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ESTROGEN INHIBITS CHLORIDE SECRETION CAUSED BY CHOLERA AND *ESCHERICHIA COLI* ENTEROTOXINS IN FEMALE RAT DISTAL COLON

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ABSTRACT

Excessive Cl^- secretion is the driving force for secretory diarrhea. 17β-estradiol has been shown to inhibit Cl^- secretion in rat distal colon through a nongenomic pathway. We examined whether 17β-estradiol inhibits Cl^- secretion in an animal model of secretory diarrhea and the downstream effectors involved. The effect of 17β-estradiol on cholera toxin and heat-stable enterotoxin induced Cl^- secretion in rat colonic mucosal sheets was studied by current-voltage clamping. Selective permeabilization of apical or basolateral membranes with amphotericin B or nystatin was used to isolate basolateral K^+ channel and apical Cl^- channel activity respectively. 17β-estradiol dose-dependently inhibited secretory responses to both toxins with IC_{50} values of approximately 1 nM. This effect was female-gender specific, with no inhibition observed in male tissues. 17β-estradiol responses were insensitive to the pure anti-estrogen ICI 182,720. 17β-Estradiol exerted its effects downstream of enterotoxin-induced production of second messengers (cAMP and cGMP) but was dependent on PKCδ activation. In nystatin-permeabilized tissues, apical Cl^- currents were unaffected by 17β-estradiol treatment while basolateral K^+ current was profoundly inhibited by the hormone. This current was sensitive to the specific KCNQ1 channel inhibitors chromanol 293B and HMR-1556. In conclusion, 17β-estradiol inhibits enterotoxin-induced Cl^- secretion via a PKCδ-dependent mechanism involving inhibition of basolateral KCNQ1 channels. These data elucidate mechanisms of 17β-estradiol inhibition of Cl^- secretion induced by enterotoxins in intestinal epithelia, which may be relevant for the treatment of diarrheal diseases.

Keywords: estrogen, KCNQ1 channels, chloride secretion, colonic epithelium, secretory diarrhea, enterotoxin.

1. Introduction

The generation of the electrochemical driving force required for Cl^- secretion by colonic crypt epithelial cells depends on their ability to accumulate intracellular Cl^- ions to concentrations greater than their electrochemical equilibrium [1, 2]. Cl^- enters the cell across the basolateral membrane through the activity of the Na^+-K^+-$2Cl^-$ cotransporter (NKCC1). which in turn, is driven by a strong inwardly directed electrochemical Na^+ gradient established by the basolaterally located Na^+-K^+-ATPase. In order to maintain the membrane potential at rest and during Cl^- secretion, K^+ must be recycled out of the cell across the basolateral membrane [3]. In rat distal colonic epithelia, which is a widely used animal model for the study of intestinal Cl^- secretion, the basolateral K^+ conductance is formed by at least two different types of K^+ channels, one activated by Ca^{2+}-mobilizing secretagogues and the other by cAMP-dependent agonists [4, 5]. Several studies have revealed the latter to be KCNQ1, a low-conductance (1-3 pS) basolateral K^+ channel which is activated during cAMP-stimulated Cl^- secretion and inhibited by chromanol 293B and HMR-1556 [6, 7].

New insights into the role of basolateral membrane K^+ channels in regulating intestinal Cl^- secretion have come from studies of the rapid antisecretory effect of steroid hormones, such as aldosterone and 17β-estradiol. Previous studies have shown that physiological concentrations of 17β-estradiol rapidly increase intracellular Ca^{2+} concentration [8, 9], stimulate protein kinase C (PKC) and cAMP-dependent protein kinase (PKA) activity [10], and modulate several K^+ conductances in mammalian colonic epithelia [11]. We have also reported that 17β-estradiol rapidly inhibits basal and forskolin-stimulated Cl^- secretion in rat distal colonic epithelium through inhibition of basolateral KCNQ1 channels [12,13].

These rapid responses to 17β-estradiol are insensitive to inhibitors of transcription or translation and depend on activation of PKCδ. At the same time, 17β-estradiol stimulates an ATP-sensitive basolateral K^+ conductance involved in Na^+ absorption. These findings suggest that the nongenomic effects of 17β-estradiol in the intestine lead to a rapid decrease in epithelial secretory capacity while increasing the potential for Na^+ absorption [14, 15].

Cl^- secretion from the intestinal crypt is thought to play a major role in secretory diarrhea of several etiologies [16]. Diarrheal diseases continue to be a major cause of morbidity and mortality in children and elderly people throughout the world. Oral rehydration therapy remains the mainstay of treatment for diarrhea [17]. However, in recent years significant effort has been made in the search for antisecretory drugs that will directly inhibit secretory processes within the enterocytes [18-20]. As in all secretory epithelia, the channels and transporters of the crypt epithelial cell must operate in concert to achieve vectorial ion transport. Therefore, blockade of specific basolateral K^+ conductance would be expected to inhibit the Cl^- secretory process.

In rat distal colon rapid 17β-estradiol-induced stimulation of intracellular Ca^{2+} occurs via a cholera toxin-sensitive Gαs-coupled membrane receptor distinct from the classical estrogen receptor [21]. Whether 17β-estradiol may interfere with cholera toxin (CT) and heat-stable entrotoxin from *E. coli* (STa) induced Cl^- secretion is unknown. Using the short circuit current (I_{SC}) technique to measure transepithelial ion transport, we tested the ability of 17β-estradiol to inhibit Cl^- secretion induced by CT and STa in rat distal colon. In addition, we explored potential cell signaling mechanisms and ion transporter targets involved in the antisecretory action of 17β-estradiol while Cl^- secretion was being stimulated by these bacterial toxins.

2. Experimental

2.1. Materials

All regents were purchased from Sigma-Aldrich (Dublin, Ireland) unless stated. Trans-N-[6-cyano-3,4-dihydro-3-hydroxy-2,2-dimethyl-2*H*-1-benzopyran-4-yl]-*N*-methyl-ethanesulfonamide (chromanol 293B) and 7α,17β-[9-[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol (ICI 182,780) were purchased from Tocris (Avonmouth, UK). Cholera toxin, rottlerin, clotrimazole, iberiotoxin, apamin and 2,2',3,3',4,4'-hexahydroxy-1,1'-biphenyl-6,6'-dimethanol dimethyl ether (HBDDE) were obtained from Calbiochem (Nottingham, UK). Anti PKCα and anti phospho-Thr638-PKCα antibodies were obtained from Upstate (Dublin, Ireland). The ECL plus chemiluminescent detection system was from Amersham Biosciences (Little Chalfont, UK) and Bradford reagent from Bio-Rad (Hemel Hempstead, UK). 1-[(2-chlorophenyl]diphenylmethyl]-1H-pyrazole (TRAM-34) was kindly provided by Dr. Heike Wulff (University of California at Irvine, CA). (3R,4S)-(+)-N-[3-hydroxy-2,2-dimethyl-6-(4,4,4-trifluorobutoxy)-chroman-4-yl]-N-methylethane-sulfonamide (HMR-1556) was provided by Dr. Uwe Gerlach (Aventis Pharma, Frankfurt-am-Main, Germany).

2.2. Tissue preparation

Following anesthesia Sprague-Dawley rats (300-350 g) 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 estrus stage. The distal colon was removed and fecal contents were rinsed off and the sub-mucosal layers were removed by blunt dissection. Colonic crypts were isolated for biochemical studies using the calcium chelation technique,

as previously described [8] while sheets of colonic mucosa were used for short-circuit current measurements. The Ethics Committee of the Royal College of Surgeons in Ireland approved all animal procedures.

2.3. Electrophysiology

Colonic epithelia were mounted in modified Ussing chambers (Physiologic Instruments, San Diego, CA, USA) on inserts exposing and area of 0.5 cm^2. Transepithelial potential difference was clamped to zero using an EVC-4000 voltage-clamp apparatus (World Precision Instruments, UK). The transepithelial I_{SC} was recorded using Ag-AgCl electrodes in 3M KCl agar bridges as previously described [12, 13]. All preparations were allowed to equilibrate for 30-45 min before the experiments were performed. Amiloride 50 μM was added to the apical bath in all experiments to block electrogenic Na^+ absorption. 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 an apical-to-basolateral K^+ gradient (80:5 mM), while the Na^+ concentration was maintained at 25 mM on both sides of the monolayers as previously described [13]. Ouabain (1mM) was added to the basolateral bath to inhibit Na^+-K^+-ATPase. The resulting I_{SC} is due to the movement of K^+ through channels at the basolateral membrane (I_K).

