of MaxiK β4 mRNA in female guinea pig brain. (**A**) Representative film images of RPAs of total RNA (~5 μg/lane) from oil- and EB (25 μg)-treated female guinea pigs illustrating mRNA expression of MaxiK β4 mRNA (exposure time of 16 h) in POA, vHTH, HPC, and Pit. (**B**) Distribution and quantitative analysis of MaxiK β4 mRNA in discretely dissected brain regions and Pit of female guinea pig. Each MaxiK β4 band was normalized to its corresponding cyclophilin band and quantified from each sense mRNA standard curve using a phosphorimager. The number of animal pairs in each group is given in parentheses. Bars with the same letter are not significantly different; those with different letters represent significantly different populations. See Figs. 1, 2, and 4 for abbreviations.

sensitive RPA to measure the expression levels of MaxiK mRNAs in the guinea pig. We found that, although it was heterogeneously distributed, the MaxiK α-subunit mRNA was highly expressed in all of the brain areas that were examined. These results are consistent with a number of previous reports and agree with the general view that the mRNA encoding the pore-forming protein within the MaxiK channel, Slo subunit, is broadly expressed in the mammalian brain *(8,12,13,15,25,26)*. By contrast, we measured low levels of β1 and β2 mRNAs in the brain, where we found greater expression of the MaxiK β4 mRNA. These findings, together with previous reports, indicate that the molecular composition of mammalian central nervous system MaxiK channel is predominately composed of the α- and β4-subunits *(15,16,25)*.

Coexpression of various β-subunits with a MaxiK α-subunit alters the functional properties of the channel as measured in in vitro expression systems *(8,27,28)*. For example, β2 induces inactivation of the MaxiK α channel, and the β4-subunit slows the activation kinetics as compared to MaxiK β1 *(8,27)*. Therefore, the differential distribution of MaxiK channel subunits may explain the functional diversities of the MaxiK channels in different cell types *(6,25,27)*.

In the mammalian brain, the GnRH neuronal system regulates reproduction by controlling the pituitary and gonadal function and also by integrating the feedback control of sex steroid hormones *(29)*. These hormones have a profound impact on the cellular physiology in the POA, hypothalamus, hippocampus, amygdala, and pituitary *(29–33)*. In previous studies, we found that estrogen acutely alters the functional expression of potassium channels in hypothalamic neurons *(34)*. We also documented that hypothalamic SK3 mRNA levels are increased in estrogen-treated animals, and that estrogen potentiates the α-adrenergic inhibition of an apamin-sensitive after-hyperpolarization (AHP) (SK2/3) current in POA neurons *(35,36)*. Electrophysiologic recordings have further established that 17β-estradiol is capable of increasing the MaxiK current and thereby inhibiting neurons in the area postrema region of the brain *(19)*. In addition, physiologically relevant concentrations of estradiol can activate MaxiK channels in a Chinese hamster ovary cell line expressing α and β4 mRNAs *(8)*. Based on these and other observations, we had predicted that estrogen treatment would alter the mRNA expression of the α- and/or β4-subunit in the hypothalamus and other steroid-sensitive regions such as the hippocampus, amygdala, and pituitary.

