Female Gender-specific Inhibition of KCNQ1 Channels and Chloride Secretion by 17β-Estradiol in Rat Distal Colonic Crypts

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The estrogen sex steroid 17β-estradiol rapidly inhibits secretagogue-stimulated cAMP-dependent Cl− secretion in the female rat distal colonic crypt by the inhibition of basolateral K+ channels. In Ussing chamber studies, both the anti-secretory response and inhibition of basolateral K+ current was shown to be attenuated by pretreatment with rottlerin, a PKCδ-specific inhibitor. In whole cell patch-clamp analysis, 17β-estradiol inhibited a chromanol 293B-sensitive KCNQ1 channel current in isolated female rat distal colonic crypts. Estrogen had no effect on KCNQ1 channel currents in colonic crypts isolated from male rats. Female distal colonic crypts expressed a significantly higher amount of PKCδ in comparison to male tissue. PKCδ and PKA were activated at 5 min in response to 17β-estradiol in female distal colonic crypts only. Both PKCδ- and PKA-associated with the KCNQ1 channel in response to 17β-estradiol in female distal colonic crypts, and no associations were observed in crypts from males. PKA activation, association with KCNQ1, and phosphorylation of the channel were regulated by PKCδ as the responses were blocked by pretreatment with rottlerin. Taken together, our experiments have identified the molecular targets underlying the anti-secretory response to estrogen involving the inhibition of KCNQ1 channel activity via PKCδ- and PKA-dependent signaling pathways. This is a novel gender-specific mechanism of regulation of an ion channel by estrogen. The anti-secretory response described in this study provides molecular insights whereby estrogen causes fluid retention effects in the female during periods of high circulating plasma estrogen levels.

Fluid and electrolyte secretion is an important function in the distal colon as it regulates whole body fluid homeostasis and also maintains mucosal hydration (1). Chloride secretion in the distal colon occurs as a two-step transport process. Cl− is transported into the cell basolaterally with Na+ and K+ via the Na+/K+2Cl− isomorph (NKCC1) co-transporter. Cl− is secreted across the apical membrane into the lumen via a chloride ion channel, the cystic fibrosis transmembrane conductance regulator (CFTR). Secretion of Cl− ions is driven by the activity of Na+/K+-ATPase located in the basolateral membrane. Basolateral K+ channel currents carry out the K+ recycling required to establish the favorable membrane electrical potential for Cl− secretion. The activity of ion transporters involved in Cl− secretion may be modulated by secretagogues acting through cAMP activity or intracellular Ca2+ concentration. Disorders resulting in increased Cl− secretion, for example in bacterial or viral infections, are a primary cause of secretory diarrhea (2).

The biologically active estrogen 17β-estradiol (E2) plays an important role in the normal development and maturation of the female. In addition to this classical role, E2 has also been implicated in the regulation of whole body fluid and electrolyte balance (3, 4). The distal colon has recently been recognized as a target of E2. The colonic crypts express both isoforms of the nuclear estrogen receptor, ERα and ERβ, similar to classical E2-responsive tissues such as uterus and breast tissue (5). We have previously demonstrated that E2 inhibits secretagogue stimulated Cl− secretion in the rat distal colonic crypt (6). The anti-secretory response to E2 was rapid, occurring within 10 min and was dependent on protein kinase C (PKC). In human distal colonic tissue, blocking basolateral K+ channel activity decreases forskolin-stimulated Cl− secretion (7). In rat distal colonic basolateral K+ conductance is formed by at least two different types of K+ channels, activated by Ca2+ or cAMP-dependent agonists (8). Electrophysiological studies in these cells have revealed the candidate K+ channel involved in Cl− secretion to be KCNQ1, a low conductance (1–3 pS) K+ channel, which is activated during cAMP-stimulated Cl− secretion (9). Other types of K+ channels have also been implicated in chloride secretion, including the Ca2+-dependent KCNN4 channel (10).

The rat KCNQ1 channel was cloned from colonic tissue and expression was demonstrated in both the crypt and surface cells (9). KCNQ1 is a voltage-gated channel, which plays a crucial role in controlling salt and water homeostasis in a number of epithelia (11). KCNQ1 is located in the basolateral membrane.
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of the rat distal colonic crypt (8) and is regulated by forming a complex with the β-subunit KCNE3 (MiRP2) to form the basolateral K⁺ conductance that is required for transepithelial cAMP-stimulated chloride secretion (12). We propose KCNQ1 as a candidate membrane target for E2 in the inhibition of Cl⁻ secretion in the rat distal colonic crypt. A previous study in cardiac tissue demonstrated a concentration-dependent inhibition of the KCNQ1/KCNE1-mediated K⁺ current by E2 (13).

PKCδ is a serine/threonine kinase belonging to the novel PKC subgroup and plays a key role in cell cycle progression, transcriptional regulation and tissue remodeling (14). In the gastrointestinal tract PKCδ has been implicated in the maintenance of the epithelial barrier integrity (15) and is an important regulator of Cl⁻ secretion (16). We have previously demonstrated that physiological concentrations of E2 (0.01–100 nM) produced a rapid (15 min) stimulation of PKC and PKA activities in female rat distal colonic crypts (17).