To evaluate apical membrane Cl^- conductance, tissues were permeabilized by basolateral addition of nystatin (200 μg/ml) in the presence of an apical-to-basolateral (126:6 mM) Cl^- gradient (sodium gluconate substituted for NaCl in the serosal medium). Because the cystic fibrosis transmembrane conductance regulator (CFTR) protein will conduct Cl^- in either direction, the Cl^- concentration gradient was set to drive Cl^- movement from the apical to the basolateral side of the epithelia to eliminate the contributions of NKCC1 and Na^+-K^+-

ATPase to the I_{SC}. Under these conditions, the I_{SC} represents the Cl^- current (I_{Cl}) as Cl^- moves down its concentration gradient through Cl^- channels in the apical membrane.

2.4. Protein kinase C activation

To evaluate the effect of 17β-estradiol on PKC activity the level of serine-638 autophosphorylation in PKCα was measure as an index of kinase activation. Briefly, isolated distal colonic crypts resuspended 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 x g, 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, protease inhibitor cocktail (Roche Applied Science) and phosphatase inhibitors). Following incubation the samples were clarified at 12,000 x g for 10 min. The cleared supernatant was collected and the protein content was quantified by the Bradford method. 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 x g for 2 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 (phospho-Thr638-PKCα 1:1000 or PKCα 1:1000) 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.

2.5. Statistical analysis

Statistical analysis of the data was obtained by analysis using *t*-test for analysis between two groups. One-way ANOVA and Tukeys post-hoc test was used for multiple analyses of groups. *P*-values of 0.05 and less were considered to be significant. Data are expressed as means ± SEM unless specifically stated to be from a representative experiment. Number of samples (*n*) refers to the number of animals used in each experiment.

3. Results

3.1. 17β-estradiol inhibits Cl^- secretion evoked by bacterial toxins

Several species of bacteria induce secretory diarrhea, including *Vibrio cholerae* and *Escherichia coli*. These pathogens do so by alteration of ion transport, disruption of tight junctions, and activation of an inflammatory response. We first set out to examine the effect of 17β-estradiol on intestinal secretion elicited by CT and STa. Addition of 1 μg/mL CT to the luminal side of the epithelia resulted in a sustained increase in I_{SC} (Fig. 1A). This effect occurred with a delay of approximately 90 min before the current slowly increased, usually reaching a plateau in 2 hours. In contrast, apical addition of 50 ng/mL STa induced an almost instantaneous and sustained increase in I_{SC} (Fig. 1C). Physiological concentrations (0.1 – 10 nM) of 17β-estradiol reduced the secretory response to both toxins in a concentration-dependent manner (Fig. 1B, 1D) with maximal inhibition observed at 10 nM (58 ± 5 % inhibition of STa-induced I_{SC}, n = 8; 50 ± 4 % inhibition of CT-induced I_{SC}, n = 8). The half-maximal concentration for the antisecretory effects of 17β-estradiol (EC_{50}) was 0.7 ± 0.2 nM for STa-induced secretion and 0.9 ± 0.3 nM for CT-induced secretion (n = 8). When 17β-estradiol (10 nM) was added to tissues in the absence of toxins, the I_{SC} significantly decreased by 7 ± 2 μA/cm^2 ($P < 0.05$, n = 8) (Fig. 2A). Preincubation for 15 min with 17β-estradiol profoundly inhibited the subsequent CT or STa activated I_{SC} response (Fig 2A, 2B).

These responses were ~60 % less than that observed in control tissues (Fig. 2B). The antisecretory effect of 17β-estradiol on enterotoxin-induced secretion was observed to be female gender specific. Inhibition of enterotoxin-induced currents was only observed in colonic tissues from female rats with no significant response in male tissues (Fig. 3) even at concentrations of 17β-estradiol as high as 100 nM.

3.2. Signaling pathways involved in the antisecretory response to 17β-estradiol

Previous data from our laboratory suggest that the rapid onset of the 17β-estradiol antisecretory effect is incompatible with a genomic signaling pathway associated with estrogenic action [8-13]. In this study, the effects of 17β-estradiol on enterotoxin-induced Cl^- secretion were found to be insensitive to inhibitors of transcription or translation such as actinomycin D (5 μM) and cycloheximide (20 μM) respectively (data not shown), confirming the nongenomic nature of this rapid antisecretory effect of 17β-estradiol. The inhibitory effect of 17β-estradiol on STA or CT induced secretion was not prevented by 30 min pre-incubation with the pure antiestrogen ICI 182,780 (10 μM) suggesting a signaling pathway independent of nuclear estrogen receptors (Fig. 4A, 4B). The selectivity of 17β-estradiol action was tested by treating colonic epithelia with other sex steroids such as testosterone, progesterone and the biologically inactive 17α-estradiol. We found that none of the other steroids tested had the ability to mimic the antisecretory actions of 17β-estradiol on either STa or CT induced secretory responses (Fig. 4C, 4D).

CT induces intestinal secretion via an excessive increase in intracellular cAMP, due to irreversible activation of adenylyl cyclase, while STa enhances intracellular cGMP production, leading to activation of protein kinase GII (PKGII) [16]. Since our data indicate that the inhibitory effect of 17β-estradiol is independent of the second messenger pathway

involved, we further investigated whether the target of 17β-estradiol occurs proximal or distal to cyclic nucleotide generation. Therefore, electrogenic Cl^- secretion was induced by means of the cell permeable nucleotide analogs, 8Br-cAMP (1 mM) or 8Br-cGMP (1 mM). These compounds induced rapid and sustained increases in I_{SC} and in both cases, the currents were reduced by 17β-estradiol (Fig. 5). Thus, the antisecretory effect of 17β-estradiol on Cl^- secretion appears to occur distal to the synthesis of either cAMP or cGMP.

We have previously demonstrated that 17β-estradiol induced a female-specific rapid increase in PKCδ activity, measured as kinase autophosphorylation (13). Pretreatment of the crypts with 10 μM rottlerin, a concentration known to selectively inhibit PKCδ [23] abolished the hormone-induced activation of PKCδ (13). Here we found that the inhibitory action of 17β-estradiol on STa induced secretion was abolished after 15 min pre-incubation with 10 μM rottlerin (Fig. 6A). We also found that treatment of colonic crypts with 17β-estradiol (10nM) had no effect on PKCα activity measured as kinase autophosphorylation (data not shown). Moreover, the PKCα inhibitor HBDDE (100 μM) failed to prevent the effect of 17β-estradiol on enterotoxin-induced secretion (Fig. 6B). These results indicate that the rapid activation of PKCδ by 17β-estradiol is required for inhibition of Cl^- secretion induced by enterotoxins.

Several studies have shown that transactivation of the epidermal growth factor receptor (EGFR) reduces the extent of G_q-protein coupled receptor induced Cl^- secretion while increasing secretory responses mediated by G_s-protein coupled receptors in colonic epithelial cells [24, 25]. Recent evidence has also suggested EGFR can be transactivated by steroid hormones in a nongenomic fashion [26-29]. To determine if transactivation of the EGFR plays a role in antisecretory effect of 17β-estradiol, we examined the effects of the EGFR inhibitor, tyrphostin-AG1478, on I_{SC} responses to forskolin (10 μM). Pretreatment

with tyrphostin-AG1478 (1 μM), failed to prevent the antisecretory effect of 17β-estradiol on I_{SC} responses induced by forskolin. Control I_{SC} responses to forskolin were 32.8 ± 5.5 μA/cm^2 compared with 17.3 ± 4.5 μA/cm^2 after 17β-estradiol treatment and 25.1 ± 5.1 in tyrphostin-pretreated cells (n = 4; P = 0.072).

