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Sexual Dimorphism and Estrogen Regulation of KCNE3 Expression Modulates the Functional Properties of KCNQ1 K+ Channels

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Citation
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Running title: Estrogen regulation of KCNQ1:KCNE3 in colonic epithelia

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Non-standard abbreviations: \(I_{SC}\), short-circuit current; \(I_K\), basolateral potassium current; \(I/V\), current/voltage; CFTR, cystic fibrosis transmembrane conductance regulator
ABSTRACT

The KCNQ1 potassium channel associates with various KCNE ancillary subunits that drastically affect channel gating and pharmacology. Co-assembly with KCNE3 produces a current with nearly instantaneous activation, some time-dependent activation at very positive potentials, a linear current voltage relationship and a 10-fold higher sensitivity to chromanol 293B. KCNQ1:KCNE3 channels are expressed in colonic crypts and mediate basolateral K⁺ recycling required for Cl⁻ secretion. We have previously reported the female-specific anti-secretory effects of estrogen via KCNQ1:KCNE3 channel inhibition in colonic crypts. This study was designed to determine whether gender and estrogen regulate the expression and function of KCNQ1 and KCNE3 in rat distal colon. Colonic crypts were isolated from Sprague-Dawley rats and used for whole-cell patch-clamp and to extract total RNA and protein. Sheets of epithelium were used for short-circuit current recordings. KCNE1 and KCNE3 mRNA and protein abundance was significantly higher in male than female crypts. No expression of KCNE2 was found and no difference was observed in KCNQ1 expression between male and female (at estrous) colonic crypts. Male crypts showed a 2.2-fold higher level of association of KCNQ1 and KCNE3 compared to female cells. In female colonic crypts, KCNQ1 and KCNE3 protein expression fluctuated throughout the estrous cycle and 17β-estradiol (E2 10 nM) produced a rapid (<15 min) dissociation of KCNQ1 and KCNE3 in female crypts only. Whole-cell K⁺ currents showed a linear current-voltage relationship in male crypts, while K⁺ currents in colonic crypts isolated from females displayed voltage-dependent outward rectification. Currents in isolated male crypts and epithelial sheets were 10-fold more sensitive to specific KCNQ1 inhibitors, such as chromanol 293B and HMR-1556, than in female. The effect of E2 on K⁺ currents mediated by KCNQ1 with or without different β-subunits was assayed from current-voltage relations elicited in CHO cells transfected with KCNQ1 and KCNE3 or KCNE1 cDNA. E2 (100 nM) reduced the currents mediated by the KCNQ1:KCNE3 potassium channel and had no effect on currents via KCNQ1:KCNE1 or KCNQ1 alone. Currents mediated by the complex formed by KCNQ1 and the mutant KCNE3-S82A β-subunit showed rapid rundown and insensitivity to E2. Together, these data suggest that estrogen regulates the expression of the KCNE1 and KCNE3 and with it the gating and pharmacological properties of the K⁺ conductance required for Cl⁻ secretion. The decreased association of the KCNQ1:KCNE3 channel complex promoted by estrogen exposure underlies the molecular mechanism for the sexual dimorphism and estrous cycle dependence of the anti-secretory actions of estrogen in the intestine.
INTRODUCTION

KCNQ proteins form a family of five voltage-gated K\(^+\) channels, which play an important role in controlling the K\(^+\) current in several tissues (Robbins, 2001; Jespersen et al, 2005). The proteins have six transmembrane domains and can form functional homomeric channels in vitro. While KCNQ2-5 can associate with each other to form channel hetero-complexes, KCNQ1 does not form heteromers with other KCNQ subunits (Robbins, 2001; Jespersen et al, 2005). However, KCNQ1 associates with β-subunits of the KCNE family (Jespersen et al, 2005). The KCNE type I transmembrane peptides are a family of tissue specific β-subunits that associate with and fine tune the electrical and pharmacologic properties of several voltage-gated K\(^+\) channels (McCrossan & Abbott, 2004). In expression systems, KCNQ1 channels have affinity for all five KCNE peptides forming K\(^+\) conducting complexes with different voltage activation, gating kinetics, unitary conductance, and pharmacology (Melman et al, 2002a; McCrossan & Abbott, 2004). Of these complexes, the electrical properties and physiological roles of the KCNQ1:KCNE1 and KCNQ1:KCNE3 complexes have been well established (Barhanin et al, 1996; Schroeder et al, 2000).

Homomeric KCNQ1 expression induces a voltage-dependent, slowly activating and slowly deactivating outward K\(^+\) current (Barhanin et al, 1996; Tristani-Firouzi & Sanguinetti, 1998). When KCNQ1 is expressed together with KCNE1 the activation kinetics are considerably slowed by 10-fold, the unitary conductance is approximately 4-fold greater than that of homomeric KCNQ1 and the voltage-dependency shifts toward more positive potentials, giving a current resembling the slow delayed rectifier cardiac current \(I_{Kr}\) (Barhanin et al, 1996; Tristani-Firouzi & Sanguinetti, 1998; Yang & Sigworth, 1998; Schroeder et al, 2000). In contrast, association with KCNE3 accelerates gating kinetics, eliminates voltage dependence and causes a fraction of the channels to remain open at all voltages (Schroeder et al, 2000; Mazhari et al, 2002; McCrossan & Abbott, 2004). These differences suggest that KCNE1 and KCNE3 interact with critical components of the channel gating machinery (Melman et al, 2001; 2002b; McCrossan & Abbott, 2004).

In the colon, KCNQ1 forms heteromultimeric channels with KCNE3, and localize at the basolateral membrane (Schroeder et al, 2000; Dedek & Waldegger, 2001; Kunzelmann et al, 2001; Vallon et al, 2005; Preston et al, 2010). These heteromers are constitutively open at the negative membrane voltages of intestinal epithelial cells and can be further activated by the action of cAMP (Warth et al, 1996; Schroeder et al, 2000). KCNQ1:KCNE3 complexes have been shown to mediate cAMP-activated Cl\(^-\) secretion in small and large intestine, by recycling K\(^+\) that is transported into the cell by basolateral Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter and Na\(^+\)-K\(^+\)-ATPase (Kunzelmann & Mall, 2002; Jespersen et al, 2005; Liao et al, 2005). Furthermore, these channels are responsible for hyperpolarization required to maintain Cl\(^-\) secretion.

Activation or inhibition of K\(^+\) channels in epithelia, particularly those secreting Cl\(^-\), has potentially useful therapeutic applications in diseases as diverse as cystic fibrosis, where Cl\(^-\) secretion is deficient and secretory diarrhea, where it is in excess (Kunzelmann & Mall, 2002; Field, 2003). Previously it has been shown that KCNQ1 channels are sensitive to chromanol 293B; however their sensitivity seems to vary from tissue to tissue. It is known that association with KCNE subunits modulates the sensitivity of KCNQ1 to both activators and inhibitors, such as mefenamic acid and chromanol 293B, respectively (Lerche et al, 2000; Unsöld et al, 2000). Association of
KCNQ1 channels with KCNE3 produces a 10-fold increase in the sensitivity of channel currents to chromanol 293B. Thus, the selectivity of this inhibitor is dependent on the type and level of expression of KCNE subunits colocalized with KCNQ1.