The phosphorylation of ion channels is a major mechanism for their regulation. Indeed it has been demonstrated recently that KCNQ1 can be phosphorylated at Ser⁷⁷ in the N terminus of the channel by PKA (18).

In the present study, we examined the female gender-specific regulation of the KCNQ1 channel by E2 and the role of PKA and PKCδ as mediators of the rapid anti-secretory response to estrogen in rat distal colonic crypts.

EXPERIMENTAL PROCEDURES

Materials—Phospho-PKCδ (Ser⁶⁴⁵) antibody was obtained from Cell Signaling Technologies. Phosphoserine (Clone IC8) antibody was obtained from Calbiochem. Total PKCδ, total PKA regulatory subunit isoform I (PKARI), and total PKA catalytic subunit isoform I (PKACI) antibodies from BD Transduction. Anti-rabbit anti-goat horseradish peroxidase-linked antibodies from Sigma-Aldrich. Total KCNQ1 antibody was obtained from Santa Cruz Biotechnology. Total KCNQ1 antibody was obtained from Sigma-Aldrich and Santa Cruz Biotechnology. Protein-G Sepharose beads and the ECL plus detection system were from Amersham Biosciences and Bradford reagent from Bio-Rad. Chromanol 293B was obtained from Tocris and rotterin from Calbiochem. All other reagents were obtained from Sigma-Aldrich.

Animals—Male (~350 g) and female (~300 g) Sprague-Dawley rats at three-months-old were used for all experiments. Animals were kept on a 12-h light, 12-h dark cycle, and were given ad libitum access to food and water. Following anesthesia rats were killed by cervical dislocation. Cervical smears were obtained from female rats, and the stage of the estrous cycle was determined histologically as previously described (19). All female rats were used at the estrus stage of the cycle where estradiol is present at a circulating level of ~75 pg/ml and progesterone at ~32.5 pg/ml (20).

The distal colon was removed to below the pelvic rim. The faecal contents were rinsed and distal colonic crypts were isolated as previously described (17). Isolations and treatments were carried out at room temperature to avoid colonic crypt disintegration (17, 21). Sheets of colonic mucosa were obtained by blunt dissection for transepithelial transport measurements. All procedures were approved by the RCSI Ethics Committee.

Transepithelial Transport Studies—Colonic epithelia were mounted in Ussing chambers (Physiologic Instruments) on inserts exposing an area of 0.5 cm². Transepithelial potential difference was clamped to 0 mV using an EVC-4000 voltage-clamp apparatus (World Precision Instruments). The transepithelial short circuit current (ISc) was recorded using Ag-AgCl electrodes in 3 mM KCl agar bridges. Apical and basolateral baths were filled with Krebs bicarbonate buffer (in mM: 120 NaCl, 25 NaHCO₃, 3.3 K₂HPO₄, 0.8 K₂HPO₄, 1.2 MgCl₂, 1.2 CaCl₂, 10 glucose), pH 7.4 maintained at 37 °C by heated water jackets and oxygenated with a 95% O₂/5% CO₂ mixture. All preparations were allowed to equilibrate for 30–45 min before the experiments were performed. The Iₛₜₜ was defined as positive for anion flow from the basolateral to apical chamber and for cation flow in the opposite direction.

To investigate the activity of basolateral K⁺ channels in Ussing chamber experiments, the apical membrane was permeabilized by addition of 10 μM of the K⁺ ionophore amphotericin B in the presence of a mucosal to serosal K⁺ gradient established by the following bath solutions: apical (in mM), 145 K-gluconate, 3 K₂HPO₄, 0.8 K₂HPO₄, 1.2 Mg(gluconate)₂, 4 Ca(gluconate)₂, 10 glucose, and 10 HEPES; basolateral, 145 Na-gluconate, 3.3 NaHPO₄, 0.8 Na₂HPO₄, 1.2 Mg(gluconate)₂, 4 Ca(gluconate)₂, 10 glucose, and 10 HEPES. Ouabain (1 mM) was added to the serosal bath to inhibit Na⁺-K⁺-ATPase. The resulting Iₛₜₜ was generated by the movement of K⁺ through channels in the basolateral membrane (Iₛₜₜ) as the current collapses to zero in equimolar K⁺ solutions bathing both sides of the epithelium. For measurement of Iₛₜₜ current-voltage relationships, currents were elicited in asymmetrical K⁺ gluconate solutions by imposition of 1-s test potentials between ~100 and +100 mV in 20-mV increments.