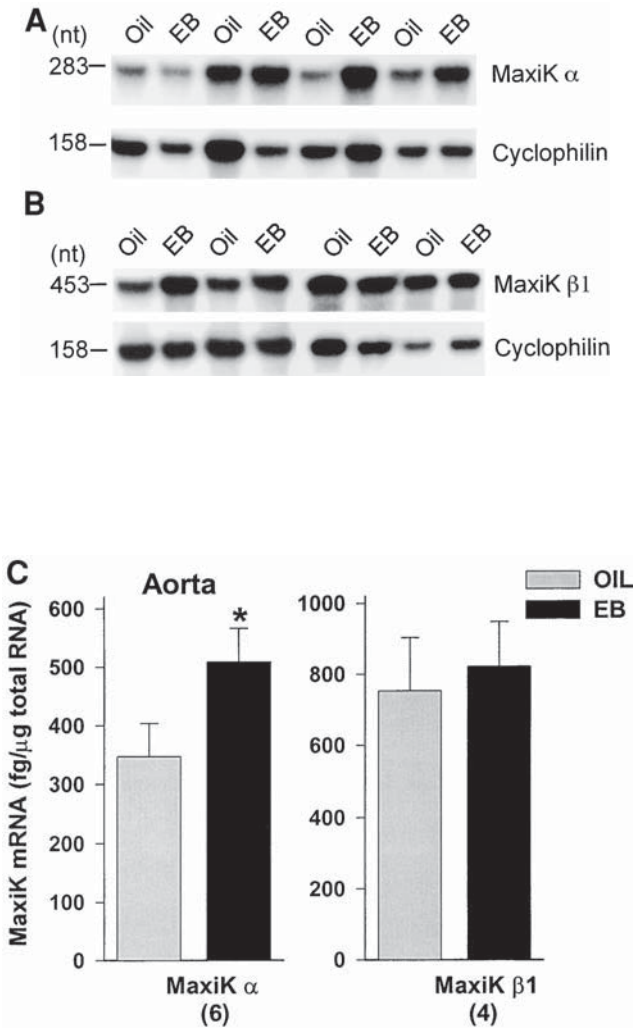

Fig. 6. Estradiol upregulates MaxiK α, but not β1, mRNA in the aorta. (**A,B**) Representative film images of RPAs of total RNA (~5 μg/lane) from individual oil- and EB (25 μg)-treated female guinea pigs illustrating mRNA expression of MaxiK α (A) and MaxiK β1 (B) (exposure time of 16 h; cyclophilin exposure time of 6–8 h) in aorta. (**C**) Quantitative analysis of mRNA expression of MaxiK α- and β1-subunits in aorta. *$p < 0.02$, oil vs EB. The number of animal pairs in each group is given in parentheses.

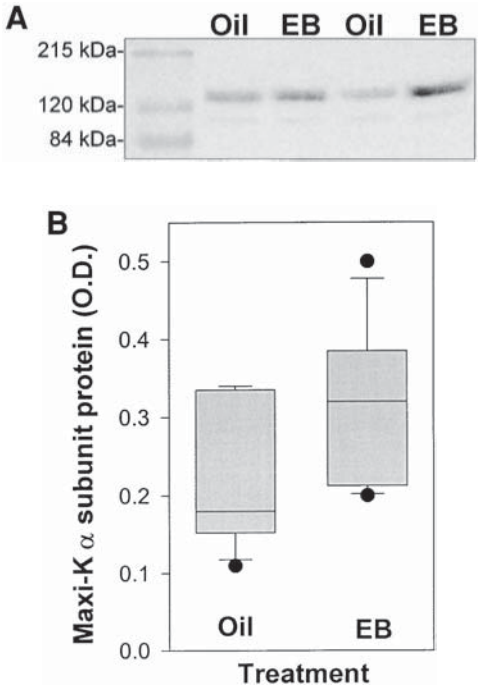

Fig. 7. Estradiol upregulates MaxiK α protein in aorta. Six to seven micrograms of guinea pig aorta membranes was fractionated by electrophoresis on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel and reacted with an antibody to a glutathione S-transferase fusion protein corresponding to residues 1098–1196 of the mouse MaxiK α-subunit. (**A**) Representative immunoblot from two sets of oil- and EB-treated animals. Bands of ~125 kDa were detected in membranes from oil- and EB-treated guinea pigs. The specificity of the antibody was confirmed by immunostaining of membrane fractions and control peptide with a preabsorbed antibody (3 μg of peptide/μg of antibody), which eliminated the positive signals (data not shown). (**B**) Box plot of immunoreactive MaxiK α-subunit density in the aorta from oil- and EB-treated OVX animals ($n = 7$ each). The median is the center line in the box. The edges of the box represent the 75th and 25th percentiles of the distribution (intraquartiles). The vertical lines show the extent of the overall distribution, and the solid circles represent the farthest outliers of the population. There was a significant increase ($p < 0.02$; paired Student's *t*-test) in MaxiK α-subunit density in the EB-treated compared with oil-treated animals.