In previous studies we have found gender differences in the anti-secretory response to 17β-estradiol in colon, which involves basolateral K⁺ channel inhibition (McNamara et al., 2000; Condliffe et al., 2001; O’Mahony et al., 2007, 2009). In this report, we examine the effect of gender on the expression of KCNE3 subunits in rat colonic crypt cells and the effect of such differences on the electrical properties of the KCNQ1 conductance in crypt cells and colonic epithelia. In addition we demonstrate that 17β-estradiol is capable of inhibiting heterologously expressed KCNQ1:KCNE3 channels, an effect that requires the presence of the KCNE3 subunit and could necessitate its phosphorylation.
METHODS

Animals
All experiments in animals were conducted in accordance with the Department of Environment legislation and approved by The Ethics Committee of the Royal College of Surgeons in Ireland. Sprague-Dawley rats (300-350 g) were kept on a 12-hour light, 12-hour dark cycle and were given *ad libitum* access to food and water. Following halothane anaesthesia rats were killed by cervical dislocation. Cervical smears were obtained from female rats and the estrous cycle stage was determined. All female rats were used at the estrous stage unless stated otherwise. The distal colon was removed and faecal contents were rinsed off. Colonic crypts were isolated using the calcium chelation technique, as previously described (Doolan & Harvey, 1996) while sheets of colonic mucosa were obtained by blunt dissection.

RNA isolation and semi-quantitative RT-PCR
Total RNA was isolated from colonic crypts using Qiagen RNeasy kit (West Sussex, UK) following manufacturer’s instructions. The mRNAs were reverse-transcribed into cDNAs by using the Improm-II™ Reverse Transcriptase System (Promega, Southampton, UK) following manufacturer’s protocol. Table 1 shows the primers used for semi-quantitative RT-PCR analysis of KCNQ1 and KCNE subunits expression; GADPH was used as an internal control. PCR reactions were performed in a final volume of 25 µl containing 1 µl of synthesized cDNA, 12.5 µl of GoTaq® Green Master Mix (Promega, Southampton, UK), and 0.5 µM of each pair of primers. Cycle parameters were 30-s denaturation step at 94°C, 30-s annealing step at 55°C, and 1-min extension at 72°C. Before examining each gene, a standard curve of cycle number within the linear range for PCR amplification was generated. It was determined by semi-quantitative RT-PCR analysis that 25 cycles were optimal for KCNQ1 and KCNE subunits. The RT-PCR amplification products were analyzed by 1% agarose gel electrophoresis and stained with ethidium bromide.

Co-immunoprecipitation and Western blotting
Isolated colonic crypts were lysed and subjected to standard 10 % SDS-PAGE and Western blotting. Immunoblots were developed using specific antibodies against rat KCNQ1 and KCNE subunits. Co-immunoprecipitation assays were carried out as previously described by Dilly *et al* (2004).

Patch-Clamp
Freshly isolated colonic crypts were transferred into a 1 ml perfusion chamber mounted on an inverted microscope (TE 2000-S, Nikon Ltd, Ireland). Patch pipettes were pulled and fire-polished using a programmable horizontal puller (DMZ-Universal, Zeitz-Instruments GmbH, Germany) and had an electrical resistance of 2-5 MΩ when filled with K+ solutions. The patch-clamp apparatus consisted of a CV-203BU head stage connected to an Axopatch 200B series amplifier (Axon Instruments, USA). Patch-clamp experiments were performed at 37°C in the standard whole-cell recording configuration, recorded membrane currents were filtered at 1 kHz through an 8 pole, low-pass Bessel filter and digitized at 5 kHz. The voltage clamp protocol consisted of a series of voltage steps from −100 mV to +100 mV in 20 mV increments from an initial holding potential of −50 mV. Colonic crypts were perfused at a rate of 1 ml/min in a standard bath
solution containing (in mM): NaCl 140, KCl 5.4, MgCl$_2$ 1, CaCl$_2$ 1.25, HEPES 10, glucose 12.2, pH 7.4. The patch pipette solution contained: K-gluconate 95, KCl 30, Na$_2$ATP 4.8, KH$_2$PO$_4$ 1.2, EGTA 1, Ca(gluconate)$_2$ 0.73, MgCl$_2$ 1, ATP 3, D-Glucose 5, pH 7.2. The protocols for patch-clamp and data analysis were established with routines using pClamp 9.2 software (Axon Instruments, USA).

**KCNQ1: KCNEx channel complex transfection and current recording in CHO cells.**
Constructs: hKCNQ1, hKCNE3 and hKCNE1 were kindly provided by Dr. Steve A. Goldstein (Institute for Translational Medicine University of Chicago, USA). hKCNQ1 was subcloned into the pCR3.1 vector. The ancillary subunits hKCNE3 and hKCNE1 were subcloned into the pIRES-CD8 vector provided by Dr. Florian Lesage (Institut de Pharmacologie Moléculaire et Cellulaire, Sophia Antipolis, Valbonne, France). Mutations in hKCNE3 were produced by Pfu-based mutagenesis (QuickChange Kit; Stratagene, La Jolla, CA, USA) by standard protocols and the amplified regions were confirmed by sequencing (Cid et al., 2000).

Electrophysiological assays: Chinese hamster ovary (CHO) cells (American Type Cell Culture; USA) were cultured in Ham’s F12 medium and transiently transfected with cDNAs for hKCNQ1 and the corresponding ancillary subunit hKCNE1 or hKCNE3, CD8 (1 μg, 1 μg, 0.3 μg, respectively) by electroporation. Transfected cells were plated in culture dishes and cultured in an incubator with 5% CO$_2$ until use (24-48 h). Dynabeads CD8 (Invitrogen Dynal ASA, Oslo, Norway) were used to visually identify transfected cells (Díaz & Sepúlveda, 1995).

Cells in culture dishes were placed on the stage of an inverted microscope (DM IL Type DFC290, Leica), and the culture medium was replaced by a standard bathing solution containing (in mM): 135 Na-gluconate, 4 KCl, 1 K-gluconate, 2 CaCl$_2$, 1 MgCl$_2$, 33 sucrose, and 10 HEPES/Tris, pH 7.4. Experiments were performed in the voltage-clamp whole-cell mode of the patch-clamp technique. All measurements were made at room temperature (22 ± 2°C). Pipettes (borosilicate, Harvard Apparatus, UK) were pulled to give a resistance of 3-5 MΩ when filled with the pipette solution which contained (in mM): 6 KCl, 134 K-gluconate, 1 MgCl$_2$, 5 EGTA, 1 Na$_3$ATP, and 10 HEPES, pH 7.2.

Potentials were corrected for liquid junction shifts. Electrophysiological data were acquired via an Axopatch 200B amplifier and a Digidata 1320A digitizer (Axon Instruments, Foster City, CA). Further data analysis was done using features of SigmaPlot v. 11 (Systat Software Inc., San José, CA, USA).