Patch-clamp—A small aliquot (100 μl) of freshly isolated colonic crypts was transferred into 1 ml of superfusion chamber mounted on the stage of an inverted microscope (TE 2000-S, Nikon Ltd). Pipettes were prepared from capillary glass (GC150F-10, Harvard Apparatus Ltd, Edenbridge, UK). Patch pipettes were pulled and fire-polished using a programmable horizontal puller (DMZ-Universal, Zeitz-Instruments GmbH) 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 (Axon Instruments Incorporated) connected to an Axopatch 200B series amplifier (Axon Instruments). Patch-clamp experiments were performed at 37 °C in the standard whole cell recording configuration (22) and 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 superfused at a rate of 1 ml/min in a standard bath solution containing (in mM): NaCl 140, KCl 5.4, MgCl₂ 1, CaCl₂ 1.25, HEPES 10, glucose 12.2, buffered at pH 7.4 with NaOH. The patch pipette solution contained (mM): K-gluconate 95, KCl 30, Na₂ATP 4.8, KH₂PO₄ 1.2, EGTA 1, Ca-gluconate 0.73, MgCl₂ 1, ATP 3, d-glucose 5 (pH 7.2). Once whole cell access to the inside of the cell was...
obtained, cells were allowed to stabilize for up to 10 min before the experiment began. The protocols for patch-clamp and data analysis were established with routines using pClamp 9.2 software (Axon Instruments), and data were stored for subsequent analysis.

**Co-immunoprecipitation and Western Blotting**—Isolated distal colonic crypts suspended in Krebs solution were treated with drugs for the indicated time points. The samples were then immediately centrifuged for 30 s at 4 °C at 4,000 rpm, and the supernatant removed. Cells were lysed by hypotonic shock on ice for 45 min (lysis buffer: 20 mM Tris, pH 7.4, 0.5% Nonident P-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, leupeptin 1 μg/μl, 500 mM dithiothreitol, 5 mM phenylmethylsulfonyl fluoride, complete mini EDTA-free protease inhibitor mixture tablets (1 tablet/7 ml of lysis buffer; Roche Applied Science) and phosphatase inhibitors). Following incubation the samples were clarified at 12,000 rpm for 10 min. The cleared supernatant was collected and the protein content was quantified by the Bradford method (23). For activation assays 50 μg of sample were combined with 2× Laemmli buffer, boiled at 95 °C for 5 min, and spun at 12,000 rpm for 2 min. All KCNQ1 immunoprecipitations were carried out using lysis buffers as described previously (24). 500 μg of sample was removed for co-immunoprecipitation of KCNQ1 and associated kinases. KCNQ1 was immunoprecipitated as follows: 1 μg of total KCNQ1 antibody was added to 500 μg of sample and incubated on a rotor for one hour at 4 °C. 40 μl of washed protein G-Sepharose beads were combined with the immunocomplex. The samples were then incubated overnight on a rotor at 4 °C. Complexes were spun at 12,000 rpm for 10 min and washed three times with ice-cold sterile 1× phosphate-buffered saline. The supernatant was removed, and 35 μl of Laemmli buffer were added and samples were boiled at 95 °C for 5 min and spun at 14,000 rpm for 5 min. Western blot analysis was carried out as standard. Protein was transferred to polyvinylidene difluoride membranes, blocked in 1× TBS with Tween (0.3%) (1× TBST) and 5% nonfat dry milk for 1 h. Membranes were incubated with the appropriate primary antibody overnight at 4 °C and incubated for 1 h at room temperature with the appropriate secondary antibody. Membranes were washed in 1× TBST 0.3% three times for 15 min. Bands were detected using autoradiographic film and chemiluminescence.

**PKA Activation Assay**—PKA activation was detected using PepTag Assay (Promega) for non-radioactive detection of cAMP-dependent protein kinase according to the manufacturer’s instructions with minor modifications. 2.5 μl of the F-Kemptide PepTag and 2.5 μl of the cAMP activator solution were added instead of 5 μl.

**RNA Preparation and Reverse Transcriptase (RT) PCR**—Total RNA was extracted from male and female rat distal colonic crypt preparations using Qiagen RNEasy kit (Qiagen). Single-strand cDNA was synthesized using the Improm II reverse transcriptase kit (Promega). cDNA was quantified and corrected for loading into RT-PCR reaction mixes. PKCδ was amplified using the following primers: forward; 5'-ctaccctctgctgctggcc-3' and reverse; 5'-ctgccctgctgccgctggc-3'. β-Actin was amplified using the following primers: forward; 5'-ctcactctgactcctgccgctg-3' and reverse; 5'-ctactctgactcctgccgctg-3'. GoTaq® polymerase mix from Promega was used in the amplification. Touch-down PCR was used to amplify PKCδ cDNA for 25 cycles over an annealing temperature range of 65–55 °C. β-Actin was amplified for 25 cycles at an annealing temperature of 52 °C. The RT-PCR product was analyzed on a 1.5% 1× Tris acetate-EDTA (TAE) agarose gel and imaged using a UV light source.

**Statistical and Densitometric Analysis**—Data are presented as mean ± S.E. for a series of the indicated number of experiments. Statistical analysis of the data was obtained by analysis using paired Student’s t test for analysis between two groups. One-way analysis of variance and Tukeys post-hoc test was used for multiple analysis of more than two groups. Patch-clamp data analysis was performed using Clampfit software of the p-clamp suite version 9.2 and Origin 7.5 (OriginLab Corp.). Densitometric analysis of Western blots, PKA and RT-PCR images were performed using GeneTools software (SYNGENE).