Although we found that most of these regions display high to moderate levels of mRNA expression for both the α- and β4-subunit, the mRNAs did not appear to be regulated by estrogen treatment. In the OVX guinea pig, a single injection of estradiol induces both inhibition and stimulation of LH release *(37)*, referred to as the estrogen-negative and-positive feedback, respectively. Using this paradigm, we found that, 24 h after a single injection of EB, the expression levels of both MaxiK α and β4 mRNAs in the POA, vHTH, hippocampus, amygdala, and pituitary were similar in both oil- and EB-treated OVX animals. These observations suggest that the estrogen's negative feedback may not involve the regulation of MaxiK α/β4 mRNAs in the hypothalamus.

Based on previous studies in the hypothalamus, the apamin-sensitive afterhyperpolarization (SK2/3) current (I_{AHP}), which in these neurons mediates the medium I_{AHP}, is regulated by norepinephrine and by estrogen *(36)*. It is therefore possible that in these neurons the MaxiK channel is not regulated by estrogen. Similarly, in the hippocampal CA1 pyramidal neurons, MaxiK channels are known to mediate the fast I_{AHP} with spike broadening during repetitive firing in pyramidal cells *(3,4,38,39)*. Again, it is the medium and slow I_{AHP} that are regulated by transmitters and/or estrogen *(4,33,40)*. This may explain the lack of estrogen effect on MaxiK channels in this brain region.

MaxiK channel α- and β4-subunit mRNA expression was relatively low in the pituitary, and there was no effect of estrogen on the expression of either subunit. This could be related to the recent reports that MaxiK channels are highly expressed in pituitary somatotropes and corticotropes, in

which these channels are of high functional significance and found to be regulated by corticosteroids and corticotropin-releasing factor *(41,42)*. By contrast, gonadotropes express few MaxiK channels and exhibit single spiking activity with comparatively low calcium entry *(41,42)*. The major channels in gonadotropes include the T-type calcium channel and SK channels *(42)*.

In the periphery, we found that β1 mRNA was highly expressed in smooth muscle–enriched tissues such as the aorta, uterus, bladder, and colon. By contrast, both β2- and β4-subunits were found in low quantities in these tissues. These findings are in agreement with previous observations that in smooth muscle the MaxiK channel is composed primarily of the α- and β1-subunits, with little or no β2- or β4-subunit expression *(12,27,43)*. However, MaxiK β2 has been found to be highly expressed in the kidney and placenta, illustrating further the tissue-specific localization of the β-subunit *(27)*.

One of the more interesting aspects of our findings is that estrogen increased the mRNA expression and protein levels of the MaxiK α-subunit in the aorta. This is the first indication that estrogen treatment leads to increased expression of the MaxiK α-subunit in the aorta, although in the uterus, estrogen causes similar effects *(44)*. It has been known for some time that estrogen both acutely and after long-term treatment may cause vasodilation and thus increased blood flow *(45)*. The chronic, more long-term treatment with estrogen results in vasodilation and reduced contractility by a mechanism found to involve increased synthesis of NO synthase (NOS) in the endothelial lining of blood vessels *(45)*. Also acutely, estrogen causes rapid vasodilation by activating endothelial NO production *(46)*. In addition, a number of studies have found that estrogen applied acutely, albeit in high concentrations (10 μ*M*), causes vasodilation by a mechanism that does not include NOS, but by direct action on myocytes *(45,47)*. Acute estrogen increases the open probability of MaxiK channels by a mechanism that is dependent on the expression of the β-subunit together with the α-subunit *(18,48)*. Therefore, it has been proposed that estrogen may cause this effect by an action on the MaxiK β-subunit since estrogen binds to the β-subunit in vitro *(18,45)*. Interestingly, in contrast to acute in vitro studies, we did not find an effect of estrogen in vivo on the mRNA expression of the MaxiK β1-subunit, but we did measure an increase in the expression of MaxiK α mRNA and protein. Based on our findings, we would argue that after a longer time period, estrogen leads to increased synthesis of the pore-forming α-subunit of the MaxiK channel, which may participate in the vasodilatory and cardioprotective effects of estrogen. The mechanism by which estrogen caused this increase in the MaxiK α-subunit expression and the functional consequences are currently not clear and need to be further evaluated. Although estrogen receptor-α (ER-α) and ER-β have been described in aortic endothelial and smooth muscle cells in various species including human *(45)*, an estrogen response element has not yet been found in the MaxiK α or β genes. Since the effects of estrogen are tissue specific, it is most likely that estrogen acts via upregulation of kinase activity to increase gene expression and subsequent channel protein *(33,49,50)*.