Protocols: Holding voltage was -80 mV unless otherwise stated. Currents were elicited by potentials ranging from -100 mV to +80 mV, in 20 mV increments, for hKCNQ1 alone, hKCNQ1-hKCNE3 and the corresponding mutants hKCNQ1-hKCNE3S82A and hKCNQ1-hKCNE3S82D. hKCNQ1-hKCNE1 channel currents were studied in the -80 mV to +100 mV range. Deactivating tail currents were recorded at -30 mV after a series of either 200 ms or 1 s activating pulses. In each figure we show representative current traces for each combination of subunits and constructs. For 17β-estradiol inhibition studies, a protocol of pulses designed to obtain a current-voltage relation as described above was given immediately after gaining access to the cell. Then, an 8 min experiment was run using a repetitive protocol consisting in pulses to -40, 0, +40 and...
+100 mV from a -80 mV holding potential with a 10-s period, to obtain a time course during which 17β-estradiol was added. After this a further current-voltage protocol was taken.

**Transepithelial transport studies**

Colonic epithelia were mounted in Ussing chambers (Physiologic Instruments, San Diego, CA, USA) on inserts exposing and area of 0.5 cm². Transepithelial potential difference was clamped to zero using an EVC-4000 voltage-clamp apparatus (World Precision Instruments, UK). The transepithelial short-circuit current ($I_{SC}$) was recorded using Ag-AgCl electrodes in 3 M KCl agar bridges as previously described (Condliffe et al, 2001). All preparations were allowed to equilibrate for 30-45 min before the experiments were performed. The $I_{SC}$ was defined as positive for anion flow from the basolateral to apical chamber.

To investigate the activity of basolateral K⁺ channels in isolation, the apical membrane was permeabilized by addition of 10 μM amphotericin B in the presence of a mucosal to serosal K⁺ gradient as previously described (McNamara et al, 2000). Ouabain (1 mM) was added to the basolateral bath to inhibit Na⁺-K⁺-ATPase. The resulting $I_{SC}$ is due to the movement of K⁺ through channels through the basolateral membrane ($I_K$). For measurement of $I_K$ current-voltage relationships, currents were elicited by imposition of 1 sec test potentials between -100 and +100 mV in 20 mV increments, from an initial holding potential of -40 mV.

**Statistical analysis**

Densitometric analysis was performed using Genetools software (Syngene, Cambridge UK). Statistical analysis was performed using $t$-test for analysis between two groups, and ANOVA and Tukeys post-hoc test for analysis of multiple groups. $P$-values of 0.05 and less were considered to be significant. Number of samples ($n$) refers to number of animals. Data is expressed as mean ± SEM.

**Materials**

All antibodies were from Santa Cruz Biotechnology (CA, USA). Protein-G sepharose beads and ECL-plus detection system were from Amersham Biosciences (Buckinghamshire, UK). Chromanol 293B was from Tocris (Avonmouth, UK). (3R,4S)-(+) N-[3-hydroxy-2,2-dimethyl-6-(4,4,4-trifluorobutoxy)chroman-4-yl]-N-methyl-ethanesulfonamide (HMR-1556) was kindly provided by Dr. Uwe Gerlach (Aventis Pharma Deutschland, Frankfurt-am-Main, Germany). All other drugs were from Sigma-Aldrich (Dublin, Ireland). Drugs were dissolved in DMSO or water as stock solutions and diluted appropriately. The final DMSO concentration in all experiments was less than 0.1%.
RESULTS

Gender comparison of KCNQ1 and KCNE subunit expression in colonic crypts
To determine expression levels of KCNQ1 and KCNE subunits semi-quantitative RT-PCR analysis was carried out. Figure 1A compares KCNQ1 expression levels between male and female (estrous) colonic crypt cells. No significant difference was found in transcript levels between male and females (male 1.08 ± 0.05 fold-higher, n = 5, P > 0.05). We then examined whether gender differences exist in basal expression levels of KCNE subunits. Expression levels for KCNE1 and KCNE3 were 4.8 ± 0.6 and 10.4 ± 1.9 fold higher respectively compared to transcript levels in female crypt cells (P < 0.01, n = 5) (Figure 1B, 1D). No expression of KCNE2 was found in either male or female colonic crypts (Figure 1C).

To verify gender differences in expression of KCNQ1 and KCNE subunits we examined protein levels in colonic crypts from male and female (estrous) rats. In agreement with our RT-PCR experiments, no significant difference was found for basal protein expression of KCNQ1 (male 1.1 ± 0.03 fold, n = 4) (Figure 2A). However, KCNE1 and KCNE3 protein levels were significantly higher in male colonic crypts (KCNE1 6.2 ± 0.5, KCNE3 14.9 ± 0.9, P < 0.01, n = 4) (Figure 2B, 2D). No expression of KCNE2 was detected on either male or female colonic crypts (Figure 2C).

In colonic crypts association of KCNQ1 with KCNE3 results in a channel complex responsible for K+ recycling at the basolateral membrane. Therefore, we compared the level of association of KCNQ1 with KCNE3 between male and female colonic crypts by immunoprecipitating KCNQ1 and measuring the amount of KCNE3 in the immunoprecipitate. Male cells showed a higher level of association between KCNQ1 and KCNE3 compared to female cells (2.25 ± 0.21 fold higher, P < 0.01, n = 3) (Figure 3A).

The effects of estrogen on the association of KCNQ1:KCNE3 were examined by co-immunoprecipitation of the channel complex in isolated rat colonic crypts (Figure 3B, 3C). E2 produced a rapid decrease in the association between KCNQ1 and KCNE3 (33.9 ± 8.7% of control, P < 0.01, n = 3) within 15 min exposure in colonic crypts isolated from female rats but had no effects in crypts from male rats (101.2 ± 1.5% of control).

The estrous cycle dependence of KCNQ1 and KCNE3 expression was also examined in female rat colonic crypts. KCNQ1 expression fluctuates throughout the estrous cycle being maximal at estrous and metaestrous while it is at its lowest during proestrous and diestrous (Figure 4A). Similarly, KCNE3 expression fluctuates throughout the estrous cycle. However, KCNE3 expression pattern significantly differ from that of KCNQ1. KCNE3 expression was maximal at proestrous and progressively decreasing reaching minimal levels at metaestrous and diestrous (Figure 4B).

Gender differences in whole-cell currents in colonic crypt cells
To study gender differences in KCNQ1 gating properties whole-cell patch-clamp experiments were performed on epithelial cells derived from single isolated colonic crypts of rat colon. In both, male and female colonic crypt cells whole-cell currents were activated by depolarizing clamp voltages. The whole-cell currents showed a reversal potential (E_{rev}) of approximately -67 mV (E_{rev} male = -68.2 ± 2.7, E_{rev} female = -66.2 ± 3.0), which is close to the predicted E_{rev} for a K+ conductance (~61.5 mV). These currents were sensitive to chromanol 293B, a specific KCNQ1 channel inhibitor with
almost complete inhibition observed at 100 µM. The currents were activated by increasing temperature from 22 °C to 37 °C (data not shown). The KCNQ1 channel activity is temperature sensitive and increasing the temperature is known to activate the channel (Unsöld et al., 2000). Taken together, these results demonstrate the functional activity of KCNQ1 channels in rat distal colonic crypts.