**RESULTS**

**17β-Estradiol Inhibits Cl⁻ Secretion Evoked by Forskolin in Rat Colonic Epithelia**—We first set out to examine the effect of 17β-estradiol (E2) on intestinal secretion elicited by the cAMP agonist forskolin. Basolateral addition of 20 μM forskolin induced an almost instantaneous and sustained increase in $I_{SC}$ (Fig. 1A). E2 (10 nM) reduced the forskolin-effect on $I_{SC}$ by 60±6% ($n = 5$, $p < 0.01$). The estrogen inhibition of forskolin-induced secretion was observed to be gender dependent. E2 inhibition of forskolin-induced chloride secretion was only observed in tissues from female rats with no significant response to E2 in male tissues (female 60 ± 6% $I_{SC}$ decrease versus male 6 ± 3%, $p < 0.01$, $n = 5$) (Fig. 1A). All Using chamber values are maximal inhibition achieved at E2 treatment between 15 and 20 min.

Previous data from our group have demonstrated direct and rapid activation of PKCδ in response to E2 (25). We therefore investigated a potential role for this kinase in mediating the anti-secretory effects of E2. We used rottlerin as an inhibitor of PKCδ as it has been reported to inhibit this kinase more potently than other kinases. A previous publication on the inhibitor reported that it inhibits the PKC isoforms at different IC₅₀ concentrations; PKCδ: 3–6 μM, PKCA, PKCβ and PKCγ: 30–42 μM, PKCε, PKCζ and PKCθ: 80–100 μM (26). Pretreatment of colonic mucosa with rottlerin (10 μM) completely abolished the anti-secretory action of E2 (E2: 59 ± 6%, E2 + rottlerin, 5 ± 3%, $n = 5$, $p < 0.01$) (Fig. 1B). These results indicate that PKCδ activity is required for the E2-mediated inhibition of forskolin-induced Cl⁻ secretion.

**The Effect of 17β-Estradiol on Basolateral Membrane K⁺ Currents in Rat Colonic Epithelia**—Basolaterally directed K⁺ currents were generated in female rat colonic epithelia permeabilized apically by pretreatment with amphotericin B. Fig. 2A shows the current/voltage ($I/V$) relationship for apically permeabilized epithelia before and after the addition of the cAMP-dependent agonist forskolin (10 μM). Treatment with forskolin-activated outward currents (apical to basolateral) that displayed slight outward rectification at positive transepithelial voltages and shifted apparent reversal potentials to more nega-
tive values consistent with activation of basolateral membrane $K^+$ current $I_{KC}$. Treatment with chromanol 293B (10 μM), a specific KCNQ1 channel blocker, inhibited both the basal and forskolin-activated basolateral membrane $K^+$ current suggesting the main component of this current is due to KCNQ1 channel activity (Fig. 2A). Treatment with E2 (10 nM) produced a marked inhibition of forskolin-stimulated $I_{KC}$ at $V_p = +100$ mV; forskolin $185 \pm 19 \mu A/cm^2$; forskolin + E2 $44 \pm 6 \mu A/cm^2$, $n = 3$, $p < 0.01$) and subsequent addition of chromanol 293B had little effect on the remaining $K^+$ current (Fig. 2B). Pretreatment of colonic epithelia with rottlerin (10 μM) for 15 min before addition of forskolin did not modify the increase in $I_{KC}$ induced by forskolin but prevented the inhibition by E2 (at $V_p = +100$ mV), forskolin + rottlerin $182 \pm 17 \mu A/cm^2$; forskolin + rottlerin + E2 $158 \pm 21 \mu A/cm^2$, $n = 3$, $p < 0.01$) (Fig. 2C). These results suggest that the anti-secretory effect of 17β-estradiol is mediated by the inhibition of basolateral KCNQ1 channels via PKC6.

The Effect of Chromanol 293B on Whole Cell KCNQ1 Channel Current in Female Rat Colonic Crypts—Membrane currents were recorded in the whole cell mode of the patch-clamp technique from colonic crypt cells at 37 °C. The mean maximal whole cell current under control conditions was $817 \pm 84 \mu A$ (at $V_p = 100$ mV, $n = 4$, $p < 0.01$) and subsequent addition of chromanol 293B (100 μM) produced a reduction of currents by 91% (corresponding to a reduction to $69 \pm 50 \mu A$, at $V_p = +100$ mV, $n = 4$, $p < 0.01$). Consistent with the decrease in whole cell current, chromanol 293B also reduced the whole cell conductance ($G_c$) at $+100$ mV.
The KCNQ1 channel activity is temperature-sensitive and increasing the temperature to 37 °C is known to activate the channel (27). Supplementary data show the mean maximal whole cell KCNQ1 currents recorded at room temperature (22 °C), Vp = +100 mV and at 37 °C prior to the addition of chromanol 293B (100 μM). Chromanol 293B inhibited the mean maximal whole cell KCNQ1 currents and reduced the K+ current toward the level measured at room temperature (22 °C). The mean maximal temperature-stimulated increase in current was 822 ± 52 pA (n = 4, p < 0.02) and chromanol 293B addition produced a reduction of current by 69% (corresponding to 252 ± 45 pA), (n = 4, p < 0.05). Taken together, these results demonstrate the functional activity of KCNQ1 channels in female rat distal colonic crypts.

