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Nongenomic Effects of Estrogens on Epithelial Chloride Secretion

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A dissertation

presented to

the faculty of the Department of Physiology

James H. Quillen College of Medicine

East Tennessee State University

In partial fulfillment

of the requirements for the degree

Doctor of Philosophy in Biomedical Sciences

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by

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August 2004

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Keywords: estrogen, nongenomic, epithelia, CFTR

## ABSTRACT

### Nongenomic Effects of Estrogens on Epithelial Chloride Secretion

by

Sabyasachi Moulik

The human colonic cell line T84, a model for studying epithelial chloride secretion and cystic fibrosis chloride channel (CFTR) function, was used to investigate the regulatory role of estrogens in transepithelial ion transport. Estrogens and other steroid hormones do not stimulate chloride secretion by themselves. However, 17  $\beta$ -estradiol (17 $\beta$ -E2) rapidly (within seconds to minutes) potentiates carbachol- and thapsigargin-stimulated chloride secretion measured as short circuit current in voltage-clamped T84 monolayer cultures. The cholinergic agonist carbachol and the SR Ca<sup>2+</sup> ATPase inhibitor thapsigargin stimulate chloride secretion by elevating intracellular calcium. 17  $\alpha$ -estradiol, a stereoisomer that does not activate nuclear estrogen receptors, is equipotent with 17 $\beta$ -E2. Other non-estrogen steroids produce much less, if any, potentiation of calcium-stimulated chloride secretion. The estrogen receptor antagonist tamoxifen does not block 17 $\beta$ -E2 potentiation of calcium-stimulated chloride secretion, indicating that the classical nuclear estrogen receptors are not involved. Potentiation is greater when 17 $\beta$ -E2 is applied to the apical membrane than to the basolateral membrane. 17 $\beta$ -E2 effects on chloride secretion coincide with an increase in monolayer electrical conductance, which is consistent with activation of one or more ion channel species. Potentiation is not blocked by the chloride channel blockers DIDS and NPPB but is abolished by the PKA inhibitor H89, suggesting that 17 $\beta$ -E2 potentiation depends on the activity of CFTR but not other types of apical membrane chloride channels. 17 $\beta$ -E2 does not increase the activity of calcium-activated

potassium channels in the basolateral membrane as measured in nystatin-permeabilized monolayers.  $17\beta$ -E2 effects are not blocked by the MAP kinase kinase inhibitor PD 98059 or by the PKC inhibitor bisindolylmaleamide, suggesting that these signal transduction pathways are not involved.  $17\beta$ -E2 potentiation requires extracellular  $\text{Ca}^{2+}$ . Paradoxically,  $17\beta$ -E2 reduces the rise in intracellular free  $\text{Ca}^{2+}$  levels in thapsigargin-stimulated T84 cells, as measured by fura-2 fluorescence. From my studies I conclude that  $17\beta$ -E2 causes an increase in the sensitivity of T84 cells to calcium-elevating secretagogues. This effect may be due to nongenomic actions of  $17\beta$ -E2 on CFTR function and/or the activity of store-operated calcium channels that leads to a change in CFTR functional regulation.

## DEDICATION

This dissertation is dedicated to my parents, and all those  
wonderful people who showed me the way.

## ACKNOWLEDGEMENTS

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## ABBREVIATIONS

17 $\alpha$ -E2	17 $\alpha$ -estradiol
17 $\beta$ -E2	17 $\beta$ -estradiol
AF-1	activation function 1
AF-2	activation function 2
BIM	bisindolylmaleimide
cAMP	cyclic AMP
cGMP	cyclic GMP
CFTR	cystic fibrosis transmembrane conductance regulator
DAG	diacylglycerol
ER $\alpha$	estrogen receptor $\alpha$
ER $\beta$	estrogen receptor $\beta$
Ins(1,4,5)P <sub>3</sub>	inositol trisphosphate
Ins(3,4,5,6)P <sub>4</sub>	inositol tetrakisphosphate
I <sub>sc</sub>	short-circuit current
MAP kinase	mitogen-activated protein kinase
MEK	MAP kinase kinase
NBCS	new born calf serum
PIP <sub>2</sub>	phosphatidylinositol bisphosphate
PKA	protein kinase A
PKC	protein kinase C
R <sub>te</sub>	transepithelial electrical resistance

SERM

selective estrogen receptor modulator

## CHAPTER 1

### INTRODUCTION

#### Steroid Hormone Action

Steroid hormones are lipophilic, low-molecular weight compounds synthesized from cholesterol. These hormones mediate a wide variety of vital physiological functions ranging from acting as anti-inflammatory agents to regulating events during pregnancy. Steroid hormones are synthesized mainly by endocrine glands such as the gonads (testis and ovary), the adrenals, and (during gestation) by the fetoplacental unit and are then released into the blood circulation. They can be grouped into one of three classes: the sex steroids, the adrenal steroids, and vitamin D<sub>3</sub> (Evans 1988). The sex steroids (estrogens, progesterone, and testosterone), influence the sexual differentiation of the genitalia, determine secondary sexual characteristics during development and sexual maturation, contribute to the maintenance of their functional state in adulthood, and control or modulate sexual behaviour. The principal adrenal steroids are the mineralocorticoids and glucocorticoids. The important mineralocorticoid, aldosterone, participates in potassium excretion and sodium reabsorption. Cortisol, the major adrenal glucocorticoid in humans, stimulates conversion of proteins to carbohydrates, raises blood sugar levels, and promotes glycogen storage in the liver. The adrenal cortex secretes androgenic hormones androstenedione, testosterone, and dehydroepiandrosterone. The adrenal androgen dehydroepiandrosterone is the primary source of androgen in women. Vitamin D<sub>3</sub> plays an important role in calcium and phosphate regulation.

The classic pathway for steroid hormone action involves binding of hormone to specific intracellular receptors. The receptor-steroid complex interacts with hormone-responsive elements

within target genes, and initiates new transcription and protein synthesis. The resulting new proteins influence various cellular functions. Genomic steroid effects are characterized by delayed onset of action (hours) and sensitivity to blockers of transcription (e.g. actinomycin D) and protein synthesis (e.g. cycloheximide). Over the past decade, there has been increasing evidence of rapid (seconds to minutes) nongenomic effects of steroids, which are initiated at the plasma membrane and result in the activation of intracellular second messenger pathways (Schmidt and others 2000; Harvey and others 2001). Examples of nongenomic effects of steroids include rapid aldosterone effects on lymphocytes, renal cells and vascular smooth muscle cells (Koppel and others 2003; Boldyreff and Wehling 2004; Uhrenholt and others 2004); vitamin D<sub>3</sub> effects on osteoblasts (Erben and others 2002); progesterone action on primate coronary arteries (Minshall and others 2002); and estrogen action on neurons (Abraham and others 2004), and vascular smooth muscle cells (Mendelsohn 2002a). The rapid nongenomic steroid signaling is characterized by several of the following criteria: 1) responses occur within seconds to minutes, actions too rapid to be compatible with RNA and protein synthesis; 2) responses refractory to inhibitors of transcription and translation; and 3) responses can be initiated by steroids conjugated to macromolecules (e.g. bovine serum albumin) that are too large to enter cells.

### Estrogens and Estrogen Receptors

Estrogens are sex steroids that exist mainly in three molecular forms: estradiol, estrone, and estriol. In premenopausal women, most of the circulating estrone is derived from estradiol. In postmenopausal women estrone is the predominant plasma estrogen and is derived from the conversion of androcortical androstenedione in the liver. Estriol is formed in the liver as a conversion product of estradiol and estrone. The biological potency of estradiol is much higher



than the other molecular forms, and hence estradiol is physiologically the most important estrogen.

For many years it was thought that only a single form of the nuclear estrogen receptor, ER $\alpha$ , existed (Greene and others 1984). More recently, multiple laboratories independently reported discovery of a second estrogen receptor (ER $\beta$ ) in rat (Kuiper and others 1996), mouse (Tremblay and others 1997), and human (Mosselman and others 1996). Of the classical estrogen receptors (ER $\alpha$  and ER $\beta$ ), ER $\alpha$  is the predominant receptor in most target organs (Lubahn and others 1993). These two receptors are not isoforms of a single receptor gene but reside on different chromosomes (ER $\beta$  is localized on human chromosome 14, and ER $\alpha$  is localized on chromosome 6) and are distinct proteins (Enmark and others 1997).