3.3. Effect of 17β-estradiol on ion channel activity

Apical membrane Cl^- channels – Nystatin, added to the basolateral bathing solution of control tissues, increased the Cl^- current (I_{Cl}) by 10 ± 3 μA/cm^2 (n = 7). This likely reflects an unmasking of a constitutively active apical Cl^- conductance. Subsequent addition of CT and STa increased the I_{Cl} to 82 ± 5 μA/cm^2 (n = 4) and 56 ± 4 μA/cm^2 (n = 4), respectively. Pre-incubation with 17β-estradiol (10 nM) for 15 min had no significant effect on either the nystatin or enterotoxin + nystatin induced I_{Cl} (Fig. 7A) (nystatin + 17β-estradiol 8 ± 5 μA/cm^2; CT + nystatin + 17β-estradiol 77 ± 6 μA/cm^2; STa + nystatin + 17β-estradiol 51 ± 5 μA/cm^2; $P > 0.05$, n = 4) . These results indicate that 17β-estradiol neither inhibits the basal activity of apical Cl^- channels nor of enterotoxin stimulated apical Cl^- channels.

Basolateral membrane K^+ channels – When amphotericin B was added to the apical bath in the presence of an apical to basolateral directed K^+ gradient (80:5 mM) and ouabain (1 mM) in the basolateral solution, the K^+ dependent current (I_K) immediately began to increase and reached maximal levels within 15 min (from 6 ± 2 to 81 ± 15 μA/cm^2, n = 6). The I_K decreased rapidly by ~60% (to 27 ± 5 μA/cm^2, n = 6) following addition of 17β-estradiol at the peak of the amphotericin-induced response (Fig. 7B). Pretreatment of colonic epithelia with rottlerin for 15 min before addition of amphotericin B did not modify the increase in I_K induced by the ionophore but prevented the inhibition of I_K by 17β-estradiol (Fig. 7B). Thus PKCδ activity is required for estrogen inhibition of basolateral membrane K^+ channel activity.

In order to functionally identify the K^+ channel involved we tested the effects of selective K^+ channel inhibitors on the basal and enterotoxin-stimulated I_K. As shown in Fig. 8A, when tissues were pretreated with 17β-estradiol (10 nM) for 15 min., the basal I_K was reduced by ~65 % of control values (81 ± 15 compared to 25 ± 2 μA/cm^2, n = 6). The I_K currents remaining after amphotericin B treatment alone and after estrogen treatment were largely inhibited by basolateral application of chromanol 293B (50 μM), a KCNQ1 channel inhibitor. Basolateral addition of $BaCl_2$ (2 mM) or the more selective KCNQ1 inhibitor HMR-1556 (10 μM) also produced an inhibition of the spontaneous I_K similar to that of chromanol 293B (Fig. 8B). Other specific K^+ channel inhibitors (of large (BK) and small (SK) conductance, Ca^{2+}-dependent K^+ channels) such as iberiotoxin (BK, 200 nM), apamin (SK, 500 nM) and TRAM-34 (KCNN4, 500 nM) did not affect I_K (Fig. 8B). These results indicate that the basolateral membrane I_K is largely generated by a K^+ conductance with a pharmacological profile similar to KCNQ1 channels and this channel is a target of 17β-estradiol.

Pretreatment with CT significantly increased I_K to 127 ± 10 μA/cm^2 (n = 3, P < 0.01) (Fig. 8C), a result in line with the well known stimulatory effect of cAMP on basolateral KCNQ1 channels via a PKA dependent pathway [7]. In contrast, pretreatment with STa had no significant effect on I_K (85 ± 8 μA/cm^2; n = 3, P = 0.24) (Fig. 8C) suggesting that the cGMP/PKG pathways does not stimulate KCNQ1 channels but rather acts on the apical Cl^- conductive pathway. Addition of 17β-estradiol decreased the CT-stimulated I_K and also inhibited the basal I_K in the presence of STa (Fig. 8C). Both KCNQ1 and KCNN4 channels are functionally expressed in the female rat colon but it appears from our results that only the KCNQ1 channel is the target for estrogen inhibition of basolateral membrane K^+ current. In order to determine the relative roles of these channels in sustaining the toxin-induced Cl^- secretion, we tested the responses of the CT and STa-induced I_{SC} to clotrimazole and

chromanol 293B. As shown in Fig. 8D, only the KCNQ1 channel inhibitor produced a marked inhibition of enterotoxin-induced Cl^- secretion whereas clotrimazole (10 μM), a specific inhibitor of Ca^{2+}-dependent KCNN4 channels had no effect on Cl^- secretion. Taken together, these results demonstrate the key role of KCNQ1 channels in mediating enterotoxin-induced secretion and the targeting of this channel by 17β-estradiol to affect an antisecretory response.

4. Discussion

The initiation of the secretory response induced by *E. coli* and *V. cholerae* is dependent on toxin binding to receptors on the surface of intestinal enterocytes. After binding, cyclic nucleotides levels are increased, which ultimately lead to secretion by crypt cells of fluid and electrolytes into the intestine lumen, and watery diarrhea. Any substance capable of decreasing net fluid secretion, by either inhibiting the secretory process or by enhancing fluid absorption, is a potential antidiarrheal drug. Oral rehydration therapy has a significant impact on morbidity and mortality of patients with acute diarrhea; however, oral rehydration therapy does not interfere with the secretory process nor diminish diarrhea [17]. Therefore, new, safe and more effective antisecretory drugs are being sought. Several inhibitors of ion transport and intestinal secretion have been described. In animal models, loperamide and berberine inhibit secretion by CT [30,31]. Berberine, chlorpromazine, and indomethacin also reduce secretion induced by STa [31,32]. Despite the efficacy of these drugs in animal models, none are routinely used in humans because of adverse side effects of the high concentrations required for efficacy.

In this study, the sex steroid 17β-estradiol rapidly decreased Cl^- secretion induced by CT and STa; two of the major causes of secretory diarrhea in humans. Physiological concentrations of 17β-estradiol reduced basal Cl^- secretion, prevented CT and STa

stimulation of Cl^- secretion and rapidly decreased Cl^- secretion pre-stimulated by these toxins in the female rat distal colon. These results provide the first evidence for an antisecretory effect of 17β-estradiol on enterotoxin-induced Cl^- secretion in the intestine, and point to a potential role for manipulation of 17β-estradiol-dependent signaling pathways and ion transporter targets in the treatment of secretory diarrheas.

The rapid antisecretory responses to 17β-estradiol are likely to be mediated by a novel receptor protein in colonic epithelial cells [21,33]. This is supported by the acute onset of the effect and by experiments, which demonstrated that pre-incubation with inhibitors of transcription and translation, or the pure antiestrogen ICI 182,170 [34] did not prevent the antisecretory effect of 17β-estradiol. Although several studies have suggested that rapid effects of 17β-estradiol are mediated by a novel estrogen receptor, the molecular identity of this receptor remains elusive. Over the last decade, the G-protein coupled receptor-30 protein (GPR30) [35] has emerge as a candidate receptor for the non-genomic effects of 17β-estradiol in several tissues and cell lines. The role of GPR30 in mediating the anti-secretory effects of 17β-estradiol in female rat distal colon will be the subject of future studies, however, the lack of inhibition by ICI182,720 suggests a different signaling pathway.

The effect of estrogen to inhibit secretion displays sexual dichotomy and was only observed in colonic tissue isolated from female rats. In female rat colonic crypts, 17β-estradiol stimulates a rapid increase in intracellular Ca^{2+}, PKA and PKC activity while having no effect on these second messengers in male colon [8, 10]. Previous studies from our laboratory have shown that in a cell free system assay, 17β-estradiol can directly activate the PKCα and PKCδ isoforms through binding to their C2 domain [10,22]. However, we have shown that activation of PKCδ but not PKCα is required for 17β-estradiol to reduce Cl^- secretion and that female colonic epithelia express significantly higher levels of these PKC isoforms compared to males [13]. Since estrogens can freely diffuse across cell membranes, it

is plausible that PKCδ may be directly activated by 17β-estradiol independent of other signalling pathways. Thus, PKCδ may constitute a novel receptor for estrogens. Therefore, the mechanism for gender specificity of 17β-estradiol antisecretory action may be related to PKCδ expression levels or differences in the K^+ channel protein components. Alternatively, colonocytes from male animals may not express the most proximal component of the 17β-estradiol nongenomic signaling pathway, the putative novel estrogen receptor. Up to date there is no PKCδ specific inhibitors, therefore our study relied on using the selective PKCδ inhibitor rottlerin. However, recent studies have shown that this inhibitor is capable of inhibiting other protein kinases [36]. Thus, our results must be analyzed from that perspective. Unfortunately, primary tissues are extremely hard to transfect with iRNA in order to inhibit the expression of PKCδ.