In male colonic crypt cells whole-cell currents were instantaneous, with rapid deactivation and did not exhibit any delayed activated component (Figure 5A). The current/voltage (I/V) relationship for these currents was linear with no outward rectification. There was no significant difference between whole-cell inward ($\gamma_{in}$) and outward conductance ($\gamma_{out}$) ($\gamma_{in} = 702.7 \pm 188.6$ pS vs. $\gamma_{out} = 613.5 \pm 126.9$ pS, $P < 0.01$, $n = 7$). Their linear I/V relationship led to significant currents at voltages more negative than -60 mV (Figure 5B). Thus this conductance should be constitutively active at the negative membrane voltages of colonic epithelial cells. On the other hand, whole-cell currents measurements in female colonic crypt cells showed currents that activate slowly upon depolarization to voltages more positive than -60 mV and showed rapid deactivation (Figure 5A). The I/V relationship for these currents showed voltage-dependence and outward rectification ($\gamma_{in} = 76.0 \pm 15.6$ pS vs. $\gamma_{out} = 662.4 \pm 97.3$ pS, $P < 0.01$, $n = 9$) (Figure 5B). At voltages below -60 mV whole-cell currents were near zero suggesting this conductance is largely inactive at the membrane voltages exhibit by colonic epithelial cells.

**Gender differences in basolateral $K^+$ currents in rat colonic epithelia**
Basolateral $K^+$ currents ($I_K$) were generated in amphotericin B apically permeabilized rat colonic epithelium mounted in Ussing chambers. Treatment with chromanol 293B (100 µM) produced marked inhibition of basal and forskolin (10 µM) activated $I_K$, suggesting the main component of this current is due to KCNQ1 channel activity. In male tissues treatment with forskolin activated outward currents that displayed an almost linear $I_K$/V relationship (inward $I_K$ 128.0 ± 10.5 µS, outward $I_K$ 143.8 ± 9.8 µS) with significant current at voltages below -40 mV (Figure 6). In contrast, female epithelia showed forskolin-activated $I_K$ with strong outward rectification (inward $I_K$ 31.0 ± 4.2 µS, outward $I_K$ 109.8 ± 7.8 µS) and a voltage-dependent $I_K$/V relationship. At voltages below -40 mV the current amplitude was significantly lower in female than in male colonic epithelia (Figure 6).

**Effect of gender on KCNQ1 sensitivity to chromanol 293B**
Coexpression of KCNQ1 channels with KCNE3 results in a 10-fold increase in the sensitivity to chromanol 293B inhibition. In this study, we compared the effects of chromanol 293B on KCNQ1 currents between male and female rats in patch-clamp recordings of whole-cell currents in colonic crypt cells and in Ussing chamber measurements of basolateral $K^+$ currents. Whole-cell currents from male colonic crypts were significantly more sensitive to chromanol 293B than currents from female colonic crypts. Figure 7A shows concentration-response curves for inhibition by chromanol 293B of whole-cell currents in colonic crypt cells from male and female rats. The $IC_{50}$ for chromanol 293B in male cells was 1.45 ± 0.15 µM while $IC_{50}$ in female crypt cell was 12.57 ± 1.74 µM; this represents a 10-fold higher sensitivity in male crypts compare to female ($P < 0.05$, $n = 5$).

A similar gender difference in the sensitivity to chromanol 293B was observed in basolateral $K^+$ currents between male and female colonic epithelia. Figure 7B shows
concentration-response curves for inhibition of basolateral K⁺ currents by chromanol 293B in male and female colonic epithelia. IC₅₀ for chromanol 293B in male colonic epithelia was 1.33 ± 0.17 µM while IC₅₀ in female colonic epithelia was 14.40 ± 2.67 µM. Male tissues showed a 10-fold higher sensitivity to chromanol 293B compared to female epithelia (P < 0.05, n = 5).

Effect of gender on forskolin-induced I₅₋ₐ sensitivity to chromanol 293B
We also sought to examine the responses of intact colonic epithelia to chromanol 293B. In basal conditions, Iₛₑ was inhibited by chromanol 293B, however, no significance gender difference was observed between male and female colonic epithelia (data not shown). Because basal currents in colonic epithelia are relatively low we stimulated short-circuit currents with forskolin (10 µM). The forskolin-activated Iₛₑ inhibition response curves for chromanol 293B were significantly different between male and female colonic epithelia (Figure 8A). IC₅₀ for chromanol 293B in male epithelia was 1.07 ± 0.10 µM while IC₅₀ in female epithelia was 25.17 ± 5.09 µM; this represents a 10-fold higher sensitivity in male colonic epithelia compared to female tissues (P < 0.05, n = 5).

In recent studies, several groups have reported that chromanol 293B is also an inhibitor of the cystic fibrosis transmembrane regulator (CFTR) channel and that the effect of chromanol 293B on Iₛₑ in intact colonic epithelia represents a mixture of its effect on both CFTR and KCNQ1 (Liao et al., 2005). Therefore, we repeated this experiment using the chromanol compound HMR-1556 a more potent and selective inhibitor of the KCNQ1 channel that has no effect on CFTR currents (Lerche et al., 2000). Like chromanol 293B, HMR-1556 also displays a 10-fold higher selectivity for KCNQ1 channels associated with KCNE3 subunits (Lerche et al., 2000). Figure 8B compares inhibition curves for HMR-1556 on forskolin-activated Iₛₑ showing a 10-fold higher sensitivity in male colonic epithelia compared to female tissues (IC₅₀ male 54.7 ± 7.5 nM; female 457 ± 17.9 nM, P < 0.05, n = 5). However, unlike chromanol 293B, HMR-1556 only inhibited 60% of total Iₛₑ. Thus these experiments confirm the results obtained with chromanol 293B showing a gender difference in KCNQ1 channels at the whole tissue level.