The ER $\alpha$  and ER $\beta$  receptors belong to the steroid/ thyroid nuclear receptor superfamily (Figure 1), members of which share a common structure characterized by the N-terminal A/B domain, the DNA-binding C domain and the ligand-binding D/E/F domain (Tsai and O'Malley 1994; Mangelsdorf and others 1995; Robinson-Rechavi and others 2003). In the absence of ligand, estrogen receptors are bound to several proteins in the nucleus, such as the heat shock proteins, to form a complex (Pratt and Toft 1997). After estrogen binding, a conformational change occurs in the receptor structure that triggers the dissociation of the heterocomplex, dimerization of liganded receptors, interaction between receptor and specific DNA sequences, recruitment of co-activators, or other transcription factors and formation of a preinitiation complex in target genes (Nilsson and others 2001).

The N-terminal domain of ER $\alpha$  and ER $\beta$  contains a ligand-independent transcriptional activation function, the AF-1 (Kraus and others 1995; McInerney and Katzenellenbogen 1996). This region is involved in protein-protein interaction and transcription activation (McInerney and

others 1996; Onate and others 1998). Using an estrogen response element based reporter construct, it is seen that the activity of AF-1 in ER $\alpha$  is many times greater than in ER $\beta$  (Cowley and Parker 1999).

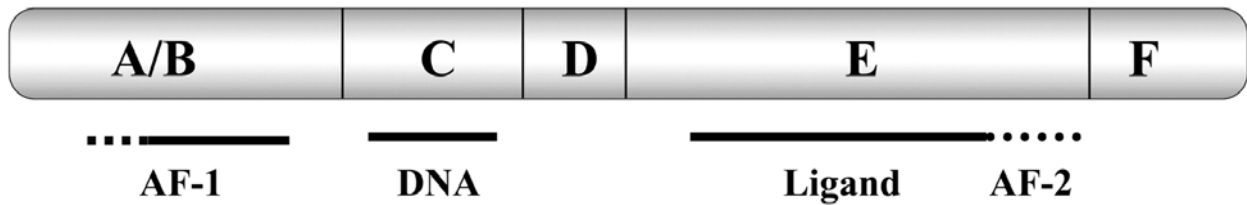


Figure 1 Diagrammatic Representation of the Domain Structure of Nuclear Receptors. The A/B domain at the N terminus contains the AF-1 site where other transcription factors interact. The C/D domain contains the two-zinc finger structure that binds to DNA, and the C/F domain contains the ligand binding pocket as well as the AF-2 domain (Nilsson and others 2001).

The DNA-binding domain is highly conserved in the steroid/thyroid nuclear receptor superfamily containing two zinc fingers that are important for receptor dimerization and DNA binding (Franco and others 2003; Meza and others 2003). The DNA binding domains of ER $\alpha$  and ER $\beta$  are highly homologous. The P box, a sequence that is critical for target-DNA recognition and specificity, is identical in both receptors (Vanacker and others 1999). Thus ER $\alpha$  and ER $\beta$  bind to various estrogen response elements with similar specificity and affinity. The D region of the ligand-binding domain serves as a linker between the DNA-binding domain and the ligand-binding domain. The E/F region of the ligand-binding domain participates in ligand binding, nuclear localization, receptor dimerization, and transcriptional regulation (Tsai and O'Malley 1994; Horwitz and others 1996). ER $\alpha$  and ER $\beta$  have similar binding affinities for 17  $\beta$ -estradiol, with K<sub>d</sub> values of 0.2 and 0.6 nM, respectively (Kuiper and others 1996). The ligand-

binding domain also contains a second transcriptional activation function, AF-2 that differs from AF-1 in that AF-2 activity is ligand-dependent (Webster and others 1988).

There is striking difference between the two receptors in their response to selective estrogen receptor modulators (SERMs) like tamoxifen, raloxifene, and pure antiestrogens like ICI 164384 (Lonard and Smith 2002). One mechanism by which these compounds inhibit estrogen receptor action is by competition with 17  $\beta$ -estradiol for binding to the estrogen receptor. It is thought that SERMs and antiestrogens promote a conformational change different from that induced by 17  $\beta$ -estradiol (Beekman and others 1993; McDonnell 1999). SERMs, like tamoxifen, have agonistic activity in certain cells, such as chicken embryo fibroblasts (Berry and others 1990) and MDA-231 human breast cancer cells (Montano and others 1995). Tamoxifen can show some agonistic activity with ER $\alpha$  but exhibits no agonistic activity with ER $\beta$  (McInerney and others 1998). The cell and promoter dependence of tamoxifen agonism has been attributed to the cell and promoter specificity of AF-1 activity (Berry and others 1990; Metzger and others 1992; Tzukerman and others 1994). The activity of AF-1 is many times more in ER $\alpha$  than in ER $\beta$  (Cowley and Parker 1999). This explains the agonistic activity of tamoxifen in cells containing pure ER $\alpha$ . Pure antagonists like ICI 164384 act by promoting rapid degradation of estrogen receptors (Dauvois and others 1992).

### Nongenomic Action of Estrogens

Many tissues traditionally considered ‘non-targets’ for classical estrogen action are now known to be regulated by nongenomic mechanisms. In the cardiovascular and central nervous system, estrogen induces rapid vasodilatation (Mendelsohn 2002a; Mendelsohn 2002b) and neuronal excitability in the cerebellum, cerebral cortex, and the pyramidal neurons of the

hippocampus (McEwen and Alves 1999). Increases in cAMP in response to estrogen in vascular smooth muscle cells (Farhat and others 1996a; Farhat and others 1996b), breast cancer cells, and uterine cells (Aronica and others 1994) are caused by a nongenomic mechanism. In primary cultured rabbit kidney proximal tubule cells 17  $\beta$ -estradiol-BSA conjugate rapidly stimulates  $\text{Ca}^{2+}$  uptake, which is mediated by cAMP and PKC pathways (Han and others 2000). 17  $\beta$ -estradiol can also rapidly activate extracellular-regulated kinases (Erk1/Erk2) in breast cancer cells (Improta-Brears and others 1999; Fernando and Wimalasena 2004). Recent reviews have focused on the nongenomic actions of estrogen (Coleman and Smith 2001; Simoncini and Genazzani 2003).

Nongenomic actions of estrogens are often associated with cell membrane receptors such as G protein coupled receptors in osteoblasts (Le Mellay and others 1997) and cell membrane ion channels in vascular smooth muscle cells (White and others 1995). Many of the membrane-delimited estrogen responses are mediated by the activation of phospholipase C/adenylyl cyclase pathways via the modulation of G protein-coupled receptors (Kelly and others 1999). There is increasing evidence that rapid effects of 17  $\beta$ -estradiol occurs after the sex steroid binds to a plasma membrane estrogen receptor. Plasma membrane receptors have been demonstrated by immunohistochemistry (Russell and others 2000) and immunoblotting (Chambliss and others 2000) of isolated plasma membrane domains. Membrane estrogen receptors that are structurally related or identical to the classical estrogen receptors have been localized in several cell types such as pituitary tumor cells (Monje and Boland 1999; Watson and others 1999) and Chinese hamster ovary cells (Razandi and others 1999). The membrane and classical estrogen receptors originate from a single transcript and have near-identical affinities for 17  $\beta$ -estradiol (Razandi and others 1999). Membrane estrogen receptors have been detected in GH3/B6 rat pituitary

tumor cells by directing antibodies against epitopes of the classical estrogen receptors (Pappas and others 1995). In the distal colonic crypts of female rats and female human, membrane-associated ER $\alpha$  and ER $\beta$  was observed (Doolan and others 2000b). In many cell types membrane estrogen receptors can activate the  $\alpha$  subunit of GTP-binding proteins leading to downstream signal transduction (Levin 1999).

The rapid effects of estrogen in many cells have been attributed to a population of membrane estrogen receptors (Figure 2) embedded in caveolar rafts and other plasma membrane domains (Kim and others 1999; Chambliss and others 2000; Razandi and others 2002). Caveolae are small (50-100 nm diameter) flask-shaped subcompartments of the plasma membranes. Caveolae are structurally composed of a family of proteins, known as caveolins. Caveolin 1 and 2 are found in many cells expressing caveolae (Anderson 1998). Caveolin 1 contains a cytosolic, N-terminal scaffolding domain that binds to signaling molecules and inhibits their usual activation after growth factor ligation of receptors (Okamoto and others 1998). Within the caveolae, an estrogen receptor interaction with other signaling molecules may be important for the propagation of signal transduction. There is also evidence of a plasma membrane protein called the 'steroid hormone recognition and effector complex' that actively mediates rapid, nongenomic responses to steroids (Daufeldt and others 2003).