The molecular target for the 17β-estradiol antisecretory effect appears not to be CFTR or other apical Cl^- channels but rather the K^+ channel responsible for K^+ recycling at the basolateral membrane. Pharmacological characterization of this K^+ current suggests that it is generated by KCNQ1 channels. KCNQ channels are a family of voltage-dependent, cAMP- and Ca^{2+}-dependent K^+ channels, first characterized in cardiac tissue [6,7,37]. In colonocytes, the KCNQ1 isoform is localized at the basolateral membrane and participates in K^+ recycling required for Cl^- secretion induced by cAMP [1,6,38,39]. In colonic epithelia KCNQ1 is coexpressed with the regulatory KCNE3 subunit. Association of KCNQ1 with KCNE3 at the basolateral membrane of colonocytes produces constitutively open channels that regulate potassium recycling required to maintain the driving force for chloride secretion[40]. Studies on KCNE3 knockout mice have shown that decreased expression of this subunit impairs the secretory capacity in colon and trachea [41]. Experiments using chromanol 293B, as an inhibitor of KCNQ1 channels have shown that this compound can completely block cAMP-induced Cl^- secretion. Our data shows that basolateral KCNQ1 currents are stimulated by the

cAMP-dependent CT but insensitive to stimulation by the cGMP-dependent STa secretagogues. Although modulation of basolateral KCNQ1 channel activity by 17β-estradiol may underlie the inhibition of basal and enterotoxin-stimulated Cl^- secretion, the hormone does not completely inhibit the enterotoxin-induced Cl^- secretion but rather reduces it by up to 60%. Recent studies [42] using the HMR-1556 compound, a more selective inhibitor of KCNQ1 [43,44], have shown that KCNQ1 channels are not essential for activation of Cl^- secretion and contribute up to 50 % of K^+ recycling during cAMP-induced secretion. Pretreatment of colonic epithelia with the PKCδ inhibitor rottlerin suppressed the inhibitory effect of 17β-estradiol on basolateral K^+ currents. Therefore, we propose that 17β-estradiol decreases basal and enterotoxin-induced Cl^- secretion by activating PKCδ, which phosphorylates basolateral KCNQ1 channels to reduce K^+ recycling and thereby decreases the electrochemical gradient required for Cl^- secretion [13]. In agreement with these findings, Reenstra has shown that the phorbol ester PMA inhibits the basolateral K^+ conductance of T84 cells and that this effect reduces transepithelial Cl^- secretion [45]. The precise mechanism through which 17β-estradiol-induced phosphorylation of basolateral KCNQ1 channels inhibits channel activity in rat colonic crypts is unknown and will be an important goal of future studies. One possibility to be considered is reduction of channel number at the plasma membrane.

Our finding that 17β-estradiol has a marked inhibitory effect on basolateral KCNQ1 channels in rat colonic crypts highlights this component of the intestinal Cl^- secretory process as a target for new antidiarrheal strategies. Identification of the mechanism of hormonal regulation of basolateral K^+ channels in colonic epithelia and a better understanding of the signaling processes involved may provide a basis for the development of new antisecretory drugs with greater potency and specificity.

Several groups have explored the selective blockade of basolateral K^+ channels as a means of regulating epithelial Cl^- secretion. The antifungal antibiotic, clotrimazole, has also been shown to prevent fluid and electrolyte secretion in rabbit and mouse intestine triggered by Ca^{2+}- and cAMP-mediated agonists via specific inhibition of basolateral K^+ conductances [46]. Furthermore, levamisole and other phenylimidazothiazoles inhibit Ca^{2+}- and cAMP-activated Cl^- secretion in T84 cell monolayers and isolated human distal colon, apparently by blocking basolateral K^+ channels [47].

In the present study, we have demonstrated that 17β-estradiol can rapidly inhibit enterotoxin-induced Cl^- secretion in female distal colonic epithelium. This suggests 17β-estradiol may also alter colonic ion transport during high estrogen states such as pregnancy and certain phases of the menstrual cycle [48,50], and during use of oral contraceptives [49] and estrogen treatment [50,51]. A molecular target of the anti-secretory effects of estrogen has been identified as the basolateral KCNQ1 K^+ channel in rat colon [52]. This channel has been shown to be rate-limiting for secretion in colon and airway epithelia [53]. Moreover, estrogen enhances amiloride-sensitive Na^+ absorption in mouse renal cortical collecting duct and distal colon (Robles, Flores, Thomas and Harvey, unpublished data). Therefore, we conclude that the ability of 17β-estradiol to limit secretion and enhance absorption in the major organs regulating whole body fluid and electrolyte balance [51] may contribute in the well-known fluid retentive properties of this hormone.

The results of this study have three major implications. Firstly, it demonstrates that estrogen is protective in the female against enterotoxins causing secretory diarrhoea. This may have an evolutionary advantage to protect reproductive females against fluid loss during the implantation window. Secondly, it highlights the key role of the KCNQ1 channel in sustaining enterotoxin-induced secretion and that this channel is the ion transport target underpinning the rapid inhibitory effects of 17β-estradiol on Cl^- secretion. Finally, it offers a

cellular and molecular explanation for the whole body salt and water retention responses in high estrogen states.

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FIGURE LEGENDS

Figure 1. Effect of 17β-estradiol on enterotoxin-induced Cl^- secretion. (A, C). Representative recordings showing the effect of cholera toxin CT (1 μg/mL) and heat stable enterotoxin STa (50 ng/mL) pretreatment respectively on I_{SC} in female rat distal colonic epithelium followed by application of 10 nM 17β-estradiol (red line) or a methanol vehicle 0.01 % (blue line). (B, D) Mean ± SEM change in I_{SC} after treatment with different 17β-estradiol concentrations following exposure to cholera toxin or STa Values were measured after maximal inhibition was achieved by each dose of 17β-estradiol and are compared to parallel vehicle controls (methanol) from the same animal. Maximal inhibition was achieved after a period of 30-45 min after 17β-estradiol treatment. The reported values are the mean of 5 min recording at the plateau of the response. (n = 8 for each group]. * $P < 0.05$, ** $P < 0.01$ compared to control.

Figure 2. 17β-estradiol pretreatment inhibits enterotoxin-induced Cl^- secretion. (A) Representative recording of the effect of 15 min pretreatment with 10 nM 17β-estradiol (red line) on the STa-induced Cl^- secretion compared to a vehicle control (blue line). (B) Mean ± SEM (n = 8 for each group] change in I_{SC} measured at the peak of enterotoxin-induced

secretion following 17β-estradiol (10 nM) pretreatment and compared to vehicle control (** $P < 0.01$ compared to control).

Figure 3. Effect of 17β-estradiol on enterotoxin-induced Cl^- secretion is gender specific. (A) Representative recording of the effect of 10 nM 17β-estradiol on STa-induced I_{SC} in male (blue line) and female (red line] rat colonic epithelia. (B) Gender specificity of 17β-estradiol (10 nM] inhibition of enterotoxin-induced secretion in female (black bars] compared to male (grey bars] rat colonic tissues. Values are mean ± SEM of $n = 6$ animals for each group, (** $P < 0.01$).