Estrogen inhibits currents elicited by KCNQ1:KCNE3 channel complex expressed in CHO cells
The data presented above show prevalence in female colon basolateral membranes of a form of the main cAMP-activated K⁺ conductance consistent of KCNQ1 homomers with little participation of ancillary β-subunits, be it KCNE1 or KCNE3. In addition we show that acute addition of E2 dissociates existing KCNQ1:KCNE3 channel complexes, which in itself would lead to the type of voltage-dependence prevalent in female colonic crypt cells. Our previous data suggest that rapid, non-genomic E2 inhibition of KCNQ1:KCNE3 is signaled by gender-specific PKCδ activation (O’Mahony et al., 2007). Data presented above suggest that the E2 effect could involve a dissociation of KCNQ1 and KCNE3 with the consequent decrease of conductance at physiologically relevant voltages. To test this hypothesis we have coexpressed KCNQ1 and KCNE3 in CHO cells to study the effect of E2 on the resulting currents. CHO cells are a good expression platform for KCNQ1:KCNE3 assays (Melman et al., 2001) and show endogenous expression of PKCδ (See Supplementary Fig. 4). Fig. 9A shows currents recorded in a cell expressing KCNQ1:KCNE3 with the expected near-instantaneous or
fast activating currents consistent with channels open at the expected colonocyte membrane potential and a near-linear $I/V$ curve (Fig. 9D). Immediately after measuring the currents shown in Fig. 9A, currents were sampled at 10-s intervals as shown in Fig. 9C. There was a rundown in KCNQ1:KCNE3 currents which are shown for pulses to -40 and 0 mV. Addition of 100 nM E2 at the time shown by the arrow led to fast current inhibition (see also first bars in Fig. 10). The decrease in KCNQ1:KCNE3 current was accompanied by a change in the kinetics as can be seen in Figs. 9E, where traces at the different voltages used are shown 20 s before and 10 and 40 s after E2 addition. These changes were not observed without E2 addition, when only the slow rundown of the current was present (Fig. 10 and Supplementary Fig. 1). E2 inhibition was largest at the lower potentials and a significant slowing down of current activation occurred in the presence of the estrogen at the most depolarized voltages (Fig. 9F). A family of currents taken at the end of the experiments shows this change in kinetics (Fig. 9B), as does the corresponding $I/V$ curve (Fig. 9D) that shows marked outward rectification and inhibition at near physiological potentials.

As seen above, and as reported before (Arrighi et al., 2001), KCNE1 β-subunit is present in intestinal epithelium, therefore both KCNQ1:KCNE1 channels and KCNQ1 homomeric channels without KCNE subunit could also in principle occur in colon epithelium. We therefore tested for a possible effect of E2 on currents elicited in CHO cells transfected with KCNQ1 on its own and KCNQ1 accompanied by KCNE1 (Supplementary Figs. 2 and 3). The currents generated in CHO cells by these transfections had the expected morphology (Bendahhou et al., 2005), with acquisition of voltage-dependence for both forms of channels and markedly slowed activation for the KCNE3:KCNE1 complex. As shown in Supplementary Figs. 2B and 3B, and summarized in Fig. 10, neither of these channel forms was affected by E2 addition.

The fast effect that E2 exerts on colonic secretion through inhibition of the KCNQ1:KCNE3 is mediated by gender-specific activation of PKCδ (O'Mahony et al., 2007). Residue S82 of KCNE3 has been shown to be a functional site for PKCδ-promoted phosphorylation and modulation of the activity of KCNE3 (Abbott et al., 2006). We have explored by site-directed mutagenesis whether S82 is required for the inhibitory effect of E2 on KCNQ1:KCNE3 channels. Transfection of KCNQ1 and KCNE3-S82A led to currents very much like those obtained with the non-mutated auxiliary subunit (Fig. 11A and B). One difference with the KCNQ1:KCNE3 channels, being that KCNQ1:KCNE3-S82A rundown much more rapidly than the non-mutated channels (Fig. 11C). Nevertheless, once rundown was at its slowest, addition of E2 was without effect on the residual current (Fig. 11C and also Fig. 10). Also the kinetics of fast activation of KCNQ1:KCNE3-S82A channels was not altered in the presence of E2 (Fig. 11D and E).
DISCUSSION

In colonic epithelia, Cl⁻ secretion stimulated by either Ca²⁺- or cAMP-dependent secretagogues requires parallel activation of basolateral K⁺ channels. Basolateral K⁺ channels are responsible for recycling K⁺ that is transported into the cell by basolateral Na⁺-K⁺-2Cl⁻ cotransporters and Na⁺-K⁺-ATPase pumps and for maintaining the electrical driving force for luminal Cl⁻ secretion (Kunzelmann & Mall, 2002, Heitzmann & Warth 2008). Two types of K⁺ channels have been identified in the basolateral membrane of rat colonic crypts: a Ca²⁺-activated intermediate conductance K⁺ channel (Kunzelmann & Mall, 2002), and a cAMP-activated very small conductance (3 pS) K⁺ channel (Schroeder et al., 2000). Pharmacological data have indicated the latter to be KCNQ1 channels and that this channel is important for cAMP-dependent Cl⁻ secretion in colon (Schroeder et al., 2000).

Recent studies have shown expression of KCNQ1 in colonic crypt cells where it colocalizes with the KCNE3 regulatory subunit. Coexpression of KCNQ1 with KCNE3 produces a constitutively open channel complex that is active at the negative membrane voltage exhibited by colonic epithelial cells (Schroeder et al., 2000). The KCNE3 subunit has been shown to be critical for the KCNQ1 role in supporting Cl⁻ secretion in intestine and trachea (Preston et al., 2010). Therefore, coexpression of KCNQ1 channels with KCNE3 is an important factor in regulating channel activity and function during Cl⁻ secretion. Previous studies have also shown expression of the KCNE1 subunit, but its physiological role in colonic epithelia remains unclear.

In this study, we report a marked gender difference in the expression of KCNE1 and KCNE3 in rat distal colonic crypt cells. We found a significantly higher expression of these regulatory subunits in male cells compared to female cells. At the transcript level, male colonic cells showed a 5-fold and 10-fold higher level of KCNE1 and KCNE3 mRNA, respectively, compared to female crypt cells. At the protein level, this difference was even higher with a 6-fold and 13-fold higher protein expression of KCNE1 and KCNE3, respectively, in male cells compared to female. Interestingly, no gender difference was observed in the expression levels of the KCNQ1 channel in either mRNA or protein expression. No expression of KCNE2 subunit was found in either male or female crypt cells.

Since coassembly of KCNQ1 channels with KCNE3 leads to active channels at negative membrane potentials exhibited by colonic crypt cells, we examined the level of association between these channel proteins. Male colonic crypts showed a 2.25-fold higher level of association between KCNQ1 and KCNE3, respectively, compared to female crypts. This result suggests that male colonic epithelia contain more than twice the amount of KCNQ1/KCNE3 complex channels compared to female colonic crypts.

In female rat colonic crypts expression of KCNQ1 and KCNE3 was also found to be dependent on estrous cycle. This indicates KCNQ1:KCNE3 channel complexes association and function is likely to depend on the estrous cycle stage. During proestrus KCNQ1 expression is at its lowest while KCNE3 is maximal, this suggests that although KCNQ1 expression is low the proportion of KCNQ1:KCNE3 complexes would be high. In contrast, at metaestrus when KCNQ1 expression is maximal while KCNE3 is minimal the amount of KCNQ1:KCNE3 complexes compared to other KCNE complexes or homomeric KCNQ1 complexes will be minimal. The variation in the degree of association between KCNQ1 and KCNE3 proteins in male and female crypts, and throughout the estrous cycle in the female, points to an endocrine modulation of the
channel complex assembly. Our finding that 17β-estradiol promotes the dissociation of KCNQ1:KCNE3 in female crypts and not in male supports this conclusion. The 60% reduction in the association between KCNQ1 and KCNE3 produced by E2 in female colonic crypts compares favourably with the 56% lower KCNE3 expression observed in colonic crypts isolated from female rats relative to males.