#### Rapid Effects of Aldosterone and Estrogen on Colonic Epithelium

A major target for aldosterone action is the mammalian distal colon. The Na<sup>+</sup>/H<sup>+</sup> exchanger in the basolateral cell membrane of the colonic epithelia is the primary ion transport target of the nongenomic actions of aldosterone (Harvey and others 2001). In the human and rat distal colon, recent studies have demonstrated rapid nongenomic activation of Na<sup>+</sup>/H<sup>+</sup> exchange,

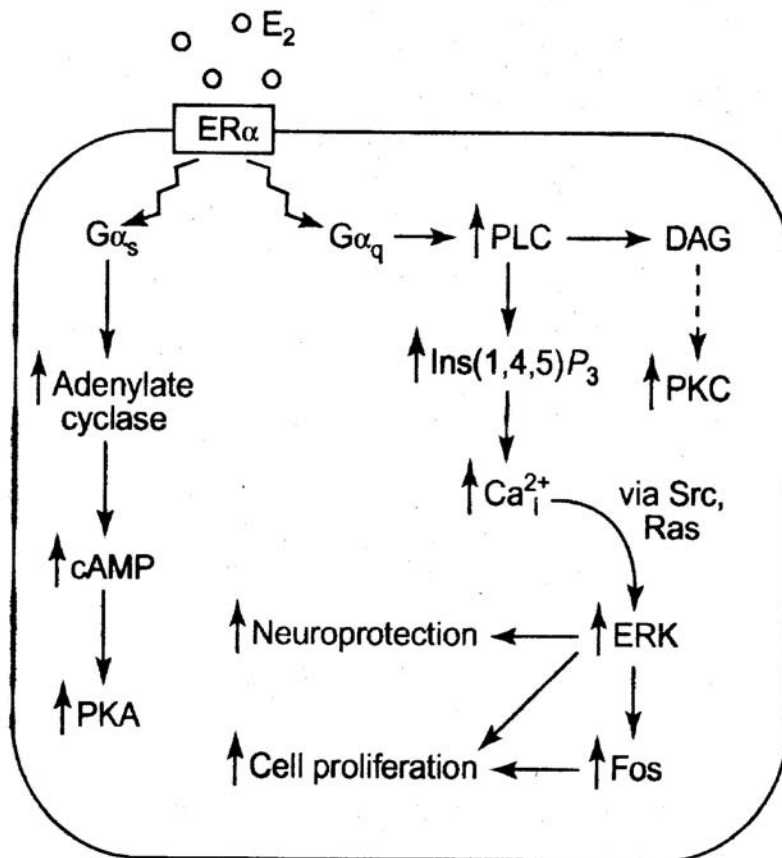


Figure 2 Schemes of Signal Transduction Resulting from Liganding of the Membrane Estrogen Receptor by Estrogen. The membrane ER activates the  $\alpha$  subunit of several GTP-binding proteins and this leads to downstream signal transduction, as shown. Membrane ER can also modulate JNK (not shown). The dashed line indicates that DAG indirectly leads to PKC activation. Abbreviations:  $Ca_i^{2+}$ , intracellular  $Ca^{2+}$ ; DAG, diacylglycerol;  $E_2$ , estradiol; ER, estrogen receptor; ERK, extracellular-signal-regulated protein kinase;  $G_{\alpha_s}/G_{\alpha_q}$ , G proteins;  $Ins(1,4,5)P_3$ , inositol (1,4,5) trisphosphate; JNK, Jun kinase; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C (Levin 1999).

K<sup>+</sup> recycling, and PKC activity by aldosterone (Doolan and Harvey 1996; Doolan and others 1998; Maguire and others 1999). The physiological function of nongenomic effects of aldosterone is to shift the balance from net secretion to net absorption in colonic epithelia (Harvey and others 2001; Harvey and others 2002).

The presence of estrogen receptors in the colon suggests that the colon is also a target for estrogen action (Thomas and others 1993). By examining the estrous-cycle-related changes in the epithelium, significant variations in both the distribution of epithelial cells through the cell cycle as well as the number of epithelial cells per unit area in mouse jejunal and colonic epithelium has been found (Cheng and Bjerknes 1988). A role of estrogens in stimulating the proliferation of colonic epithelial cells has been postulated (Cheng and Bjerknes 1988). It has also been reported that under selected conditions, growth of colonic cell line, Caco-2, is triggered by 17  $\beta$ -estradiol (Di Domenico and others 1996). Cell growth is estradiol concentration dependent and prevented by tamoxifen and the pure antagonist, ICI 182780 (Di Domenico and others 1996).

There is increasing evidence of nongenomic effects of estrogen on the gastrointestinal system. Forskolin stimulates Cl<sup>-</sup> secretion in rat distal colon by a cAMP dependent activation of apical CFTR Cl<sup>-</sup> channels (Schultheiss and others 1998). A physiological concentration of 17  $\beta$ -estradiol rapidly reduces the forskolin (20  $\mu$ M) stimulation of Cl<sup>-</sup> secretion (Condliffe and others 2001) in the distal colon of female rats. This effect can be seen after preincubating distal colonic epithelial sheets with 17  $\beta$ -estradiol for only 10 minutes, and the inhibition of Cl<sup>-</sup> secretion is not blunted by tamoxifen (Condliffe and others 2001). The presence of 17  $\alpha$ -estradiol (100 nM), a stereoisomer that does not activate nuclear estrogen receptors, does not cause inhibition of forskolin-stimulated Cl<sup>-</sup> secretion (Condliffe and others 2001). The intracellular Ca<sup>2+</sup> chelator

BAPTA (50  $\mu\text{M}$ ) or the PKC inhibitor chelerythrine chloride (1  $\mu\text{M}$ ) abolishes the antisecretory rapid effects of 17  $\beta$ -estradiol on forskolin-stimulated  $\text{Cl}^-$  secretion (Condliffe and others 2001). Additionally, in the rat distal colonic epithelium, a brief incubation of distal colonic epithelial sheets with 10-100 nM concentration of 17  $\beta$ -estradiol also significantly reduces the  $\text{Cl}^-$  secretory responses to  $\text{Ca}^{2+}$  dependent secretagogue carbachol (10  $\mu\text{M}$ ) (Condliffe and others 2001).

17  $\beta$ -estradiol rapidly increases the activity of cAMP dependent protein kinase (PKA), stimulates a rise in intracellular  $\text{Ca}^{2+}$  (Doolan and others 2000a), activates protein kinase C (Doolan and Harvey 1996; Doolan and others 2000a; Harvey and others 2002), increases intracellular alkalinization, and decreases  $\text{K}^+$  channel activity in colonic epithelium ( McNamara and others 2000; Condliffe and others 2001). All these rapid signaling responses seen in native colonic epithelial sheets are sex-steroid specific and female gender specific and observed in the low nanomolar (nM) concentrations of 17  $\beta$ -estradiol (Harvey and others 2002).

In the colonic epithelial cell line T84, micromolar ( $\mu\text{M}$ ) concentrations of 17  $\beta$ -estradiol caused a rapid and reversible inhibition of forskolin-stimulated  $\text{Cl}^-$  secretion (Singh and others 2000). Singh and others (2000) also reported that 17  $\alpha$ -estradiol, the stereoisomer that failed to bind and activate nuclear estrogen receptors was equipotent with 17  $\beta$ -estradiol, suggesting a nongenomic mechanism of steroid action. Tamoxifen and synthetic estrogens including diethylstilbesterol also inhibited forskolin-stimulated  $\text{Cl}^-$  secretion. Diethylstilbesterol and other synthetic estrogens inhibited CFTR chloride channel activity in excised membrane patches. Additionally, experiments with radioactive photoactivatable estrogen derivatives demonstrated that these compounds bind directly to CFTR expressed in Sf9 cells by gene transfection. Singh and others (2000) concluded that estrogens can interact directly with CFTR by a nongenomic mechanism to inhibit channel activity and anion transport.



## Chloride Secretion in the Colon

The main functions of the mammalian intestine are nutrient absorption, and regulation of water and electrolyte balance. The villous and surface epithelial cells are responsible for absorption, whereas the intestinal crypts are the main location of secretory epithelial cells (Welsh and others 1982). In the healthy state, the mammalian colon is primarily an absorptive organ with water, sodium, and chloride being absorbed while potassium and bicarbonate are secreted (Liedtke 1989). However, in the case of secretory diarrhea, the colon is altered from an absorptive to a secretory state.