In summary, the α/β4 and α/β1 complexes are the most prominent compositions of MaxiK channels in brain and smooth muscle of the guinea pig, respectively. During the estrogen negative feedback, MaxiK α and β4 mRNAs are not affected by estradiol in the brain and pituitary. However, estradiol upregulates MaxiK α, but not β1, mRNA and MaxiK α protein in the female guinea pig aorta. The upregulation of vascular MaxiK α-subunit by estradiol may account for some of its cardioprotective effects.

Materials and Methods

Animal Treatment and Tissue Preparation

All animals were handled according to the National Institutes of Health (NIH) Laboratory Animal Care and Use Guidelines. Male and female guinea pigs (Topeka strain) were obtained from the Oregon Health and Science University breeding colony. Animals were subjected to a 14:10 h light/dark schedule and allowed to drink tap water and to feed ad libitum. In preliminary experiments, four intact male guinea pigs were killed at 4 to 5 wk of age and used for distribution analysis in order to quantify the MaxiK subunit composition in the brain and peripheral tissues. Thereafter, 12 adult female guinea pigs (8–10 wk old) were used to study estrogen regulation of gene expression. In addition, 14 females were used to analyze MaxiK α protein levels in the aorta. The females were OVX under anesthesia (ketamine/xylazine mixture). Seven days after surgery, each OVX female was given a single injection of either 25 μg of EB solubilized in sesame oil (EB group) or vehicle (oil group). Twenty-four hours later, animals were killed by decapitation while lightly sedated with ketamine, and blood samples were collected. Brains were also removed quickly and then rinsed in chilled Sorensen's buffer. A brain slicer was used to produce 2 to 3-mm-thick frontal blocks, and brain areas such as the POA, vHTH, dHTH, hippocampus, and amygdala were dissected. The POA block included the bed nucleus of the stria terminalis, medial and lateral POAs, anterior hypothalamic nucleus, and periventricular nucleus. The vHTH is referred to as the hypothalamic area extending from, rostrally, the end of the retrochiasmatic nucleus to, caudally, the beginning of the mamillary bodies and to, dorsally, the mid-level of the third ventricle. The dHTH is referred to as the hypothalamic area laying dorsally to the vHTH block. Peripheral tissues such as the pituitary, uterus, testis, skeletal muscle, heart ventricle, and aorta (descending dorsal branch) were also collected. Tissues were rapidly frozen in isopentane and then stored at –80ºC. Total RNA was extracted using TRIzol reagents (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's directions, and the concentration was determined by spectrophotometry.

For isolation of cell membranes, the aortas from female guinea pigs were homogenized on ice in 1.0 mL of lysis buffer containing 320 m*M* sucrose, 5 m*M* Na_2PO_4 with Protease Inhibitor Cocktail (Sigma P8340; Sigma, St. Louis, MO). The homogenates were centrifuged at 6000*g* for 10 min twice, and the resulting supernatants were combined and centrifuged at 60,000*g* for 60 min to pellet cell membranes. The membrane pellets were resuspended in phosphate-buffered saline, and protein content was quantified using a Bio-Rad protein assay kit and a Power Wave x 340 Microplate Reader (Bio-Tek, Winooski, VT) utilizing KCjr software (Bio-Tek). Protein aliquots were stored at –20ºC.