E2 Effect on KCNQ1 Whole Cell Currents in Female Rat Colonic Crypts—Following membrane breakthrough to the whole cell patch-clamp configuration, the patched cells were allowed to stabilize and dialyze for 5 min. Currents were recorded over this time period before the addition of E2 to ensure stability of the current recording and to check for channel rundown. The experiments involved exposing the isolated colonic crypts to E2 (100 nm) and measuring the whole cell currents at 30-s intervals.

Fig. 3A shows the effects of E2 (100 nm) treatment over time on the whole cell current-voltage relationships. E2 application caused a decrease in whole cell current and its outward rectification over 9 min with a concomitant fall in whole cell membrane conductance at +100 mV from control 278 ± 22 pS to: 160 ± 13 pS at 1 min; 158 ± 11 pS at 3 min; 107 ± 9 pS at 5 min; 82 ± 16 pS at 7 min and 81 ± 25 pS at 9 min (n = 4, p < 0.01). The effect of E2 treatment on whole cell current over time is shown in Fig. 3B. The mean maximal whole cell currents recorded over 5 min prior to E2 addition was 526 ± 50 pA (at Vp = +100 mV). Addition of E2 (100 nm) inhibited the mean maximal current after 5 min to 248 ± 38 pA and to 73 ± 10 pA after 15 min (n = 4, p < 0.05).

Taken together, these results support the conclusion that KCNQ1 channel activity generates the main basolateral membrane K+ current, in female colonic crypts, consistent with the Ussing chamber results, and the channel is a target for E2 causing an anti-secretory response.

Gender Comparisons of PKA, PKCδ, and KCNQ1 Basal Expression Levels in Rat Distal Colonic Crypts—Total untreated cellular lysates of isolated rat distal colonic crypts were prepared, subjected to Western blot analysis, and probed using specific antibodies to endogenous levels of PKARI, PKACI, PKCδ, and KCNQ1. In all cases expression differences were normalized for loading by probing for total β-actin levels. Differences are expressed as fold values of female expression levels compared with male.

PKA isoform I is a cytoplasmic PKA, unlike isoform II which is membrane bound (28). Previous studies in rat colonic crypts demonstrated no significant activation of PKA in the membrane fractions of crypts and a significant activation of PKA in cytosolic fractions (25). Isoform I, the soluble cytosolic isoform, was therefore investigated in this study. Basal expression amounts of PKARI and PKACI were similar between male and female rat distal colonic crypts (PKACI: 1.1 ± 0.2, p > 0.05, n = 3; PKARI: 1 ± 0.2, n = 3, p > 0.05) (Fig. 4A and B).

A comparison of KCNQ1 basal expression levels between male and female distal colonic crypts was also investigated. No significant difference was found between male and female crypts for total basal expression levels of KCNQ1 (1.1 ± 0.03, n = 3, p > 0.05) (Fig. 4A).

We have found gender differences to exist in the basal expression for novel PKCδ. In comparison to male, distal colonic crypts from female rats displayed a 3.2 ± 0.42-fold higher expression of the PKCδ protein (n = 6, p < 0.01) (Fig. 4C).

To further verify the gender difference in expression of PKCδ we also amplified the messenger transcript levels of the kinase by RT-PCR. Female distal colonic crypts had a significantly higher level of the PKCδ transcript present compared with male distal colonic crypts (2.1 ± 0.1-fold higher, n = 3, p < 0.01) (Fig. 4D).

We conclude from these expression studies that a significant gender difference exists for PKCδ, with a higher expression level in female rat distal colonic crypts. No gender differences
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The anti-secretory effect of E2 is observed during proestrus. Fig. 5 shows the anti-secretory effect of 17β-estradiol (10 nM) is related to the expression levels of PKCδ in the colonic tissue. A, inhibitory effect of E2 on forskolin (20 μM) induced Cl⁻ secretion in male and female (proestrus and estrus) colonic mucosa (bar graph) compared with PKCδ protein levels (line graph and representative blot). B, PKCδ transcription is regulated by E2. Colonic crypts from male and female rats were treated with E2 for 30 min. Total mRNA was extracted and converted into cDNA. PKCδ cDNA was amplified by PCR using specific primers. The figure shows a representative agarose gel of the amplified products. Values on the graph are given as a mean fold increase compared with control values. Values are displayed as mean ± S.E. (n = 5 for A and n = 4 for B). ** and ## denotes significance (p < 0.01) between male and female values.

FIGURE 5. The anti-secretory effect of 17β-estradiol (10 nM) is related to the expression levels of PKCδ in the colonic tissue. A, inhibitory effect of E2 on forskolin (20 μM) induced Cl⁻ secretion in male and female (proestrus and estrus) colonic mucosa (bar graph) compared with PKCδ protein levels (line graph and representative blot). B, PKCδ transcription is regulated by E2. Colonic crypts from male and female rats were treated with E2 for 30 min. Total mRNA was extracted and converted into cDNA. PKCδ cDNA was amplified by PCR using specific primers. The figure shows a representative agarose gel of the amplified products. Values on the graph are given as a mean fold increase compared with control values. Values are displayed as mean ± S.E. (n = 5 for A and n = 4 for B). ** and ## denotes significance (p < 0.01) between male and female values.