The mechanism for epithelial  $\text{Cl}^-$  secretion occurs was initially proposed in the late seventies (Frizzell and others 1979). For  $\text{Cl}^-$  secretion to occur, a favorable electrochemical gradient must exist (Figure 3).  $\text{Cl}^-$  is taken up from the bloodstream across the basolateral membrane of epithelial cells via bumetanide-sensitive  $\text{Na}^+/\text{2Cl}^-/\text{K}^+$  co-transporter. This co-transporter is driven by low intracellular  $\text{Na}^+$  concentration established by the active  $\text{Na}^+/\text{K}^+$  ATPase localized at the basolateral membrane. This allows  $\text{Cl}^-$  to accumulate in the cell cytosol above its electrochemical equilibrium. When apical chloride channels are opened,  $\text{Cl}^-$  flows out of the cell down this electrochemical gradient, resulting in overall net transepithelial transfer of the anion.  $\text{Cl}^-$  exit occurs predominantly through the apical cAMP activated CFTR (cystic fibrosis transmembrane conductance regulator)  $\text{Cl}^-$  channels. The presence of basolateral  $\text{K}^+$  channels serve to recycle co-transported  $\text{K}^+$  back across the basolateral membrane, thus preventing cell depolarization (Barrett 2000). Electroneutral  $\text{NaCl}$  absorption (parallel  $\text{Na}^+/\text{H}^+$  and  $\text{Cl}^-/\text{HCO}_3^-$  exchange) dominates in the surface epithelium and is also present in the crypts. Electrogenic  $\text{Na}^+$  absorption via the epithelial  $\text{Na}^+$  channel (ENaC) takes place in the surface epithelium and upper crypts of the distal colon. The cystic fibrosis transmembrane conductance

regulator (CFTR) is expressed throughout the colonic epithelium and dominates in the crypts.  $\text{Cl}^-$  secretion is activated by stimulation of CFTR (Kunzelmann and Mall 2002).

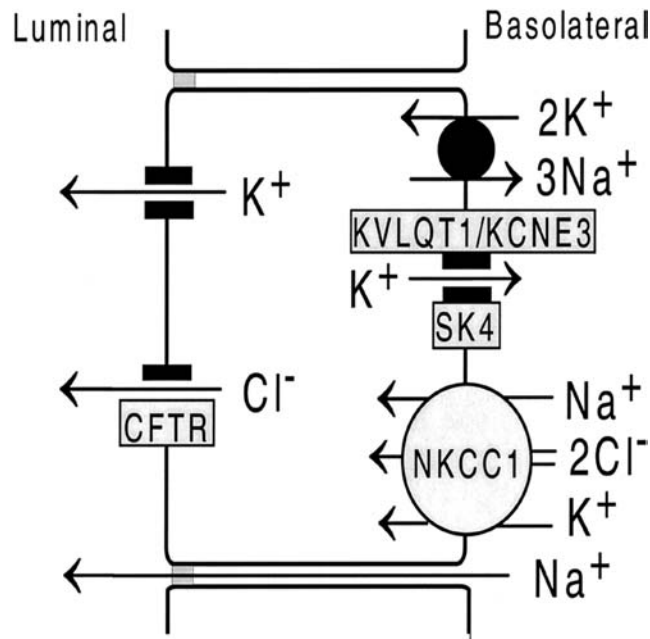


Figure 3 Cellular Model for  $\text{Cl}^-$  Secretion.  $\text{Cl}^-$  secretion is activated by cAMP-dependent stimulation of CFTR  $\text{Cl}^-$  channels, which are the predominant apical  $\text{Cl}^-$  channels in the native colon.  $\text{Cl}^-$  secretion is paralleled by secretion of  $\text{K}^+$  via apical  $\text{K}^+$  channels and  $\text{Na}^+$  transport through the paracellular shunt.  $\text{Cl}^-$  is taken up into the cells by the basolateral  $\text{Na}^+/2\text{Cl}^-/\text{K}^+$ . The rate of secretion is controlled by the activity of  $\text{Na}^+/2\text{Cl}^-/\text{K}^+$ , which is regulated through the intracellular  $\text{Cl}^-$  concentration, cell swelling, and probably phosphorylation.  $\text{Cl}^-$  transport is maintained by basolateral cAMP-activated KVLQT1/KCNE3 and  $\text{Ca}^{2+}$ -activated SK4  $\text{K}^+$  channels (Kunzelmann and Mall 2002).

The human epithelial cell line T84 is a well-established model for epithelial chloride transport (Dharmasathaphorn and others 1984). These cells can be grown on semipermeable supports to form polarized monolayers that develop high levels of transepithelial resistance similar to native colonic epithelium. T84 monolayers grown on semipermeable supports can be

mounted in Ussing chambers (named after Hans Ussing, and initially designed to study vectorial ion transport through the frog skin (Ussing and Zerahn 1951)) and used for electrophysiological studies. T84 monolayers mounted in Ussing chambers develop a spontaneous potential difference and current that can be short-circuited (Dharmasathaphorn and others 1985). The short-circuit current ( $I_{SC}$ ) is defined as the current that must be passed across the epithelial monolayer to reduce the transepithelial voltage to 0 mV.

At the apical side of the T84 cell, chloride conductance is dominated by CFTR, the cAMP-activated chloride channel. The transport systems localized in the basolateral membrane are: 1) a ouabain-inhibitable  $\text{Na}^+/\text{K}^+$  ATPase, which catalyzes the ATP-driven uptake of  $2\text{K}^+$  and efflux of  $3\text{Na}^+$  2) a bumetanide-inhibitable  $\text{Na}^+/\text{2Cl}^-/\text{K}^+$  co-transporter driven by low intracellular  $\text{Na}^+$  concentration established by  $\text{Na}^+/\text{K}^+$  ATPase, allowing chloride to accumulate in the cell cytosol above its electrochemical equilibrium 3) a cAMP-stimulated,  $\text{Ba}^{2+}$ -inhibitable  $\text{K}^+$  channel, and 4) a  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channel.

The epithelial cell line T84 secretes chloride ions in response to both cAMP- and  $\text{Ca}^{2+}$ -regulated pathways (Barrett 1993; MacVinish and others 1993). Forskolin stimulates  $\text{Cl}^-$  secretion in T84 cells by elevating intracellular cAMP, which leads to activation of protein kinase A (Riordan 1993). This causes the dissociation of protein kinase A catalytic subunit from its regulatory subunit. The catalytic subunit of protein kinase A phosphorylates the apical CFTR  $\text{Cl}^-$  channels, activating them, leading to  $\text{Cl}^-$  secretion. The  $\text{Ca}^{2+}$  ATPase inhibitor thapsigargin and the cholinergic agonist carbachol stimulate  $\text{Cl}^-$  secretion in T84 cells by raising intracellular calcium. Thapsigargin raises intracellular calcium by inhibiting  $\text{Ca}^{2+}$  ATPase of the endoplasmic reticulum. Carbachol binds to a basolateral G protein-linked muscarinic receptor and thereby activates phospholipase C. Phospholipase C acts on phosphatidylinositol bisphosphate ( $\text{PIP}_2$ ) to

yield calcium-mobilizing messenger inositol trisphosphate [Ins(1,4,5)P<sub>3</sub>] and diacylglycerol (DAG). Elevation in intracellular calcium opens basolateral K<sup>+</sup> channels providing the driving force for Cl<sup>-</sup> exit through the small population of constitutively active apical CFTR Cl<sup>-</sup> channels. Cyclic nucleotides (cAMP or cGMP) evoke a sustained increase in Cl<sup>-</sup> secretion, whereas the calcium-dependent Cl<sup>-</sup> secretion is considerably more transient due to negative regulation exerted by mediators like epidermal growth factor, protein kinase C and inositol tetrakisphosphate [Ins(3,4,5,6)P<sub>4</sub>] (Barrett 1993; Barrett 1997).

### Cystic Fibrosis Transmembrane Conductance Regulator (CFTR)

CFTR is a low-conductance chloride channel that is activated by phosphorylation and gated by ATP binding and hydrolysis. The structure of CFTR resembles other ATP-binding cassette (ABC) transporters. They have two membrane domains, each consisting of six transmembrane segments and two nucleotide-binding domains. CFTR activity is controlled by a central regulatory domain of approximately 200 amino acids (Gadsby and Nairn 1999; Schultz and others 1999). The regulatory domain contains multiple consensus phosphorylation sites for cAMP-dependent protein kinase (PKA), protein kinase C (PKC), and Type II cGMP-dependent protein kinase (Riordan and others 1989; Riordan 1993; Gadsby and Nairn 1999; Sheppard and Welsh 1999). The more extensive the phosphorylation, the greater the channel open probability of CFTR (Hwang and others 1993; Gadsby and Nairn 1999). There appears to be extensive redundancy in the regulatory PKA phosphorylation sites. Five sites are phosphorylated *in vivo*, but removal of these sites or all 10 dibasic PKA sites reduced but did not eliminate PKA activation (Chang and others 1993). CFTR is still phosphorylated after removal of the 10 dibasic sites indicating that phosphorylation at non-dibasic PKA sites is sufficient for channel activation

(Riordan 1993; Gadsby and Nairn 1999; Sheppard and Welsh 1999). PKC phosphorylation may be required for activation by PKA (Jia and others 1997). Following phosphorylation, opening and closing of the channel is controlled by ATP binding and hydrolysis and CFTR is deactivated by protein phosphatases (Riordan 1993; Gadsby and Nairn 1999; Sheppard and Welsh 1999).