17β-Estradiol Assay

Plasma levels of estradiol were measured in both oil and EB groups by RIA using an automated clinical assay instrument (Roche Diagnostics Elecsys 2010). Ruthenium-labeled tracer, coated microparticles, and electrochemiluminescence were used for quantification. A standard curve ranging from 10 to 4500 pg/mL was constructed. Water blanks read <10 pg/mL. The percentage of recovery was >96%. The intraassay coefficient of variation was <9%. Using this RIA, we confirmed that, 24 h after a single injection of EB (25 μg), the circulating estradiol levels were highly elevated in the EB-injected group (17β-estradiol = 202.3 ± 20.6 pg/mL; $n = 8$) compared with the oil-injected group (17β-estradiol = 16.6 ± 3.6 pg/mL; $n = 6$).

Cloning of Guinea Pig MaxiK Channel Subunits

GenBank database was used to search for those MaxiK channel subunit cDNA sequences that were obtained from human, monkey (*Maccaca mulatta*), rat (*Rattus norvegicus*), or rabbit (*Oryctolagus cuniculus*) tissues. For each MaxiK subunit, two or more sequences were aligned using the Align Plus software program (Scientific & Educational Software). This allowed us to determine for each MaxiK subunit the gene domains that have the highest conservation rates across species. Primers were designed using the Clone Manager 5 software (Scientific & Educational Software). The primers that were specific to MaxiK α-, β1-, β2-, and β4-subunits were designed as follows:

1. MaxiK α: Both primers were 100% homologous to both the monkey (GenBank accession no. AF026001) and rabbit (accession no. AB009312) MaxiK α sequences. The forward primer was 5'AGCCATTGAGTACAAGTCTG3', which corresponds to bp 1812–1831 and 1801–1820 of the monkey and rabbit sequences, respectively. The backward primer was 5'GGAGTCCATGTTGTCAATCT3', which corresponds to bp 2138–2157 and 2127–2146 of the monkey and rabbit sequences, respectively.
2. MaxiK β1: Both primers were 84.8 and 100% homologous to, respectively, the human (accession no. U25138) and rabbit (accession no. AF107300) MaxiK β1 sequences. The 5' primer was 5'TGTGCTGTCATCACCTACT3', which corresponds to bp 148–166 and 235–253 of the rabbit and human sequences, respectively. The 3' primer was 5'CATGGCAA TAATGAGGAG3', which corresponds to bp 586–603 and 785–802 of the rabbit and human sequences, respectively.
3. MaxiK β2: Both primers were 100% homologous to the human MaxiK β2 sequence (accession no. NM005832.1). The 5' and 3' primers were, respectively, 94.7 and 95% homologous to the rat MaxiK β2 sequence (the unpublished sequence was obtained from ref. *11*. The forward primer was 5'GACTGGCTATGATGGTGTG3', which corresponds to bp 297–315 and 152–170 of the human and rat sequences, respectively. The 3' primer was 5'GGTCAGAATAGCAG GAGAAG3', which corresponds to bp 658–677 and 513–532 of the human and rat sequences, respectively.
4. MaxiK β4: Both primers were 100% homologous to the human MaxiK β4 sequence (accession no. AF207992). The forward and backward primers were, respectively, 5'CGTG TCGCTCTTCATCTT3' (bp 87–104) and 5' CAATGCAGG ACAATC3' (bp 501–518).

Each designed primer pair was analyzed by the Blast Sequence Similarity Searching Program (blastn) using the GenBank sequence database of the National Center for Biotechnology Information (NIH) to ensure that each primer has an exclusive specificity for the MaxiK subunit of interest. All primers were synthesized by Gibco-BRL (Life Technologies) and included a 12-base (deoxy-UMP residues) extension at their 5' end.

Reverse Transcriptase/Polymerase Chain Reaction

Both reverse transcription and PCR were carried out using the PTC-100 Programmable Thermal Controller (MJ Research) and the RT/PCR GeneAmp kit reagents (Perkin Elmer, Foster City, CA). The Oligo-dT primer was used for the mRNA-co DNA first-strand synthesis. The reverse transcription reaction was performed on 200–400 ng of total RNA extracted from the following brain areas: ventral tegmental area/substantia nigra (α-subunit), POA (β1-subunit), VHTH (β2-subunit), and hippocampus (β4-subunit). PCR was carried out for 45 cycles after an initial 5-min denaturation at 94ºC. Each cycle was set as follows: 45-s denaturation at 94ºC; 45-s annealing (at 51ºC for the α, 55ºC for the β1, and 62ºC for both the β2 and β4); and 1-min 10-s extension at 72ºC. The PCR products were run for electrophoresis on 2% agarose gel, and for each product, the result was a single band around the expected size of the PCR product: 381 (α), 477 (β1), 405 (β2), and 456 bp (β4). Each PCR product was subcloned with a CloneAmp pAMP1 System cloning kit (Gibco-BRL, Life Technologies) and sequenced.