Figure 4. Effect of antiestrogen and other sex steroid hormones on enterotoxin-induced Cl^- secretion. (A, B) Mean ± SEM ($n = 6$] change in total I_{SC} caused by STa and CT in control conditions, in the presence of ICI 182,780 (10 μM], and ICI 182,780 plus 17β-estradiol (10 nM) in female rat distal colon. (C, D) Effect of steroid hormones on STa- and CT-induced secretion respectively. Mean ± SEM ($n = 6$) change in I_{SC} caused by enterotoxin in control conditions, in the presence of 17β-estradiol (10 nM), 17α-estradiol (10 nM), progesterone (10 nM) and testosterone (10 nM) (* $P < 0.05$, ** $P < 0.01$ compared to control).

Figure 5. Effect of 17β-estradiol on Cl^- secretion induced by cAMP (grey bars) and cGMP (black bars) analogs. Mean ± SEM ($n = 6$) change in total I_{SC} caused by 8Br-cAMP (1 mM) and 8Br-cGMP (1 mM) in control conditions and in the presence of 17β-estradiol (10 nM) in female rat distal colon (* $P < 0.05$ compared to control).

Figure 6. Role of PKC isoforms on the antisecretory effect of 17β-estradiol. (A) Representative recording of the effect of Rottlerin (blue line] on the 17β-estradiol antisecretory response compared to preincubation with a vehicle control (red line]. (B) Mean ± SEM (n = 6) change in I_{SC} caused by pre-incubation with PKC□ inhibitor Rottlerin (10 μM) and PKCα inhibitor HBDDE (100 μM) on the antisecretory effect of 17β-estradiol (10 nM) in STa-stimulated colonic epithelia (** $P < 0.01$ compared to control).

Figure 7. Effects on estrogen on apical Cl^- and basolateral K^+ currents in antibiotic-permeabilized colonic epithelia. (A) The basolateral membrane was permeabilized with nystatin and apical chloride currents measured in response to 17β-estradiol (10 nM). Estrogen had no effects on apical membrane Cl^- current. (B) The apical membrane was permeabilized with amphotericin B and basolateral membrane K^+ currents recorded in response to 17β-estradiol (10 nM) in the absence or presence of the PKCδ inhibitor rottlerin (10 μM). Estrogen produced a profound inhibition of basolateral K^+ current (red line) compared to vehicle control (blue line) which was largely prevented by pre-treatment with rottlerin (green line).

Figure 8. Effects of K^+ channel inhibitors on basolateral K^+ channel current and the antisecretory response to estrogen in the absence or presence of enterotoxins. (A) Effect of the KCNQ1 channel inhibitor chromanol 293B on basolateral membrane K^+ current in amphotericin-permeabilized female rat colonic epithelia. Chromanol 293B (50 μM) produced an almost complete inhibition of I_K in the absence of estrogen (blue line) and following pre-treatment with 17β-estradiol (10 nM, red line). (B) Effect of several specific K^+ channels inhibitors on I_K. Only the KCNQ1 channel inhibitors, chromanol 293B and HMR-1556 (10 μM), produced a significant inhibition of I_K as did the non-selective K^+ channel inhibitor

$BaCl_2$ (2 mM). Treatment with inhibitors of Ca^{2+}-activated K^+ channels iberiotoxin (200 nM), apamin (500 nM) and TRAM-34 (500 nM) had no effect on I_K. Values are means ± SEM $n = 6$. ** $P < 0.01$ vs. control. (C) Effect of 17β-estradiol on basolateral K^+ currents pre-stimulated with bacterial enterotoxins. Cholera toxin produced an increase in I_K which was inhibited by estrogen (E2). Heat-stable enterotoxin had no effect on I_K but this current was also inhibited by estrogen (10 nM). Values are means ± SEM, $n = 4$. ** $P < 0.01$ vs. control. (D) Effect of 10 µM clotrimazole (KCNN4 inhibitor) and 50 µM chromanol 293B (KCNQ1 inhibitor) on transepithelial chloride ion secretion induced by cholera toxin (black bars) or STa (grey bars). Only the KCNQ1 channel inhibitor produced a significant inhibition of the enterotoxin-induced secretion ($P < 0.01$ vs. control DMSO vehicle response) whereas the KCNN4 inhibitor had no effect on secretion.

Figure 1

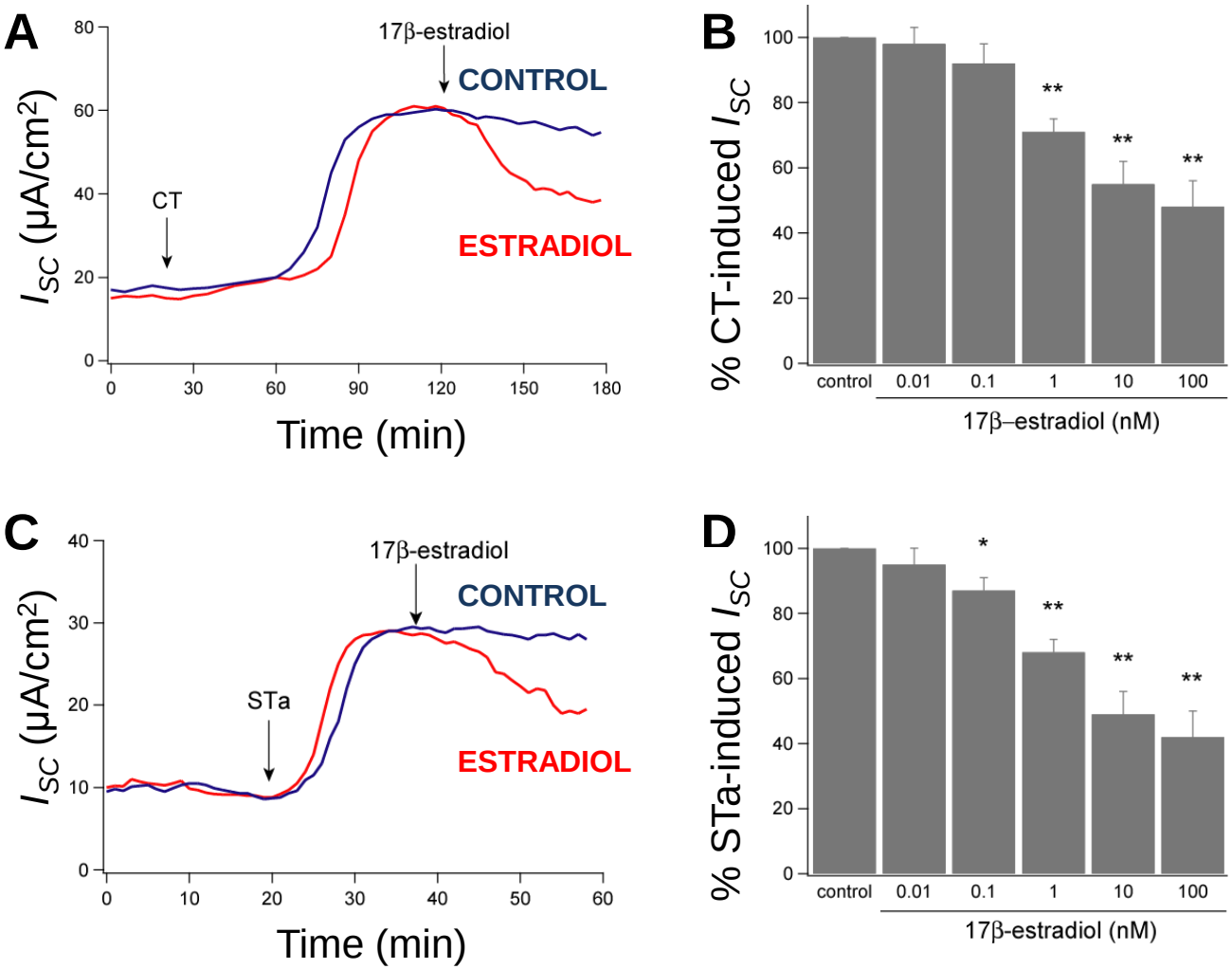
A
17β-estradiol
CONTROL
CT
ESTRADIOL
I_{SC} (μA/cm²)
Time (min)
B
% CT-induced I_{SC}
control
0.01
0.1
1
10
100
17β–estradiol (nM)
C
17β-estradiol
CONTROL
STa
ESTRADIOL
I_{SC} (μA/cm²)
Time (min)
D
% STa-induced I_{SC}
control
0.01
0.1
1
10
100
17β-estradiol (nM)