Recently, the crystallisation of wild-type human CFTR has been accomplished (Rosenberg and others 2004). Rosenberg and others (2004) have reported the formation of two-dimensional crystalline arrays of CFTR in the presence of the high affinity ligand, AMP-PMP. Electron crystallography, yielding 3D structural information to a resolution of  $\sim 2$  nm indicated that the crystallized CFTR is monomeric, has overall structural similarities to P-glycoprotein, and can assume two different conformational states, possibly reflecting differential nucleotide binding to the two nucleotide binding domains (Rosenberg and others 2004).

#### Rationale for this Investigation

It has been suggested that 17  $\beta$ -estradiol may alter colonic ion transport during high estrogen states like pregnancy and certain phases of menstrual cycle (Condliffe and others 2001). These effects might involve rapid, nongenomic actions of 17  $\beta$ -estradiol. Most studies involving rapid effects of estrogens on epithelial  $\text{Cl}^-$  secretion have been done on native colonic epithelial sheets. Studies of electrogenic  $\text{Cl}^-$  secretion across sheets of native colonic epithelia cannot separate direct effects of agonists on colonic epithelial cells from indirect effects involving enteric nerves or the release of other factors from cells within the lamina propria. To eliminate the problems associated with native colonic epithelial sheets, cell lines derived from human colonic carcinomas (T84, HT-29, and Caco-2) have been used. These cell lines grow as confluent monolayers, retain cellular polarity, form intercellular tight junctions, exhibit ion

transport, respond to hormones and neurotransmitters, and undergo morphologic and functional transformation in response to changes in their external environment. The T84 cell line is a convenient model to study colonic  $\text{Cl}^-$  secretion and crypt cell function.

I have investigated the nongenomic effects of 17  $\beta$ -estradiol on epithelial  $\text{Cl}^-$  secretion using the T84 cell line. Condliffe and others (2001) have reported that a physiological concentration of 17  $\beta$ -estradiol rapidly reduced the forskolin stimulation of  $\text{Cl}^-$  secretion in the distal colon of female rats. Additionally, in the rat distal colonic epithelium, a brief incubation of distal colonic epithelial sheets with 10-100 nM concentration of 17  $\beta$ -estradiol significantly reduced the  $\text{Cl}^-$  secretory responses to  $\text{Ca}^{2+}$  dependent secretagogue carbachol (Condliffe and others 2001). I used T84 monolayers to confirm these findings and develop a tissue culture model to study the rapid effects of 17  $\beta$ -estradiol on epithelial  $\text{Cl}^-$  secretion.

Initial experiments were developed to confirm the observations of Singh and others (2000) who have used T84 monolayers to study epithelial  $\text{Cl}^-$  secretion. Singh and others (2000) have reported that 17  $\beta$ -estradiol caused a rapid and reversible inhibition of forskolin-stimulated  $\text{Cl}^-$  secretion across T84 monolayers with a  $K_i$  of 8  $\mu\text{M}$ . 17  $\alpha$ -estradiol was equipotent with 17  $\beta$ -estradiol, arguing against a genomic-mediated mechanism of action. Their data suggested that estrogens can interact directly with CFTR to alter anion transport. My initial experiments confirmed the observations of Singh and others (2000). I found that 17  $\beta$ -estradiol inhibited forskolin-stimulated  $\text{Cl}^-$  within seconds and in a dose-dependent manner. The inhibitory effect of 17  $\beta$ -estradiol on  $\text{Cl}^-$  secretion was greater when applied to the apical side of the monolayers consistent with 17  $\beta$ -estradiol and other steroids acting as blockers of the CFTR  $\text{Cl}^-$  channels, as reported earlier (Singh and others 2000).

I have used thapsigargin and carbachol to stimulate  $\text{Cl}^-$  secretion by raising intracellular calcium. Contrary to the observations of Condliffe and others (2001), my studies have revealed that  $17\ \beta$ -estradiol potentiates  $\text{Ca}^{2+}$  dependent  $\text{Cl}^-$  secretion. Supraphysiological concentrations of  $17\ \beta$ -estradiol were required to elicit rapid responses in T84 monolayers. I have found that  $17\ \beta$ -estradiol has two contrasting effects on epithelial  $\text{Cl}^-$  secretion.  $17\ \beta$ -estradiol inhibits cAMP-stimulated chloride secretion, but potentiates calcium-stimulated chloride secretion. The inhibitory effects on cAMP-stimulated chloride secretion are consistent with steroids acting as a CFTR channel blocker (Singh and others 2000). With respect to the potentiation of calcium-stimulated chloride secretion,  $17\ \beta$ -estradiol might enhance the calcium sensitivity of CFTR activation. The mechanism of potentiation of calcium-stimulated chloride secretion has been delineated in this investigation.

A better knowledge of the nongenomic actions of estrogens on colonic epithelia will help understand epithelial ion transport and open new perspectives in the pharmacological treatment of pathological conditions like cystic fibrosis and secretory diarrhea.

## CHAPTER 2

### MATERIALS AND METHODS

#### Cell Culture

The human colonic cell line T84 was used for this investigation. Cultures were initiated from T84 stocks maintained in liquid nitrogen. Unless indicated otherwise, T84 cells were grown to confluence in T-75 culture flasks in Dulbecco's modified Eagle's medium and Ham's F-12 (1:1) (DME/F12, Sigma, St. Louis, MO) supplemented with 15 mM HEPES, 2 mM glutamine, 5% new born calf serum (NBCS, Sigma, St. Louis, MO), 100 U/ml penicillin, and 100 µg/ml streptomycin. Cultures were maintained at 37°C in an atmosphere of 5% CO<sub>2</sub>-95% air. The confluent T84 cultures were trypsinized and subcultured (1:3) at weekly intervals. Cells from passages 35-47 were used in experiments. Cultures were fed fresh medium every other day and day before use.

#### Electrophysiology

For electrophysiological studies,  $5 \times 10^5$  cells were seeded in Millicell-HA or Millicell-PCF (Millipore, Bedford, MA) culture plate inserts (12 mm diameter; 0.6 cm<sup>2</sup> surface area). Epithelial monolayer development was monitored daily by measuring transepithelial electrical resistance ( $R_{te}$ ) with an epithelial volt-ohm meter (EVOM) apparatus (World Precision Instruments). Maximal  $R_{te}$  (1500-3500 Ω.cm<sup>2</sup>) developed between days 6 and 12 of culture. To maintain sterility, EVOM electrodes were soaked in 70% ethanol for a few minutes then rinsed in sterile phosphate-buffered saline (PBS) before use.



Short-circuit current ( $I_{SC}$ ) is the current that must be passed across the epithelial monolayer to reduce the transepithelial voltage to 0 mV. For  $I_{SC}$  measurements (except experiments performed to study basolateral membrane  $K^+$  channel activity), Millicells were mounted in water-jacketed Ussing chambers and bathed in bicarbonate-buffered Ringer solution (composition in mM: 125 NaCl, 5 KCl, 1.5  $CaCl_2$ , 1.5  $MgCl_2$ , 25  $NaHCO_3$ , 5 glucose, and 0.0005% phenol red) with continuous bubbling of 95%  $O_2$  -5%  $CO_2$ . The potential difference across the epithelial monolayer was measured with calomel electrodes in 3M KCl and monitored with a current-voltage clamp (Physiologic Instruments, San Diego). Monolayer potential was clamped to 0 mV with Ag/AgCl<sub>2</sub> electrodes connected to the Ussing chambers by agar bridges. At 20 or 30 sec intervals, a biphasic pulse of 5 mV was applied and the resulting current deflection used to calculate monolayer conductance (G) by Ohm's law. Output from the current-voltage clamp was recorded on a Pentium-based microcomputer equipped with a DATAQ data acquisition board (DATAQ Instruments Inc., Akron OH) running Acquire and Analyze software (Physiologic Instruments).