Ribonuclease Protection Assay

Insert-containing pAMP1 circular plasmids were linearized using *Hind*III or *Afl*II endonuclease enzyme, which has a cut site within the insert MaxiK α at its 74-bp position. This allowed us to generate a shorter MaxiK α antisense riboprobe that was 283 nucleotides (nt) long instead of 357 nt when using *Hind*III. RPA probes were labeled with ^{32}P-

rUTP (Perkin Elmer Life Sciences) and then purified using a Fullengther Gel Apparatus (BioKey America, Aloha, OR). For each guinea pig MaxiK subunit, the circular plasmid was also linearized using *Eco*RI endonuclease and then used as a template to generate a specific sense RNA. Different concentrations of sense RNA were used to construct sense RNA standard curves for each guinea pig MaxiK channel subunit and also for a monkey cyclophilin *(51)*. RPA was performed using an RPA II kit (Ambion, Austin TX). The antisense riboprobes were incubated overnight at 42ºC with 5 or 20 μg of total RNA, or sense standard (62.5–8000 fg) RNA. Hybridization was terminated by a T1 ribonuclease digestion (900 U/sample). The protected RNA bands were isolated by electrophoresis in acrylamide gel. The gel was exposed to a Kodak X-Omat Blue XB-1 film (Perkin Elmer Life Sciences) for visualization of the protected bands. Quantification of radioactive signal was performed using a phosphorimager and Multi-Analyst software (Bio-Rad, Hercules, CA). Each MaxiK subunit band was normalized with its corresponding cyclophilin band.

Western Blot Analysis

Six to seven micrograms of aorta membranes was fractionated by electrophoresis on 12% SDS-polyacrylamide gel and transferred overnight to nitrocellulose at 4ºC. Blots were stained with Ponceau S to check for transfer and the approximate amount of protein loaded. Blots were then blocked with Tris-buffered saline plus Tween-20 (TBS-T: 50 m*M* Tris-HCl, 150 m*M* NaCl, 0.5% Triton-X100, 0.1% Tween-20, pH 7.4) containing 5% nonfat dry milk for 60 min on a rotary shaker. The blots were then reacted sequentially with a polyclonal rabbit anti-MaxiK α-subunit antibody (residues 1098–1196, accession no. A48206; Alomone) and diluted 1:300 in TBS-T containing 5% nonfat dry milk overnight at 4ºC, and a secondary antibody, donkey anti-rabbit IgG/horseradish peroxidase (1:5000) (Amersham, Piscataway, NJ) in the TBS-T solution for 60 min at room temperature. After each step, the blots were washed four times for 15 min in TBS-T. Thereafter, immunoreactive MaxiK α-subunit was detected by chemiluminescence using SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL) and visualized on Epi-Chem II Darkroom (UVP, Upland, CA) using LabWorks 4.0 software (UVP). Quantification of chemiluminescence signals was performed using a phosphorimager and Multi-Analyst software (Bio-Rad). Results are expressed as optical densities. Equal amounts of protein were used for expression comparison. To determine the specificity of the antibody, blots were reacted with antibody preabsorbed with antigenic peptide (3 μg of peptide to 1 μg of antibody). This eliminated the observed signal.

Statistical Analyses

Data are expressed as the mean ± SEM. The two groups were compared using a paired student's two-tailed *t*-test. MaxiK channel subunit distribution in different brain regions was evaluated using ANOVA followed by Newman-Keuls multiple range test. In data presented as box plots, the central line represents the median, the edges of the box represent the intraquartiles, the vertical lines show the extent of the overall distribution, and the solid circles represent the farthest outliers of the population. Differences were considered statistically significant if the probability of error was <5%.

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