Figure 2

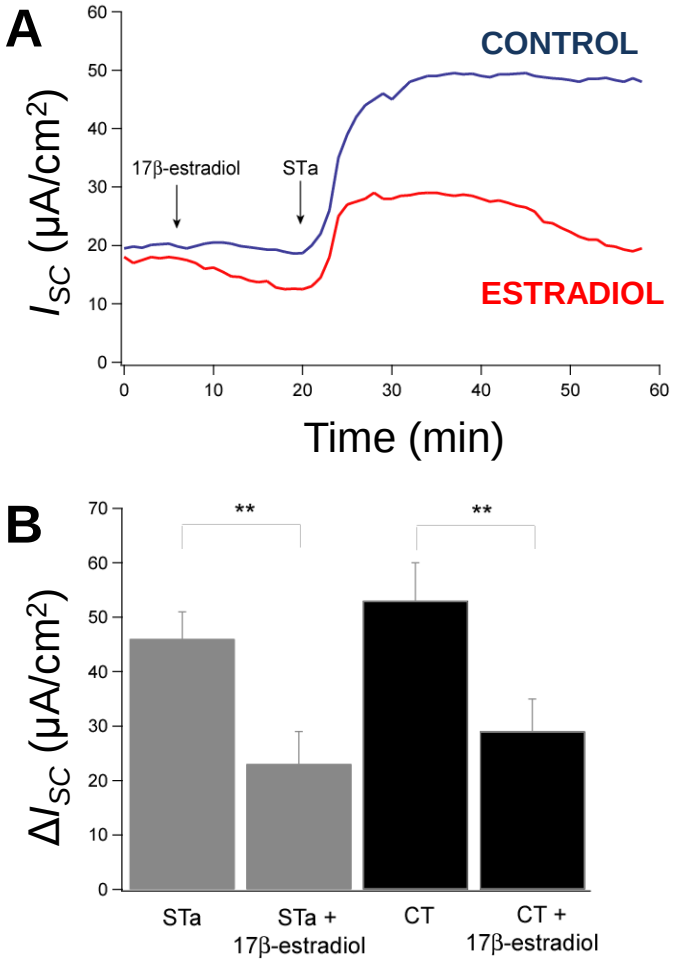
A
CONTROL
ESTRADIOL
17β-estradiol
STa
I_{SC} (μA/cm²)
Time (min)
0
10
20
30
40
50
60
B
**
**
ΔI_{SC} (μA/cm²)
70
STa
STa + 17β-estradiol
CT
CT + 17β-estradiol

Figure 3

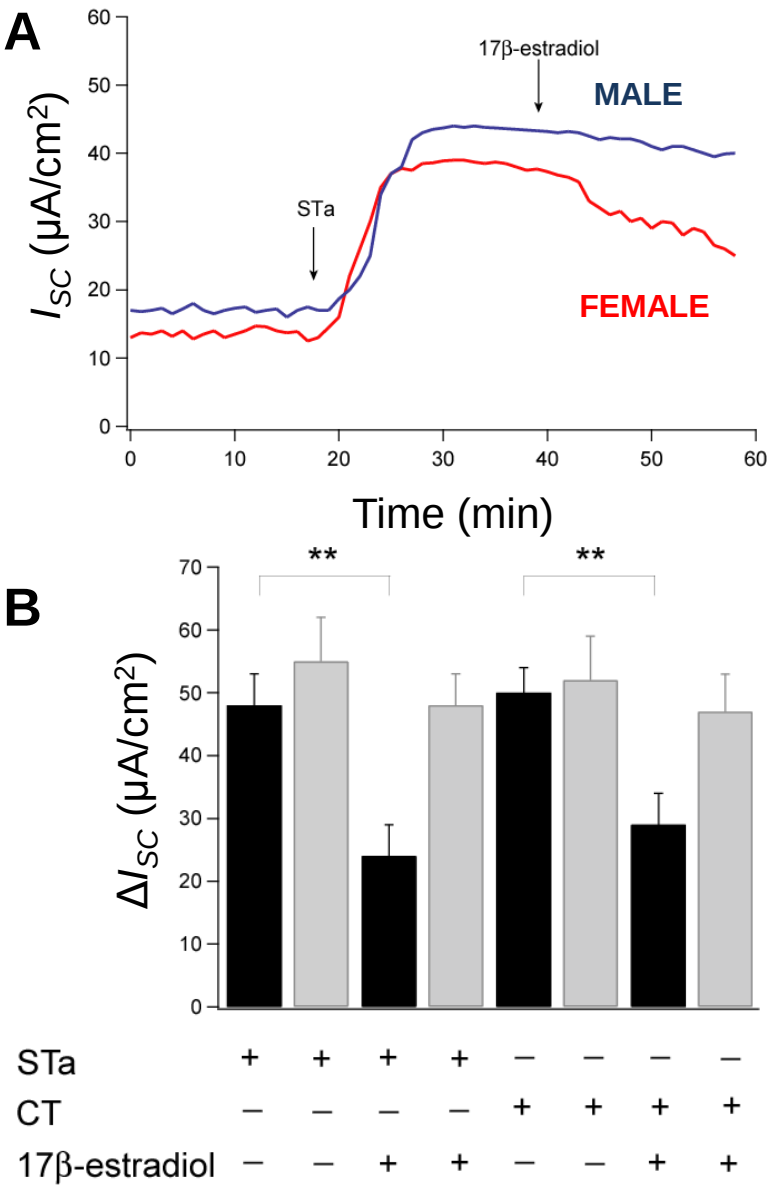

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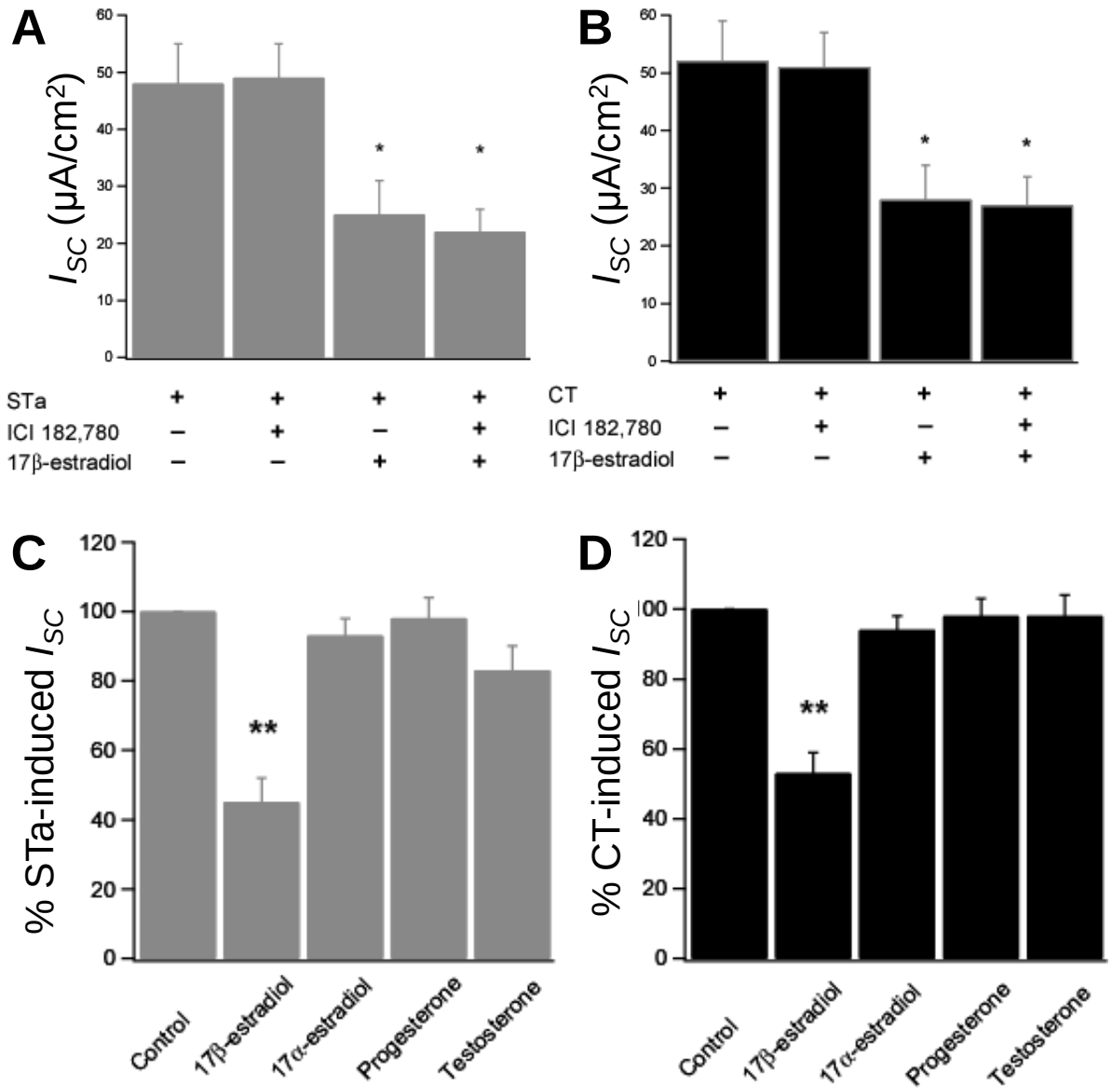
Figure 4