To stimulate cAMP dependent  $Cl^-$  secretion, 10  $\mu$ M forskolin was added to the basolateral hemichamber. Forskolin causes  $I_{SC}$  to rise sharply and plateau at a stable  $I_{SC}$  within 15 minutes.  $I_{SC}$  is fully reflective of  $Cl^-$  secretory activity (McRoberts and Barrett 1989). 17  $\beta$ -estradiol or other steroids were added from 1000X ethanol stock solutions to both the apical and basolateral hemichambers (unless noted otherwise), and the effects of steroids on forskolin-stimulated  $I_{SC}$  was noted. To study the rapid effects of steroids on  $Ca^{2+}$ -dependent  $Cl^-$  secretion, 17  $\beta$ -estradiol and other steroids were added from 1000X ethanol stock solutions to both the apical and basolateral hemichambers, unless noted otherwise. To stimulate  $Ca^{2+}$ -dependent  $Cl^-$  secretion, 100  $\mu$ M carbachol (100X in Ringer) was added to the basolateral hemichamber or 1

$\mu\text{M}$  thapsigargin (1000X in DMSO) was added to the apical hemichamber. These secretagogues cause a rise in cytosolic calcium and produce a transient increase in  $I_{\text{SC}}$  that peaks in 1 and 10 minutes respectively. Effects of steroids and other compounds on  $\text{Cl}^-$  secretion were determined by comparing effects on peak  $I_{\text{SC}}$  values after carbachol or thapsigargin addition. A vehicle control (0.1% ethanol) was included in all experiments.

Basolateral membrane  $\text{K}^+$  channel activity in T84 monolayers was determined in nystatin-permeabilized monolayers by methods similar to those described previously (Ecay and Valentich 1993). Monolayers were mounted in Ussing chambers, bathed in HEPES-buffered Ringer (composition in mM: 125 NaCl, 5 KCl, 1.5  $\text{CaCl}_2$ , 1.5  $\text{MgCl}_2$ , 10 HEPES, and 5 glucose) and voltage clamped. Standard HEPES-buffered Ringer solution was replaced with HEPES-buffered Ringer solutions formulated to produce a 140:5 mM apical to basolateral  $\text{K}^+$  gradient (apical Ringer composition in mM: 140 Kgluconate, 10 NaGluconate, 1 CaGluconate, 1  $\text{MgSO}_4$ , 10 HEPES, and 10 glucose; basolateral Ringer composition in mM: 5 Kgluconate, 135 N-methyl-D-glucamine gluconate, 10 NaGluconate, 1 CaGluconate, 1  $\text{MgSO}_4$ , 10 HEPES, and 10 glucose). Ringer solution was continuously bubbled with 100 %  $\text{O}_2$ . The apical membrane was permeabilized with 350  $\mu\text{g}/\text{ml}$  nystatin (Sigma, St. Louis, MO), and gassing of the apical hemichamber was reduced to minimize frothing. The spontaneous sodium current produced by nystatin permeabilization was blocked by addition of 50  $\mu\text{M}$  ouabain (Calbiochem, LaJolla, OR) to the basolateral hemichamber. After a 5-minute incubation, 17  $\beta$ -estradiol was added to both the apical and basolateral hemichambers, and after 20 minutes 1  $\mu\text{M}$  thapsigargin was added to activate the basolateral  $\text{K}^+$  channels. The rapid rise in  $\text{K}^+$  current ( $I_{\text{K}}$ ) was recorded.

### Measurement of Intracellular Ca<sup>2+</sup> Concentration

For studies involving measurement of intracellular free Ca<sup>2+</sup> concentration, the T84 cells were grown to confluency on sterile 150 mm plastic tissue culture dishes. In initial experiments, trypsinizing cells immediately prior to assay resulted in fragile cells, a significant fraction of which died during fura 2-acetoxymethyl ester (fura-2) loading and fluorescence measurements. Cell viability was assayed by trypan-blue exclusion. To enhance cell viability, cultures were trypsinized the day before an experiment and maintained overnight as a suspension in Sigmacote treated 150 mm plastic tissue culture dishes. Under these conditions the cells attached poorly to plastic and could be easily detached and resuspended the following day. Mild trypsinization was sometimes required to bring cells back to suspension. Approximately 5 X 10<sup>6</sup> cells were used in each experiment. Cells were washed and resuspended in HEPES saline buffer (pH 7.45, composition in mM: 145 NaCl, 5 KCl, 1 MgSO<sub>4</sub>, 2 CaCl<sub>2</sub>, 10 HEPES and 10 Glucose). Cells were loaded with 3 μM fura-2 for 30 min at 22° C. Fura-2 used in these experiments was obtained from Molecular Probes (Eugene, OR). It has been shown that the organic anion transport inhibitor probenecid (2.5 mM) suppresses fura-2 extrusion from various cells (Di Virgilio and others 1988; McDonough and Button 1989). To prevent extrusion of fura-2 from T84 cells, 2.5 mM probenecid was added to the fura-2 loaded cell suspension and the cells further incubated for 10 min. Then these cells were washed twice with HEPES saline buffer and resuspended in HEPES saline buffer containing 2.5 mM probenecid. 2.5 ml of the fura-2 loaded resuspended cell suspension was loaded in a standard 1 cm<sup>2</sup> quartz cuvette, 17 β-estradiol (or vehicle control) added, and after 20 minutes the cells were stimulated with 2 μM thapsigargin. The cuvette temperature was maintained at 37°C with constant stirring throughout the experiment. Intracellular Ca<sup>2+</sup> was measured over a period of 15 minutes. Intracellular Ca<sup>2+</sup> was

determined by measuring fura fluorescence at 510 nm, using 340/380-nm dual wavelength excitation in a SPEX Fluoromax-3 Spectrofluorometer (Jobin Yvon-Spex, Edison, NJ).

Intracellular calcium  $[Ca^{2+}]_i$  was calculated with DataMax for Windows software (Jobin Yvon-Spex, Edison, NJ) using the equation (Grynkiewicz and others 1985),  $[Ca^{2+}]_i = K_d \times b \times (R - R_{min}) / (R_{max} - R)$ , where  $R$  is the measured fluorescence ratio. Calibration was performed at the end of each experiment by lysing the cells with addition of 25  $\mu$ M digitonin to determine  $R_{max}$  (the maximum 340/380 nm ratio of fura-2 fluorescence), and  $R_{min}$  (the minimum 340/380 nm fluorescence ratio) was obtained by addition of 10 mM EGTA (pH 7.4).  $K_d$  is the dissociation constant of the fura-2/ $Ca^{2+}$  complex (140 nM), and  $b$  is the ratio of fluorescence at 380 nm at 'zero' and saturating  $Ca^{2+}$  concentration.

### Statistical Analysis

The data shown are either original traces or mean values  $\pm$  standard error of the mean (SEM). Data analysis was done using Sigmaplot and SigmaStat software (SASS, Chicago, IL). The variance amongst each group was not always equal (equal variance test failed in many experiments), hence the ANOVA analysis is not an appropriate statistical test. Statistical significance ( $p < 0.05$ ) was determined by using the Student's t-test.

## CHAPTER 3

### RESULTS

#### 17 $\beta$ -estradiol and other Steroids Inhibit Forskolin-stimulated $\text{Cl}^-$ Secretion

T84 monolayer cultures were mounted in Ussing chambers and voltage clamped at 0 mV. Short-circuit current ( $I_{\text{SC}}$ ), which is fully reflective of  $\text{Cl}^-$  secretory activity (McRoberts and Barrett 1989) was stimulated by addition of 10  $\mu\text{M}$  forskolin to the basolateral hemichamber. Prior to stimulation, the cultures exhibited a low and stable basal current (1.5-3  $\mu\text{A}/\text{cm}^2$ ). Forskolin stimulation caused  $I_{\text{SC}}$  to rise sharply and plateau to a stable value that ranged from 30-40  $\mu\text{A}/\text{cm}^2$ . 17  $\beta$ -estradiol and other steroids were added from 1000X ethanol stock solutions to both the apical and basolateral hemichambers, and the effect of these steroids on forskolin-stimulated  $I_{\text{SC}}$  was noted.

Following forskolin stimulation, addition of 17  $\alpha$ -estradiol, 17  $\beta$ -estradiol, testosterone, progesterone, or the synthetic estrogen diethylstilbestrol to both hemichambers caused an immediate decline in  $I_{\text{SC}}$ . This inhibition of forskolin-stimulated  $\text{Cl}^-$  secretion was rapid (within seconds) and dose-dependent (Figures 4, 5 and Table 1). Diethylstilbestrol was the most potent inhibitor of  $\text{Cl}^-$  secretion tested, followed by progesterone, testosterone, and the naturally occurring estrogens 17  $\alpha$ - and 17  $\beta$ -estradiol (Figure 5, Table 1). 17  $\alpha$ -estradiol, a stereoisomer that does not activate nuclear estrogen receptors, had similar potency to 17  $\beta$ -estradiol (Figure 5, Table 1), confirming that the effect was nongenomic. The inhibitory effect of 17  $\beta$ -estradiol on  $\text{Cl}^-$  secretion was greater when applied to the apical side of the monolayers (Figure 4D) consistent with 17  $\beta$ -estradiol and other steroids acting as blockers of the CFTR  $\text{Cl}^-$  channels, as reported previously (Singh and others 2000).