FIGURE 4. Male and female rat distal colonic crypts express similar levels of both PKA regulatory and catalytic isoform I subunits and the KCNQ1 channel. Female rat distal colonic crypts express higher levels of PKCα, PKCβ, and PKCγ. A and B, representative blot of PKACI and PKARI protein levels in cellular extracts from male and female distal colonic crypts. C, representative blot of PKCδ protein levels in cellular extracts from male and female distal colonic crypts. D, representative image of PKCα transcript levels from total RNA extracts from male and female distal colonic crypts. E, representative blot of KCNQ1 protein levels in cellular extracts from male and female distal colonic crypts. Values are given as mean pixel intensities of bands for male and female. Values are displayed as mean ± S.E. (n = 3 for A, B, D, and E, and n = 6 for C). ** denotes significance (p < 0.01) between male and female values.

were detected for PKACI, PKARI, or the KCNQ1 channel protein expression.

The Anti-secretory Response to 17β-Estradiol Correlates With PKCδ Expression Levels in Colonic Epithelia—Based on the gender differences in the E2 anti-secretory action and PKCδ expression, we hypothesized that the potency of E2 in reducing secretion must relate to the expression levels of PKCδ. Unpublished data from our group have shown that PKCδ expression fluctuates throughout the estrous cycle. PKCδ expression is maximal at the estrus stage while at its lowest during proestrus (estrous 2.8 ± 0.8-fold higher compared with proestrus, n = 4, p < 0.01) (Fig. 5A). No significant difference between proestrus and male colonic cells was observed (male 1.5 ± 0.4-fold compared with proestrus, n = 4, p > 0.05) (Fig. 5A). Therefore, we examined if the anti-secretory effect of E2 is observed during proestrus. Fig. 5A shows the anti-secretory effect of E2 on forskolin-stimulated Cl⁻ secretion in colonic epithelia from female rats at proestrus was significantly lower compared with rats at estrus and (estrous 58 ± 3%; proestrus 91 ± 2%, of control values; n = 5, p < 0.01) (Fig. 5A). No significant difference was observed between male colonic epithelia and female epithelia at proestrus indicating a similar profile between the tissues. These results show a direct correlation between the anti-secretory effect of E2 with the expression levels of PKCδ, confirming the regulatory role of PKCδ in E2 action.

The gender and estrous cycle differences in PKCδ expression suggests that this kinase expression may be regulated by E2 in female colonic epithelial cells. In colonic crypt cells from female rats at proestrus, exposure to E2 (10 nM) for 30 min dramatically increased transcription of PKCδ mRNA (E2 1.8 ± 0.05-fold higher than controls, n = 3, p < 0.001) (Fig. 5B). However, no significant response was observed in male colonic crypt cells.

Gender Differences in PKCδ and PKA Activation in Response to 17β-Estradiol in Rat Distal Colonic Crypts—Isolated male and female rat distal colonic crypts were exposed to E2 (10 nM) or equivalent vehicle (0.01%) for the duration of 2, 5, and 15 min. Total lysates were prepared, subjected to Western blot analysis and probed using phosphospecific antibodies to the Ser⁶⁴¹ autophosphorylation site on the PKCδ protein. In all cases differences were normalized for loading by probing total β-actin. For PKA analysis total lysates were analyzed using the PepTag assay for the non-radioactive detection of cAMP-dependent protein. Differences are expressed as fold values of treated over control.

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PKCδ was activated in female distal colonic crypts in response to E2 at 2, 5, and 15 min with a maximum activation occurring at 5 min (8.4 ± 1.2-fold higher, n = 3, p < 0.001) (Fig. 6A). No significant activation of PKCδ in male crypts was detected (1 ± 0.2, n = 3, p > 0.05) (Fig. 6B). PKA was activated in female distal colonic crypts in response to E2 at 5 min (2.8 ± 0.4-fold higher, n = 5, p < 0.001) (Fig. 7A). In contrast, PKA was not activated in the male distal colonic crypts in response to E2 (1.1 ± 0.1, n = 3, p > 0.05) (Fig. 7B). In summary, the rapid activation of PKCδ and PKA by E2 is female gender-specific.

PKCδ and PKACI Associate with the KCNQ1 Channel in Female Rat Distal Colonic Crypts in Response to 17β-estradiol—Co-immunoprecipitation experiments were preformed to establish whether PKCδ and PKACI associate with KCNQ1 in response to E2.

Following E2 (10 nM) treatment PKCδ associated with the KCNQ1 channel protein at 5 and 15 min, with maximal association at 5 min (6.2 ± 1.2-fold higher, n = 3, p < 0.001) (Fig. 8A). The timing of the association correlates with the time response of PKCδ activation (Fig. 6A). No increase in association between PKCδ and KCNQ1 was observed in the male tissue after E2 treatment (1.1 ± 0.3, n = 3, p > 0.05) (Fig. 8B).

Following E2 (10 nM) treatment, PKACI rapidly and transiently associated with KCNQ1 at 5 min (3.1 ± 0.4-fold higher, n = 3, p < 0.001) (Fig. 9A). The timing of the PKACI/KCNQ1 association was similar to the time course for PKA activation (Fig. 7A). No association of PKACI with the KCNQ1 channel was detected in the male tissue in response to E2 (0.9 ± 0.1, n = 3, p > 0.05) (Fig. 9B). These results indicate that PKCδ and PKACI form a multiprotein complex with the KCNQ1 channel in response to E2 in female colonic crypts.