A
I_{SC} (μA/cm²)
60
50
40
30
20
10
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*
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STa + + + +
ICI 182,780 – + – +
17β-estradiol – – + +
B
I_{SC} (μA/cm²)
60
50
40
30
20
10
0
*
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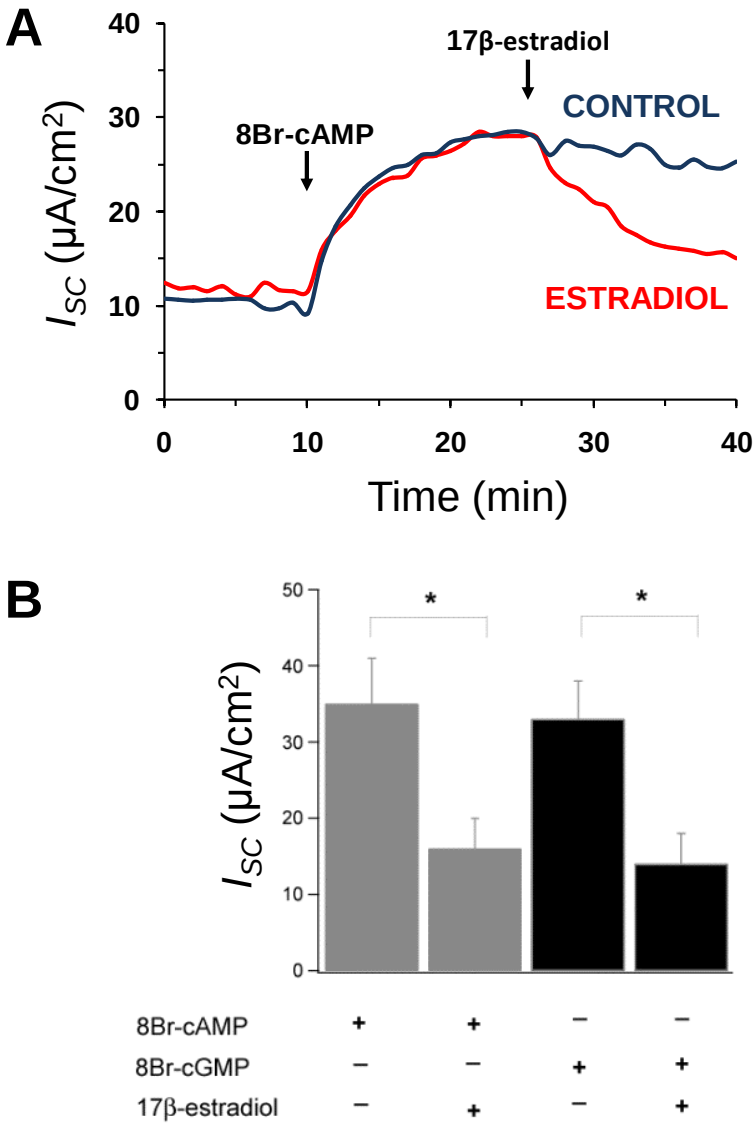
CT + + + +
ICI 182,780 – + – +
17β-estradiol – – + +
C
% STa-induced I_{SC}
120
100
80
60
40
20
0
**
Control
17β-estradiol
17α-estradiol
Progesterone
Testosterone
D
% CT-induced I_{SC}
120
100
80
60
40
20
0
**
Control
17β-estradiol
17α-estradiol
Progesterone
Testosterone

Figure 5


A
40
30
20
10
0
I_{SC} (μA/cm²)
17β-estradiol
8Br-cAMP
CONTROL
ESTRADIOL
0
10
20
30
40
Time (min)
B
50
40
30
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10
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I_{SC} (μA/cm²)
*
*
8Br-cAMP + + − −
8Br-cGMP − − + +
17β-estradiol − + − +

Figure 6

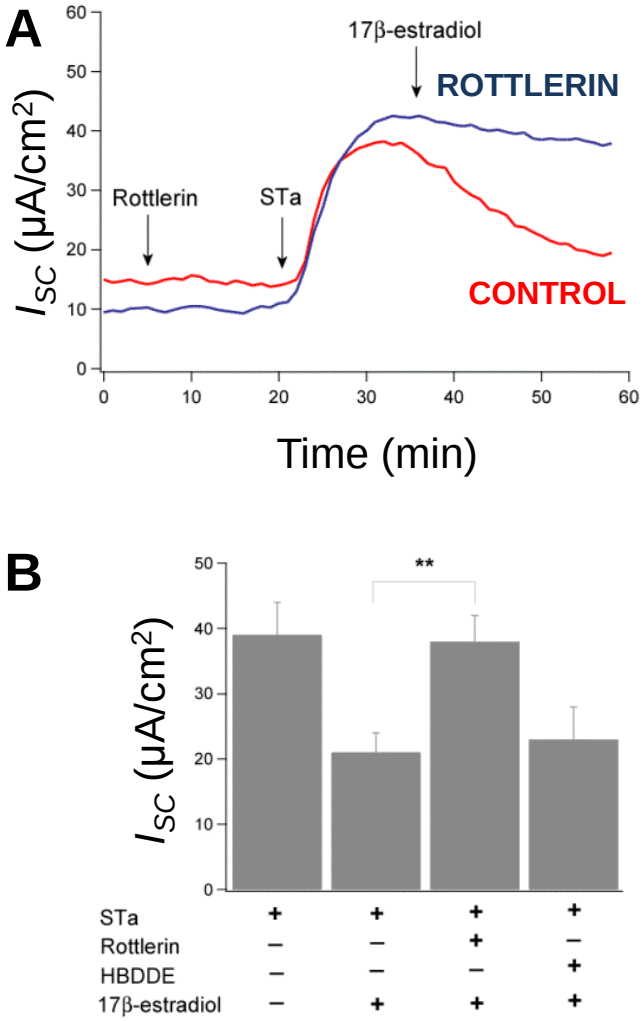

A
17β-estradiol
ROTTLERIN
Rottlerin
STa
CONTROL
I_{SC} (μA/cm²)
Time (min)
B
**
I_{SC} (μA/cm²)
STa
Rottlerin
HBDDE
17β-estradiol