Corticosterone, d-aldosterone, and the antiestrogen tamoxifen failed to inhibit forskolin-stimulated  $\text{Cl}^-$  secretion in T84 monolayers (Figure 4C, Table 1).

Figure 4 Nongenomic Effect of Steroids on Forskolin-stimulated  $\text{Cl}^-$  Secretion in T84 Monolayers. T84 monolayers were mounted in Ussing chambers, voltage clamped and  $\text{Cl}^-$  secretion stimulated by addition of  $10 \mu\text{M}$  forskolin to the basolateral hemichamber. Forskolin caused  $I_{\text{SC}}$  to rise sharply and plateau to a stable  $I_{\text{SC}}$ . At this point steroids ( $50 \mu\text{M}$ ) were added to both the apical and basolateral hemichambers, unless indicated otherwise. (A)  $17 \beta$ -estradiol ( $17\beta$ -E2) caused rapid inhibition (within seconds) of forskolin-stimulated  $\text{Cl}^-$  secretion ( $n=3$ ). (B) progesterone was a more potent inhibitor of cAMP-stimulated chloride secretion compared to  $17\beta$ -E2 ( $n=3$ ). (C) Corticosterone did not inhibit forskolin-stimulated chloride secretion ( $n=3$ ). The antiestrogen tamoxifen and d-aldosterone also failed to inhibit  $\text{Cl}^-$  secretion (data shown in Table 1). (D) The inhibitory effects of  $17\beta$ -E2 on  $\text{Cl}^-$  secretion are greater when applied to the apical side of the monolayer.

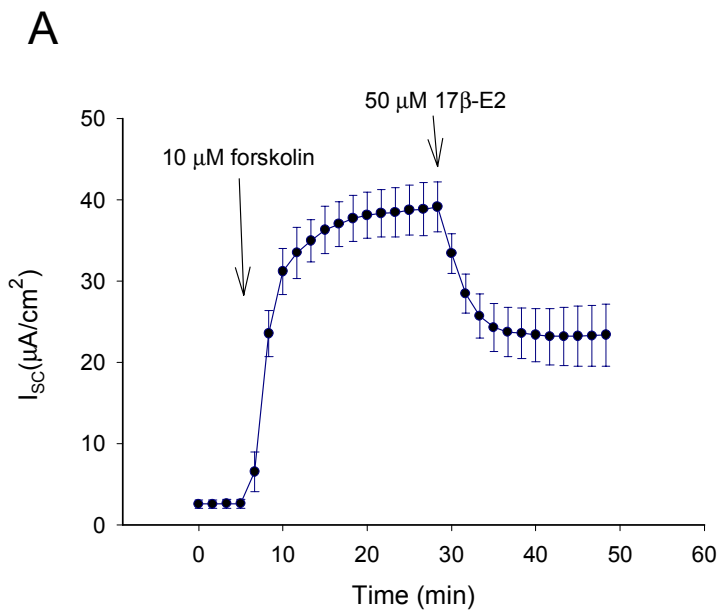
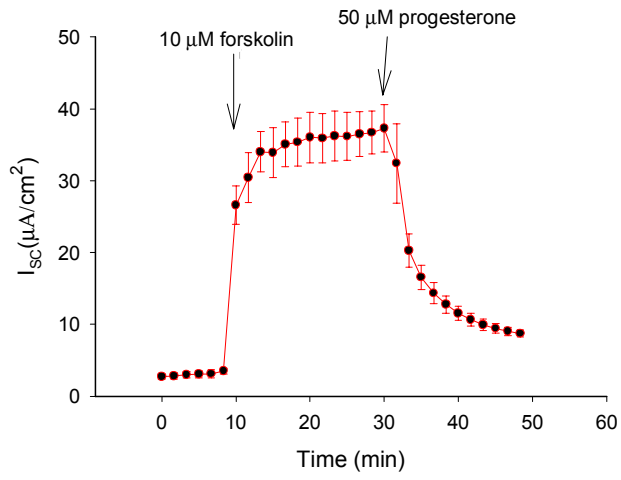
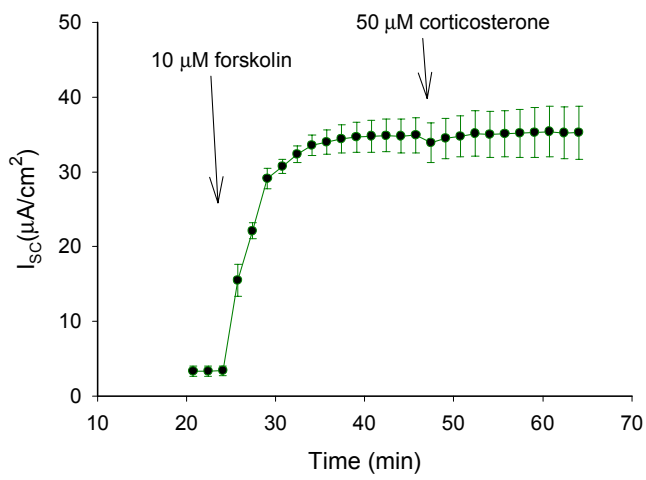
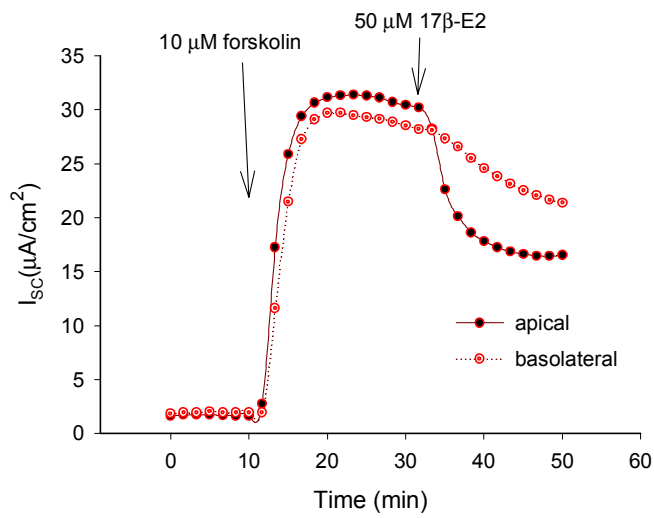


Figure 4 (continued)

**B****C****D**

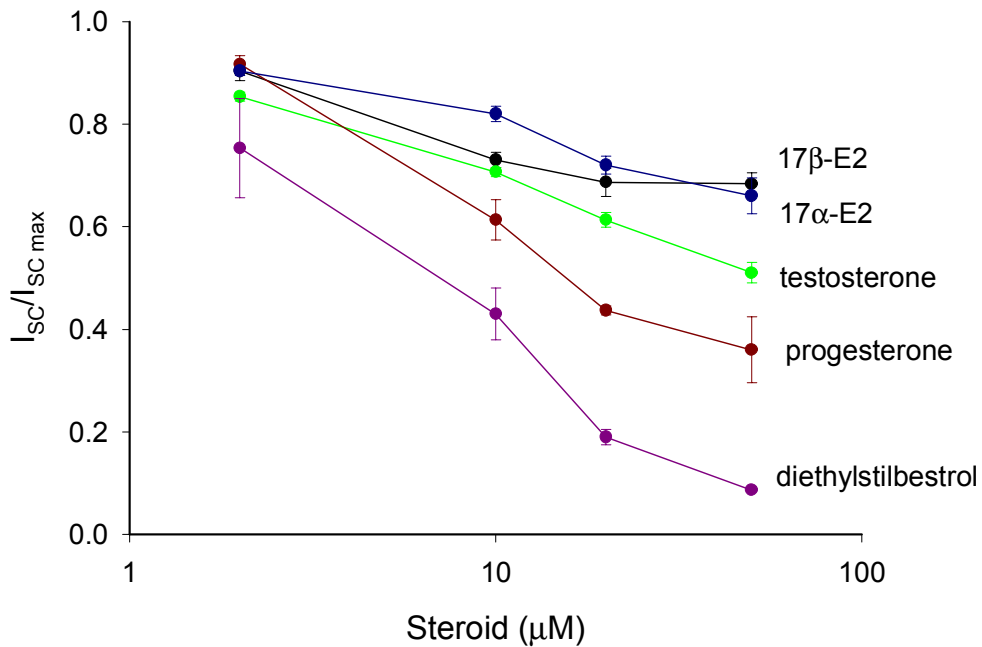


Figure 5 Treatment of T84 Monolayers with Steroid causes Dose-dependent Inhibition of Forskolin-stimulated Chloride Secretion. T84 monolayers were mounted in Ussing chambers, voltage clamped and  $\text{Cl}^-$  secretion stimulated by addition of  $10 \mu\text{M}$  forskolin to the basolateral hemichamber. Forskolin caused  $I_{SC}$  to rise sharply and plateau to a stable  $I_{SC}$ . At this point steroids (at increasing concentration) were added to both the apical and basolateral hemichambers and  $I_{SC}$  recorded.  $I_{SC} / I_{SC \text{ max}}$  were plotted against steroid concentration ( $\mu\text{M}$ ). The synthetic estrogen diethylstilbestrol is the most potent inhibitor of chloride secretion tested, followed by progesterone, testosterone, and the naturally occurring estrogens  $17 \beta$ -estradiol ( $17\beta$ -E2) and  $17 \alpha$ - estradiol ( $17\alpha$ -E2). Values shown are the mean  $\pm$  SEM,  $n=3$ .  $17\alpha$ -E2 does not activate a nuclear estrogen receptor confirming that this effect is nongenomic.