PKACI Activation and Association With the KCNQ1 Protein in Response to 17β-estradiol is PKCδ dependent—The sequence of activation of PKCδ and PKA by E2 in female distal colonic crypts was established. Total lysates were prepared and analyzed using the PepTag Assay for the non-radioactive detection of CAMP-dependent protein. PKA activity in response to E2 was abolished following 15-min pretreatment with rottlerin (10 μM) (E2 alone; 5.0 ± 0.9-fold higher, n = 3, p < 0.001; E2 + rottlerin; 1.6 ± 0.3, n = 3, p > 0.05) (Fig. 10A). Therefore, in the female rat distal colonic crypts PKA activation by E2 is downstream to PKCδ activation.

The latter result indicates that PKCδ could also regulate PKACI association with the KCNQ1 channel. PKACI associ-
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Pre-treatment with rottlerin (10 μM) reduced the E2-induced phosphorylation of the KCNQ1 channel (E2 + rottlerin, 1.4 ± 0.1; E2 + H89, 1.2 ± 0.1, n = 3, p > 0.05) (Fig. 11). These results demonstrated that the KCNQ1 channel is serine-phosphorylated in response to estrogen and is PKCα/PKA-regulated.

**DISCUSSION**

The present study demonstrates E2 inhibition of forskolin induced Cl− secretion in female rat distal colonic epithelia. The response is female gender-specific and does not occur in male tissues. Ussing chamber and patch-clamp experiments demonstrated that the inhibition of chloride secretion resulted from the blocking of chromanol 293B sensitive basolateral K+ currents. This study provides evidence that E2 inhibits a basolateral K+ current, specifically the KCNQ1 ion channel as chromanol 293B is a specific KCNQ1 channel inhibitor. The addition of chromanol 293B to E2-treated tissues did not produce a further reduction in the K+ current suggesting that E2 inhibited the entire KCNQ1 portion of the total basolateral membrane current. The E2 inhibitory effect on KCNQ1 chan-
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E2 activation of PKC and PKA has previously been reported in rat distal colonic crypts (17, 25). In the present study, we describe an earlier activation of PKCζ (at 2 min) and PKA (at 5 min) compared with the previous studies and in addition demonstrate activation at an identified stage of the estrous cycle, namely in estrus (circulating estrogen levels begin to increase during proestrus and exerts biological effects during the estrus stage). Activation of both kinases was female gender specific. Colonic crypts isolated from female rats expressed significantly higher levels of PKCζ, both at the transcript and protein level, in comparison to male rats. PKCζ activation by estrogen may be absent in the male as there may not be a sufficient amount of the kinase to induce a significant response. Gender-specific expression of PKCζ has been observed in rat liver and also a lack of activation in male liver tissue where the kinase expression was low (31). PKCζ expression is known to be estrogen regulated in the rat ovaries and human breast cancer cells (32, 33). Here we show for the first time that estradiol can stimulate the expression of PKCζ in female colonic crypts. Although male and female rat distal colonic crypts express similar levels of PKA there is no activation of this kinase in the male. This again may be due to the lower level of PKCζ expression in male colonic crypts and insufficient PKCζ activation to modulate the PKA activation by estrogen. The low expression level of PKCζ in the

A.

 vue increase in F-Kemptide phosphorylation over control

Time (min) 0 5 5 5 5
17β-estradiol - - + - -
Vehicle - - - - -
Rottlerin - - - - -

B.

 vue increase in PKA phosphorylation over control

Time (min) 0 5 5 5 5
17β-estradiol - - + - -
Vehicle - - - - -
Rottlerin - - - - -

The KCNQ1 K⁺ ion channel is serine-phosphorylated in response to 17β-estradiol. The KCNQ1 channel phosphorylation is PKCζ/ PKA-dependent. Female colonic crypts were stimulated with E2 (10 nm) for 10 min and the KCNQ1 was immunoprecipitated. Phosphorylation of the channel was detected using an antibody to phosphoserine. Pretreatment with rottlerin (10 µM) and H89 (10 µM) prevented the E2-induced phosphorylation of the channel. The graph represents densitometric analysis of specific time points of E2 treatment. Values are given as fold changes in PKA phosphorylation for female samples. Values are displayed as mean ± S.E. (n = 3). ** denotes significance (p < 0.01) between control and treatments.

FIGURE 11.