Figure 7

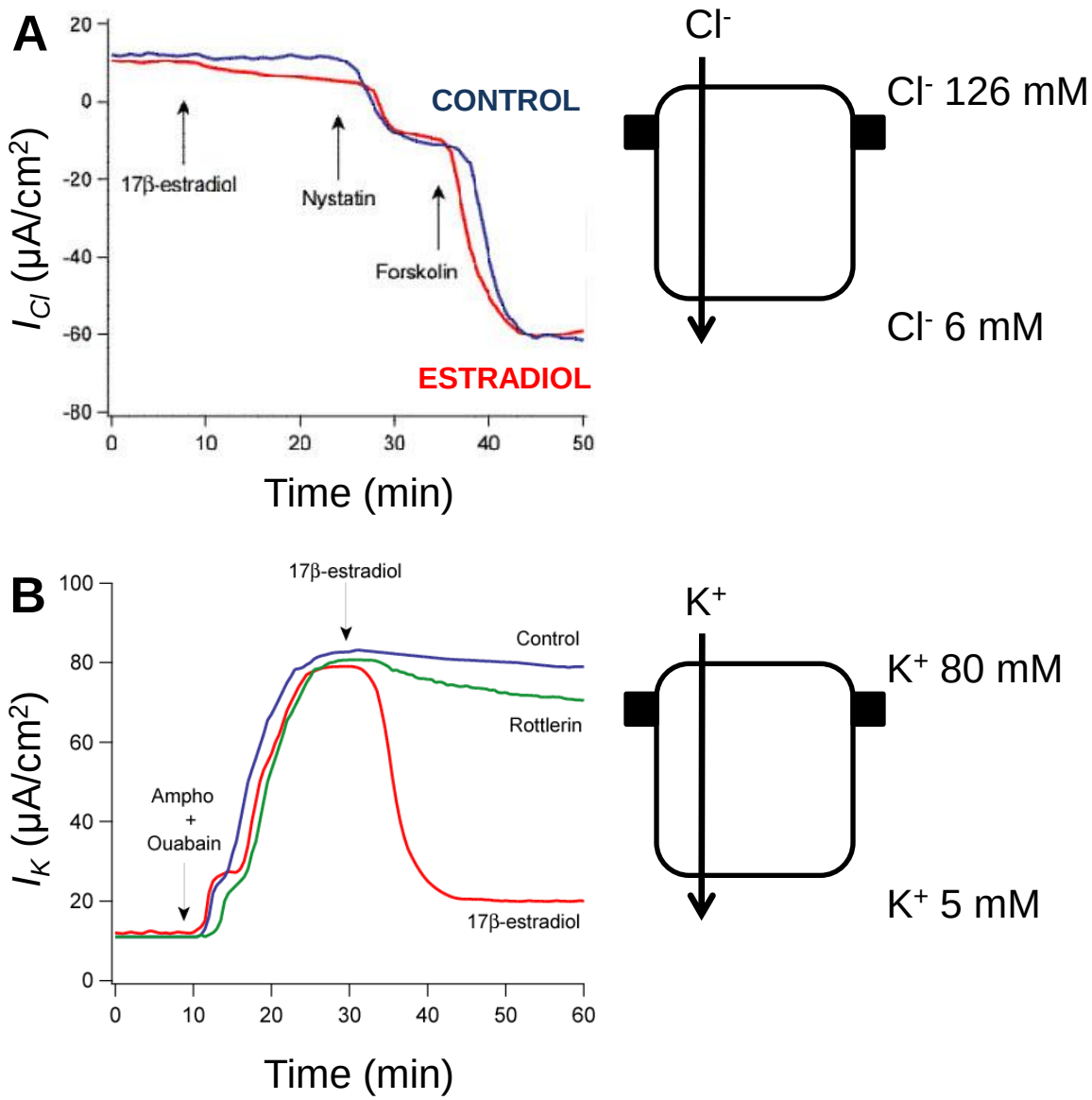
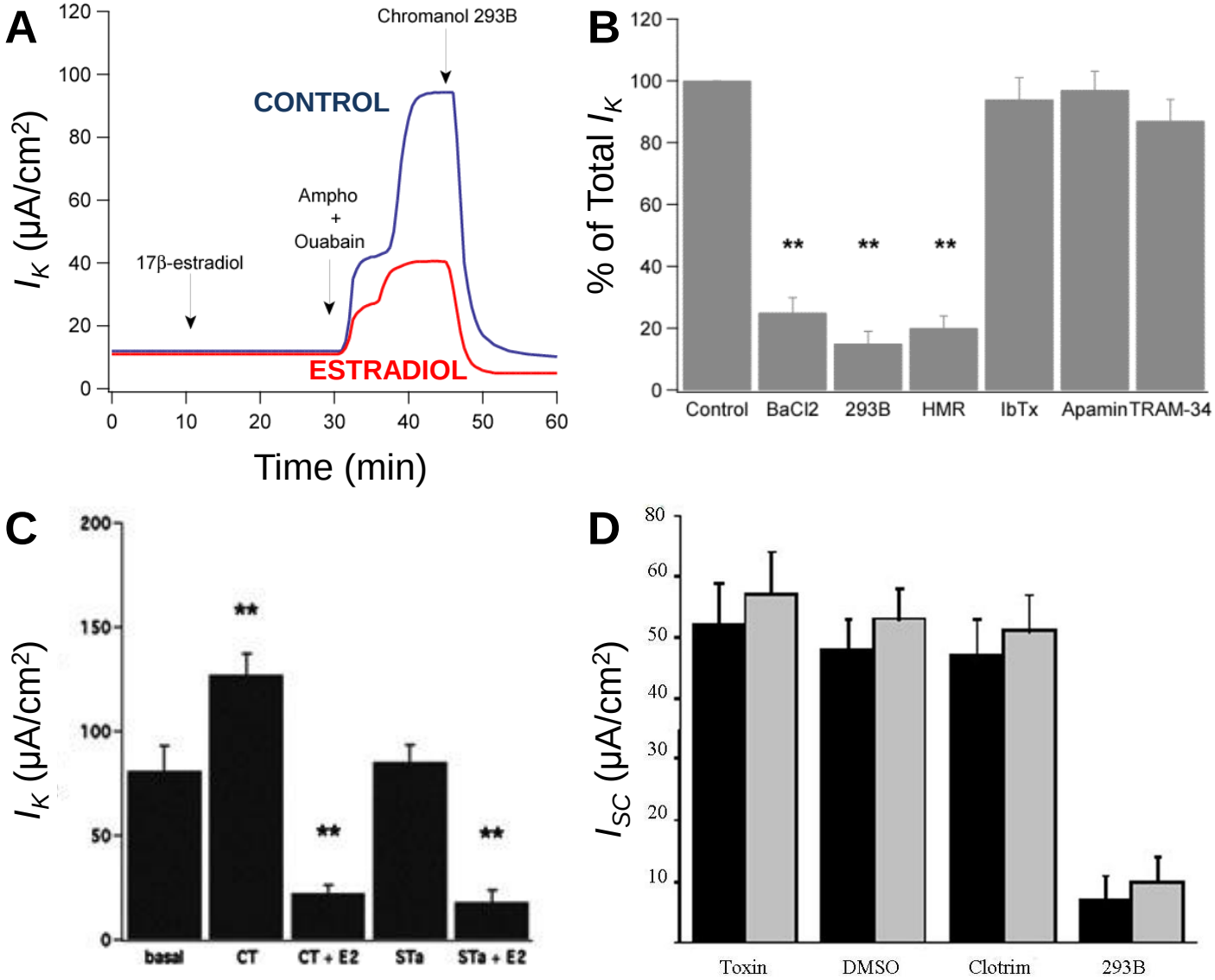

A
20
0
-20
-40
-60
-80
I_{Cl} (μA/cm²)
17β-estradiol
Nystatin
Forskolin
CONTROL
ESTRADIOL
0
10
20
30
40
50
Time (min)
Cl⁻
Cl⁻ 126 mM
Cl⁻ 6 mM
B
100
80
60
40
20
0
I_K (μA/cm²)
17β-estradiol
Control
Rottlerin
Ampho
+
Ouabain
17β-estradiol
0
10
20
30
40
50
60
Time (min)
K⁺
K⁺ 80 mM
K⁺ 5 mM

Figure 8


A
Chromanol 293B
CONTROL
Ampho
+
Ouabain
17β-estradiol
ESTRADIOL
I_K (μA/cm²)
Time (min)
B
% of Total I_K
Control
BaCl2
293B
HMR
IbTx
Apamin
TRAM-34
C
I_K (μA/cm²)
basal
CT
CT + E2
STa
STa + E2
D
I_{SC} (μA/cm²)
Toxin
DMSO
Clotrim
293B