Since KCNE3 regulates the gating properties of KCNQ1 channels we examined if the gender difference in KCNE3 expression and association with KCNQ1 led to differences in the electrical properties of this channel between male and female colonic crypts. In male colonic crypts, whole-cell current showed the typical characteristics of the KCNQ1:KCNE3 channel complex with instantaneous activation and rapid deactivation, the I/V relationship was linear with no outward rectification and significant currents were active at voltages more negative than -50 mV. In contrast, whole-cell currents from female colonic crypts showed a much slower activation kinetics, the I/V relationship showed strong outward rectification and voltage dependence reminiscent of the properties of homomeric KCNQ1 channels or KCNQ1:KCNE1 complexes. These results suggest that in male colonic crypt cells KCNQ1:KCNE3 represents the main KCNQ1 current while female colonic crypt cells may express a mixed population of homomeric KCNQ1 and heteromeric KCNQ1:KCNE1 and KCNQ1:KCNE3 channel complexes, although the latter may represent only a small fraction of the KCNQ1 conductance.

We also examined the properties of the basolateral K⁺ conductance in apically permeabilized colonic epithelia to determine gender differences in the KCNQ1 currents. Male epithelia showed basolateral K⁺ currents with a linear I/V relationship characteristic of KCNQ1:KCNE3 complexes with significant current at hyperpolarizing membrane potentials. In contrast, basolateral K⁺ currents from female colonic epithelia showed voltage-dependence and outward rectification. At membrane voltages more negative than -50 mV, currents recorded from female colonic epithelia were significantly lower than those observed in male epithelia. These data suggest that at the cellular and tissue level gender differences in KCNE3 expression leads to marked differences in the electrical properties of KCNQ1 currents.

Association of KCNQ1 channels with KCNE subunits not only affects the gating properties of the channel but also its pharmacological profile (Robbins, 2001; McCrossan & Abbott, 2004; Jespersen et al., 2005). Several studies have reported that association of KCNQ1 with KCNE increases the sensitivity to chromanol 293B (Lerche et al., 2000; Unsöld et al., 2000). Therefore, sensitivity to this blocker has been used as an index of association of KCNQ1 with KCNE subunits. In this study, we observed a significant gender difference in the sensitivity to chromanol 293B of whole-cell currents in crypt cells, apically permeabilized epithelia and intact colonic epithelia. Male colonic cells and tissues showed a 10-fold higher sensitivity to chromanol 293B compared to female cells and tissues. This difference in sensitivity to chromanol 293B is similar to that reported between KCNQ1 and KCNQ1:KCNE3 channels expressed in Xenopus oocytes (Lerche et al., 2000; Unsöld et al., 2000). Recent studies have reported that chromanol can also block the cystic fibrosis transmembrane conductance regulator (CFTR) Cl⁻ channel (Liao et al., 2005); therefore, in intact colonic epithelia we also tested the sensitivity of short-circuit currents to inhibition by HMR-1556. This compound has been shown to be more potent and selective than chromanol 293B in blocking KCNQ1 channels and also displays a 10-fold higher potency on channels associated with KCNE subunits (Lerche et al., 2000). Again, male colonic epithelial
short-circuit currents showed a 10-fold higher sensitivity to HMR-1556 compared to female colonic epithelia.

The modulatory effect of KCNE subunit expression on KCNQ1 drug sensitivity reported here is not unique for chromanol 293B and HMR-1556. It has been shown previously that coexpression of KCNE subunits with KCNQ1 channels decreases the effect of other drugs, including clofyllium and the KCNQ1 channel opener benzodiazepine R-L3 (Busch et al, 1997; Boucherot et al, 2001; Salata et al, 1998). Interestingly, it also has been shown that compared with KCNQ1 channels, the KCNQ1:KCNE complexes have higher affinity for several antiarrhythmic drugs (Kurokawa et al, 2003). Therefore, association with KCNE subunits appears to affect the general pharmacological profile of the KCNQ1 channel, and these intriguing interactions between KCNE subunits and KCNQ1 may help in the design and development of drugs that selectively target different KCNQ1 channel complexes in specific tissues.

Following the discovery that KCNQ1 co-assembles with KCNE subunits to form heteromeric channels there was much excitement about the fundamental changes of KCNQ1 channel properties brought about by these subunit interactions. Apart from the modulation of electrical and pharmacological properties of KCNQ1, several studies have reported that association with KCNE subunits regulates modulation of KCNQ1 channels by several factors. KCNE1 beta subunits can dissociate from KCNQ1 once they are at the plasma membrane (Poulsen & Klaerke, 2007). Deletion of KCNE2 beta subunits, disrupts polarized trafficking resulting in misrouting of KCNQ1 from apical to basolateral membranes in parietal cells (Roepke et al, 2011). KCNE1 mediates dynamin-dependent endocytosis of the heart IKs channel (KCNQ1-KCNE1 complex), and stimulation of PKC with the phorbol ester PMA increased KCNQ1-KCNE1 endocytosis in CHO cells expressing wild-type dynamin 2 (Kanda et al, 2011). Homomeric KCNQ1 channels show little regulation by temperature but when coexpressed with KCNE subunits they become highly sensitive to temperature (Unsöld et al, 2000). Intracellular acidification leads to a reduction of most K+ currents, and this is also the case for KCNQ1. However, coexpression with KCNE1 has been reported to reverse this effect of intracellular acidification and to mediate a net KCNQ1 current increase (Unsöld et al, 2000). In Xenopus oocytes, stimulation of rat KCNQ1 currents by an increase in intracellular Ca2+ requires coexpression of either KCNE1 or KCNE3. Kurokawa et al (2003) demonstrated in CHO cells that cAMP mediated KCNQ1 phosphorylation is independent of coassembly with KCNE1, but transduction of the channel phosphorylation into increased channel activity requires the presence of KCNE1.

We have used a CHO cell expression for KCNQ1:KCNE3 system further to understand the action of E2 on the channel complex. CHO cells have been used before in KCNQ1:KCNE3 functional assays (Melman et al., 2001). We show, on the other hand, that CHO cells express PKCδ, which we have proposed as the key player in the gender-specific signaling of the rapid estrogen effect in colonic epithelium (O’Mahony et al., 2007). Our results indicate that E2 inhibits currents mediated by KCNE3/KCNQ1 expressed in CHO cells. The effect is rapid, consistent with a non-genomic action and requires the presence of the KCNE3 subunit, as there is no observable effect when KCNQ1 is expressed on its own. The currents induced by co-expression of KCNE3 and KCNQ1 had the characteristics time- and voltage-independence expected for these channel. Strikingly, however, there was a radical change in voltage-dependence brought
about by E2. The currents become slow activating and markedly voltage-dependent, and reminiscent of those seen when KCNQ1 is expressed in the absence of auxiliary subunit. The marked voltage-dependence of the currents post-E2 treatment has as a consequence that a major collapse in conductance occurs at physiologically relevant potentials (Giraldez et al., 1988).