FIGURE 10. PKA activation and PKACI association with the KCNQ1 channel in response to 17β-estradiol (10 nm) is PKCζ-dependent in female rat distal colonic crypts. A, representative image of PKA phosphorylation of an F-Kemptide PepTag in cellular extracts from female rat distal colonic crypts. B, representative blot of PKACI association with the KCNQ1 channel in the female rat distal colonic crypts. Rottlerin (10 µM) prevented this association. The graphs represent densitometric analysis at specific time points of E2 treatment. Values are given as fold changes in PKA phosphorylation (A) and fold changes in PKACI association with KCNQ1 (B) for female crypts. Values are displayed as mean ± S.E. (n = 3 for A and B). * and ** denotes significance (p < 0.05, p < 0.01) between male and female values.
male colonic crypts may be the molecular mechanism for the sexual dimorphism in the E2 response and the failure of E2 to inhibit Cl− secretion in the male colon. This may also be the case for female proestrus colon, which also shows lower expression of PKCδ and response to E2. As a corollary, the rapid non-genomic response to estrogen in the female is dependent on the genomic regulation of the expression of the kinase PKCδ. Thus, the non-genomic response is regulated by a genomic event depending on the hormonal status of the animal. Studies in PKCδ-null mice might be helpful to further confirm the role of this kinase in modulating rapid responses to estrogen and intestinal secretion.

It is becoming increasingly evident that non-genomic effects prime latent genomic functions of protein expression and cell differentiation (30). Non-genomic and genomic cross-talk works in both directions and it is untenable to consider rapid and latent responses as unrelated physiological processes.

This is the first report of a gender-specific hormonal modulation of the KCNQ1 channel. Also, this is the first report of modulation of KCNQ1 channels by the novel PKCδ. The KCNQ1 channel is known to be regulated by multiprotein complexes containing phosphatases, protein kinases, and A Kinase Anchoring Proteins (AKAPs). It has previously been reported that PKA is capable of interacting with and phosphorylating cardiac KCNQ1 at Ser27 via an AKAP, specifically, yotiao (34). The latter study demonstrated PKA phosphorylation of the KCNQ1 channel through the recruitment of a multiprotein complex that included PKA, protein phosphatase I (PPI) and the AKAP yotiao. The requirement for the regulation of the KCNQ1 channel by a macromolecular complex may increase the specificity of the channel targeting by signaling pathways. Our results demonstrate an interaction of PKCδ and PKACI with the KCNQ1 channel in response to E2 in female rat distal colonic crypts and not in males. Phosphorylation of the KCNQ1 channel by PKA may be the molecular mechanism for E2 inhibition of KCNQ1 currents.

In this study we demonstrated that E2 regulates KCNQ1 channel function indirectly via kinases which results in inhibition of secretagogue stimulated Cl− secretion. This is an important physiological response, which provides one of the few known examples for a physiological meaning to E2-induced rapid responses. Several studies have shown variability in the response of KCNQ1 to PKA- or PKC-induced phosphorylation (18, 24). A reason for this is the requirement of accessory proteins to transduce protein phosphorylation into channel function. As an example, it has been found in cardiac cells that association of KCNQ1 with its β-subunit KCNE1 and to other proteins such as Yotiao is necessary to translate the phosphorylated KCNQ1 subunit into altered channel activity. Therefore, tissue differences in the expression of regulatory proteins could account for the differences reported in the channel response to PKA. In unpublished data from our group we have found gender differences in the expression and association of KCNE subunits in colonic epithelia. Also, PKA regulation of channel activity may be dependent on the activated isoforms of adenyl cyclase or PKA that are activated and their subcellular localization with cAMP.

An interesting paradox exists in the anti-secretory pathway. The induction of Cl− secretion by forskolin requires an increase in cAMP activity, however, E2 also increases cAMP activity in rat distal colonic crypts (25). Why, therefore, does activation of PKA by E2 not lead to increased CFTR activity and therefore further stimulate Cl− secretion rather than exert an anti-secretory effect? It has previously been reported that CFTR activation is via PKA catalytic subunit isoform II (PKACII) which is a membrane-bound insoluble isoform of the kinase (35, 36). The authors have previously shown that E2 does not activate membrane bound PKA in the rat colonic crypt (25). This fraction contains PKACII, the activator of CFTR. Our data, however have shown that it is the PKA isoform, PKACI that is activated by E2 (25), and the current study demonstrates E2 induces association of PKACI with the KCNQ1 channel. Thus CFTR is not a target for estrogen and inhibition of the KCNQ1 channel provides the molecular basis for the anti-secretory response. PKACI has been reported to be recruited by the scaffolding protein AKAP (37) and in our case this may be a mechanism for targeting PKACI to KCNQ1. Other mechanisms for membrane specific targeting of kinases have been well-described involving subcellular compartmentalization of PKA isoforms and their spatial restriction to either the basolateral or apical membrane or indeed a restriction to a particular signaling complex (38). In epithelial cells local pools of cAMP exist which result in the activation of PKA in highly localized areas of the cell (28).

In summary, the present study has identified the ion transporter target in the anti-secretory response to estrogen in distal colon and has provided a molecular explanation for its female gender-specificity. E2 inhibits KCNQ1 channel current indirectly via PKCδ and PKACI resulting in the inhibition of Cl− secretion. The anti-secretory response may also be a contributory factor by which estrogen causes whole body salt and water retention during periods of high circulating estrogen such as certain stages of the menstrual cycle (39), pregnancy (40) and during the use of oral contraceptives (41) and hormone replacement therapy (42). The regulation of the KCNQ1 channel by E2, reported here, provides important leads in the investigation into molecular mechanisms controlling intestinal secretory disorders and drug targeting.

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