As KCNE1 is also expressed in the intestinal epithelium, we tested the effect of E2 on the slowly activating KCNQ1:KCNE1 channels that are known to mediate currents responsible for slow repolarization of cardiac cells. E2 had no effect on KCNQ1:KCNE1-mediated currents in the CHO expression system pointing to a specificity for the estrogen action dependent on the presence of KCNE3.

All KCNE subunits possess a consensus site for PKC-dependent phosphorylation in a region near their only transmembrane domain which is formed by a serine residue and three basic amino acids (RSK82RK in KCNE3) and its potential importance in phosphorylation has been suggested by mutagenesis (Abbott et al., 2006). Currents elicited by KCNQ1:KCNE3-S82A had all the hallmarks of non-mutated channels, but exhibited a markedly faster rundown upon breaking into the whole-cell recording configuration mode. Addition of E2, however, was without effect showing that S82 is essential for estrogen action and suggesting that phosphorylation at that residue might be part of its mechanism of action. We hypothesize that the effect of E2 on KCNQ1:KCNE3 might be to induce the rapid dissociation of the two subunits leading to kinetics more akin to that associated with expression of KCNQ1 on its own. This would not necessarily involve a complete physical separation, as subtle mutations in KCNE3 can lead to a functional uncoupling of KCNQ1 and its auxiliary subunit (Melman et al., 2002). Nevertheless, the gender-specific dissociation of KCNQ1 and KCNE3 after E2 treatment that we document here could be related to the changes in channel kinetics observed in the heterologous expression system. The hypothesis of a dissociation of KCNQ1 and KCNE3 under the action of E2 would also explain the differences in rectification observed in the K⁺ currents between males and estrous female colonic epithelium or isolated crypts, as well as in pharmacological sensitivity.

In physiological terms, this study demonstrates that the pharmacology and gating properties of KCNQ1 in colonic epithelia are strongly dependent on gender-regulated expression of KCNE subunits. This subunit-specific pharmacology suggests that drugs that are targeted to KCNQ1 in specific organs can be more specific by considering subunit characteristics. Furthermore, the sexual dimorphism in channel kinetics and subunit expression shown here suggest that gender is an important factor in the use of these drugs for pharmacological interventions.

Because proper function of KCNQ1:KCNE3 channel is essential for maintaining the electrical driving force for electrolyte secretion, the gender specificity of the anti-secretory response to 17β-estradiol has implications for the development and use of new pharmacological tools in the treatment of Cl⁻ secretory disorders such as secretory diarrhea and cystic fibrosis.
REFERENCES


ACKNOWLEDGEMENTS

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(F), Sense; (R), antisense
FIGURE LEGENDS

Figure 1: Gender differences in KCNQ1 and KCNE subunits transcript expression. Semi-quantitative RT-PCR analysis of KCNQ1 and KCNE subunits expression in male and female rat colonic crypts. Reverse transcribed cDNA from male and female colonic crypt cells was amplified using specific primers for rat KCNQ1 (A) and rat KCNE1 (B), KCNE2 (C) and KCNE3 (D). The PCR reaction produced bands at the expected length for KCNQ1 and KCNE3 subunits. GADPH was used as an internal control to estimate cDNA loading. Values in graphs represent mean ± SEM *P < 0.01, n = 5.

Figure 2: Gender differences in KCNQ1 and KCNE subunits protein expression. Western blot analysis of KCNQ1 and KCNE proteins in male and female rat colonic crypts. Total protein was transferred to PVDF membranes after fractioning by SDS-PAGE and blotted with anti-rat KCNQ1 (A) and rat KCNE1 (B), KCNE2 (C) and KCNE3 (D) β-actin was used as an internal control to estimate protein loading. Values in graphs represent mean ± SEM *P < 0.01, n = 4.

Figure 3: Gender and Estrogen dependent differences in KCNQ1 and KCNE3 association. (A) Association of KCNQ1 and KCNE3 proteins in male and female rat colonic crypts. KCNQ1 protein was immunoprecipitated from total cellular lysate using an antibody specific to KCNQ1, then associated KCNE3 was analyzed by Western blot. KCNQ1 was used as an internal control to estimate protein loading. (A) Sexual dimorphism of KCNQ1: KCNE3 protein:protein interaction. (B) and (C) Estrogen modulated changes in KCNQ1:KCNE3 association in male (B) and female (C) colonic crypts. Colonic crypts were treated with 10 nM estrogen for 15 min (15’ E2) or untreated (control). Values in graphs represent mean ± SEM **P < 0.01, n = 3.

Figure 4: KCNQ1 and KCNE3 expression levels throughout the estrous cycle. (A) KCNQ1 expression levels in female rat distal colonic crypts were measured by Western blot. The figure shows a representative blot of KCNQ1 proteins levels at different stages of the estrous cycle. β-Actin expression was used as a protein loading control. The graph represents densitometric analysis of four individual experiments. (B) KCNE3 expression levels in female rat distal colonic crypts were measured by Western blot. The figure shows a representative blot of KCNE3 proteins levels at different stages of the estrous cycle. β-Actin expression was used as a protein loading control. The graph represents densitometric analysis of three individual experiments. Values are given as arbitrary units and expressed as mean ± SEM. **P < 0.01, ***P < 0.001 determined by ANOVA and Tukeys post hoc test. Pro, proestrous; Est, estrous; Meta, metaestrous; Dies, diestrous.

Figure 5: Gender differences in whole-cell currents in rat colonic crypts. Currents were activated by depolarizing voltage pulses applied in 20 mV increments from -100 mV to +100 mV in male and female rat distal colonic crypts. (A) Representative whole-cell current tracings of male and female colonic crypts. (B) Current-voltage (I/V) relations obtained for voltage-activated whole-cell currents in from male (filled circles) and female (open circles) rat distal colonic crypts. Values in graphs represent mean ± SEM *P < 0.01, n = 7 for male, n = 9 for female.
Figure 6: Gender differences in basolateral K⁺ conductance in rat colonic epithelia. Steady state short-circuit current/voltage relationship (Iₛ⁄V) of basal (A) and forskolin-activated (B) basolateral K⁺ currents in male (filled circles) and female (open circles) rat colonic epithelia. Currents were measured in apically permeabilized epithelia in the presence of a basolaterally directed K⁺ gradient. Values are represent mean ± SEM, n = 5.

Figure 7: Gender differences in chromanol 293B inhibitory response. Concentration-responses curve for the inhibitory effect of chromanol 293B on whole-cell currents (I₇C, A) and basolateral membrane K⁺ currents (I₉, B) from male (filled circles) and female (open circles) rat distal colonic crypts. Currents (I₇C or I₉) were normalized to control currents (I_con) recorded in the absence of chromanol 293B. Values in graphs represent mean ± SEM *P < 0.01, n = 5.

Figure 8: Gender differences in IₛC inhibition by KCNQ1 inhibitors in rat colonic epithelia. Concentration-responses curves for the inhibitory effect of chromanol 293B (A) and HMR-1556 (B) on forskolin-activated short-circuit currents (IₛC) of male (filled circles) and female (open circles) rat colonic epithelia. Currents (I₇C or I₉) were normalized to control currents (I_con) recorded in the absence of chromanol 293B or HMR-1556. Values in graphs represent mean ± SEM *P < 0.01, n = 5.

Figure 9: Effect of 17β-estradiol (E2) on currents mediated by the KCNQ1/KCNE3 potassium channel complex expressed in CHO cells. A and B: current families elicited in CHO cells transfected with KCNQ1 and KCNE3 cDNA before and after addition of 100 nM E2 respectively. Currents were elicited by square pulses taking the membrane from a holding potential of -80 mV to voltages between -100 and 80 mV in 20 mV steps. Post-pulse was to -30 mV. C: end of pulse current-voltage relations for experiments as those shown in A and B. Means ± SEM of 6 experiments without or with 17β-estradiol. Currents in A were measured before and those in B after giving a train of 3-s pulse stimulations consisting of 500-ms square pulses to -40, 0, 40 and 100 mV. The holding potential was -80 mV and the period 10-s. D: currents taken at the end of pulses to -40 and 0 mV of the train of pulses plotted for the duration of the experiment, with an arrow showing the time of 100 nM E2 addition. E: currents elicited at the different voltages during the stimulus train taken 20 s before and 10 or 40 s after E2 addition. F: times to reach 90% of maximal current as function of voltage for currents taken 20 s before and 40 s after E2 addition (means ± SEM, n=5).

Figure 10. Effect of 17β-estradiol on K⁺ currents mediated by KCNQ1 with or without different KCNE β-subunits. The effect of E2 (100 nM) was assayed as described in Figure 9. The percent effect of E2 (or that due to channel rundown) was calculated comparing the current before E2 addition and that reached 120 s after E2. The data are means ± SEM, with n=5 for KCNQ1/KCNE3, n=5 for KCNQ1/KCNE3 without addition of E2, n=4 for KCNQ1 without β-subunit, n=5 for KCNQ1/KCNE1, and n=5 for KCNQ1/KCNE3-S82A. Data are for measurements taken at -40 and 0 mV, except for the KCNQ1/KCNE1 constructs where figures correspond to measurements at 0 and 40 mV respectively.
Figure 11. Effect of 17β-estradiol (E2) on currents mediated by the potassium channel complex formed by KCNQ1 and the mutant KCNE3-S82A β-subunit.

(A): family of currents elicited in CHO cells transfected with KCNQ1 and KCNE3-S82A cDNA taken immediately after breaking into whole cell. Currents were elicited by square pulses taking the membrane from a holding potential of -80 mV to voltages between -100 and 80 mV in 20 mV steps. Post-pulse was to -30 mV. (B): end of pulse current-voltage relation for experiments as those shown in (A). Means ± SEM of 10 experiments. A train of 3-s pulse stimulations consisting of 500-ms square pulses to -40, 0, 40 and 100 mV was given to monitor current rundown and test the effect of E2. The holding potential was -70 mV and the period 10-s. In (C), currents taken at the end of pulses to -40 and 0 mV of the train of pulses are plotted for the duration of the experiment, with an arrow showing the time of 100 nM E2 addition. E: currents elicited at the different voltages during the stimulus train taken 20 s before and 10 or 40 s after E2 addition. D: times to reach 90% of maximal current as function of voltage taken 20 s before and 40 s after E2 addition (means ± SEM, n=5).
Figure 1
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Supplementary Figures

Figure 1S. Rundown of currents mediated by the KCNQ1/KCNE3 potassium channel complex. (A): a family of currents elicited by KCNQ1 and KCNE3 transfection of CHO cells obtained immediately after the establishment of the whole-cell recording configuration. Square pulses taking the membrane from a holding potential of -80 mV to voltages between -100 and 8 mV in 20 mV steps were used. Post-pulse was to -30 mV. The cell was then subjected to a train of 3-s pulse stimulations consisting of 500-ms square pulses to -40, 0, 40 and 100 mV. The holding potential was -80 mV and the period 10-s. In (B): currents taken at the end of pulses to -40 and 0 mV of the train of pulses are plotted for an 8-min period, during which no addition took place. In (C), currents elicited at the different voltages during the stimulus train taken at the times indicated by the arrows in (B).

Figure 2S. Effect of 17-β-estradiol (E2) on currents mediated by the KCNQ1 potassium channel in the absence of β-subunit. (A): a current family elicited in CHO cells transfected with KCNQ1 shortly after breaking into the whole-cell recording configuration. Currents were elicited by square pulses taking the membrane from a holding potential of -80 mV to voltages between -100 and 80 mV in 20-mV steps. Post-pulse was to -30 mV. After currents in (A) were measured, a train of 3-s pulse stimulations consisting of 500-ms square pulses to -40, 0, 40 and 100 mV was given. The holding potential was -80 mV and the period 10-s. In (B), currents taken at the end of pulses to -40 and 0 mV of the train of pulses are plotted for the duration of the experiment, with an arrow showing the time of 100 nM E2 addition.

Figure 3S. Effect of 17β-estradiol (E2) on currents mediated by the KCNQ1/KCNE1 potassium channel complex expressed in CHO cells. (A) and (B): current families elicited in CHO cells transfected with KCNQ1 and KCNE1 cDNA before and after addition of 100 nM E2 respectively. Currents were elicited by square pulses taking the membrane from a holding potential of -80 mV to voltages between -100 and 80 mV in 20 mV steps. Post-pulse was to -30 mV. Currents in (A) were measured before and those in (B) after giving a train of 9-s pulse stimulations consisting of 2-s square pulses to 0 and 40 mV from a holding potential of -80 mV. The period was 10-s. In (C), currents taken at the end of pulses to 0 and 40 mV of the train of pulses are plotted for the duration of the experiment, with an arrow showing the time of 100 nM E2 addition. In (D), times to reach 90% of maximal current as function of voltage for currents taken 20 s before and 40 s after E2 addition (means ± SEM, n=5) are shown.

Figure 4S. Western blot analysis of PKCδ expression in CHO whole cell lysate. Cells grown on plastic dishes were scraped into cold PBS followed by centrifugation at 5,000 rpm for 5 min at 4°C. The cell pellet was then suspended in cold RIPA (radioimmune precipitation assay) lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P40, 10 mM N-ethylmaleimide, 0.1 mM phenylmethylsulphonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pestatin A. After 30 min on ice, unlysed cells and nuclei were
pelletted at 12,000 rpm for 15 min at 4°C. The protein concentration of the supernatant was determined by the Bradford method. For immunoblotting, 50 µg protein was loaded onto 10% (w/v) SDS-polyacrylamide gels and electrotransferred onto nitrocellulose membranes. Blots were probed with a 1:200 dilution of anti-PKCδ rabbit polyclonal IgG antibody (C-17: sc-213, Santa Cruz) and visualized with peroxidase-labeled donkey anti-rabbit antibody (dilution 1:25,000). The band corresponding to PKCδ molecular weight is shown by an arrow. We interpret low molecular weight bands as degradation products.
Figure 1S
Figure 2S
Figure 3S