Table 1 Inhibition of Forskolin-stimulated Chloride Secretion across T84 Monolayers

Steroid Tested (50 $\mu$ M)	$I_{SC}/I_{SC\ max}$
diethylstilbestrol	.08 $\pm$ .003 (3)
progesterone	.22 $\pm$ .03 (3)
testosterone	.50 $\pm$ .01 (3)
17 $\beta$ -E2	.59 $\pm$ .05 (3)
17 $\alpha$ -E2	.66 $\pm$ .03 (3)
corticosterone	1 $\pm$ .03 (3)
d-aldosterone	1 (2)
tamoxifen	1.53 $\pm$ .26 (3)

#### 17 $\beta$ -estradiol Potentiates Thapsigargin- and Carbachol-stimulated $Cl^-$ Secretion

T84 monolayers were mounted in Ussing chambers, voltage clamped, and treated with a bilateral addition of 17  $\beta$ -estradiol. Addition of 17  $\beta$ -estradiol always caused a small (< 2  $\mu$ A/cm<sup>2</sup>) and stable increase in  $I_{SC}$ . After 15-20 minutes of treatment with 17  $\beta$ -estradiol, the monolayers were stimulated with either 1  $\mu$ M thapsigargin or 100  $\mu$ M carbachol. Stimulation of T84 monolayers with thapsigargin or carbachol resulted in  $Ca^{2+}$ -dependent  $Cl^-$  secretion. 17  $\beta$ -estradiol potentiated thapsigargin- and carbachol-stimulated  $Cl^-$  secretion. Figure 6 presents data from a typical experiment.

The potentiation of  $Ca^{2+}$  stimulated  $Cl^-$  secretion was dose-dependent, rapid, and steroid specific (Figures 7, 8, 9). 17  $\beta$ -estradiol potentiated thapsigargin-stimulated chloride secretion at a concentration as low as 5  $\mu$ M. Amongst all the other steroids tested (diethylstilbestrol, progesterone and testosterone), and the nonsteroidal selective estrogen receptor modulator

tamoxifen, no significant potentiation of thapsigargin stimulated  $\text{Cl}^-$  secretion was observed at the concentration of 5  $\mu\text{M}$  (Figure 8).

17  $\beta$ -estradiol potentiated carbachol-stimulated chloride secretion at a concentration of 50  $\mu\text{M}$ . Potentiation of carbachol-stimulated chloride secretion could be observed at 17  $\beta$ -estradiol concentration as low as 10  $\mu\text{M}$ , but this potentiation was not consistently reproducible. At 50  $\mu\text{M}$  concentration, the steroids corticosterone, d-aldosterone and nonsteroid tamoxifen did not potentiate carbachol-stimulated  $\text{Cl}^-$  secretion. Figure 9B shows the lack of an effect of corticosterone on carbachol-stimulated  $\text{I}_{\text{SC}}$ .

For potentiation of thapsigargin-stimulated  $\text{I}_{\text{SC}}$ , 17  $\alpha$ -estradiol and 17  $\beta$ -estradiol were equipotent (Figure 8B), confirming that this effect is nongenomic. Apical 17  $\beta$ -estradiol caused a significantly greater potentiation of carbachol-stimulated  $\text{Cl}^-$  secretion, than basolateral 17  $\beta$ -estradiol (Figure 9A).

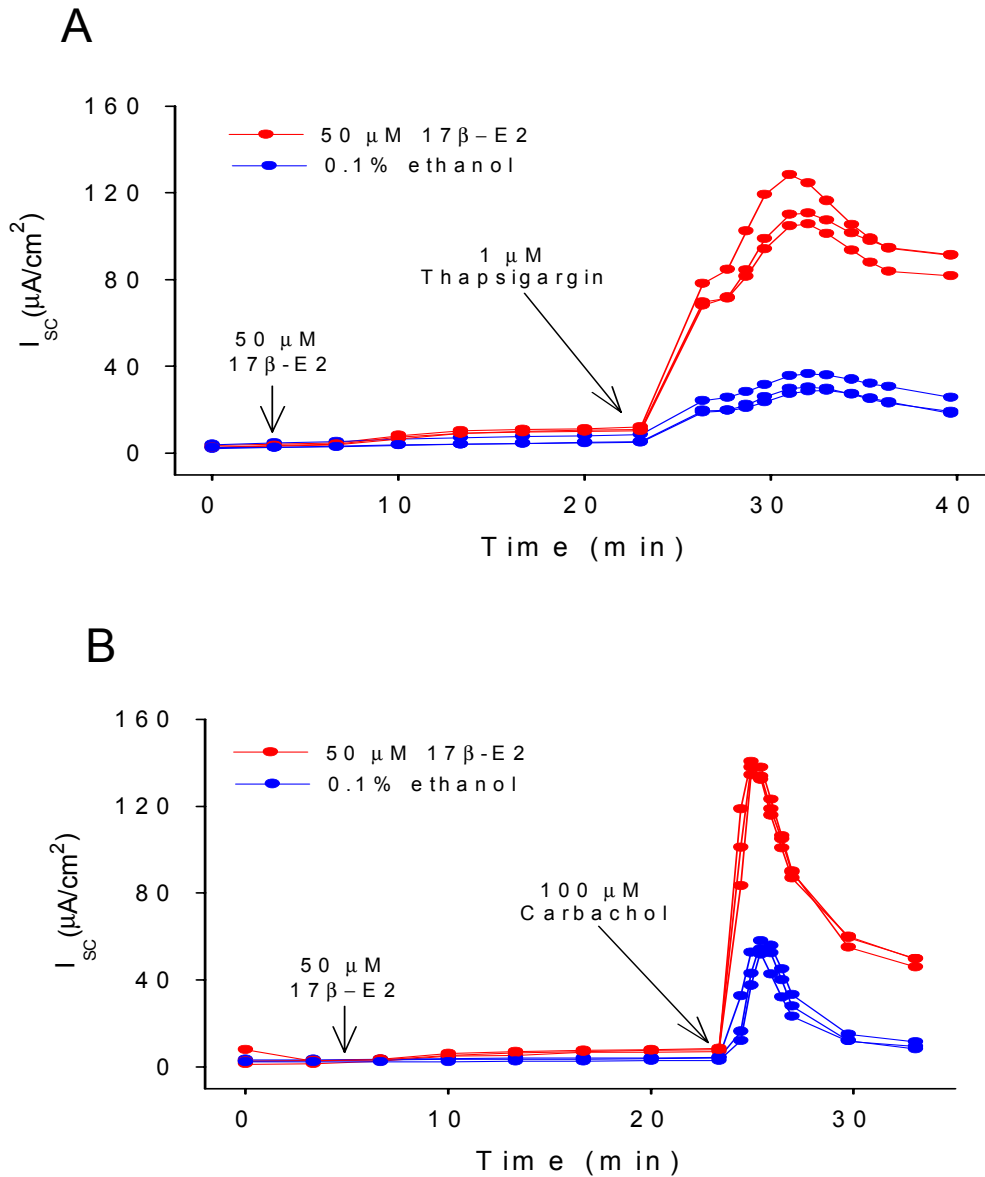


Figure 6 17  $\beta$ -estradiol Potentiates Thapsigargin-and Carbachol-stimulated  $Cl^-$  Secretion. This figure shows individual short-circuit current recording from a typical experiment. In these examples, 17  $\beta$ -estradiol (17 $\beta$ -E2) potentiated thapsigargin-stimulated  $Cl^-$  secretion ( $I_{SC}$ ) by 3.5 fold compared to the 0.1% ethanol controls ( $115.0 \pm 6.7$  vs.  $31.8 \pm 4.1$   $\mu A/cm^2$ , respectively (mean  $\pm$  SEM, n=3)). Carbachol-stimulated  $I_{SC}$  was potentiated 2.5 fold by 17 $\beta$ -E2 ( $140.7 \pm 1.0$  vs.  $56.0 \pm 1.5$   $\mu A/cm^2$  (mean  $\pm$  SEM, n=3)).

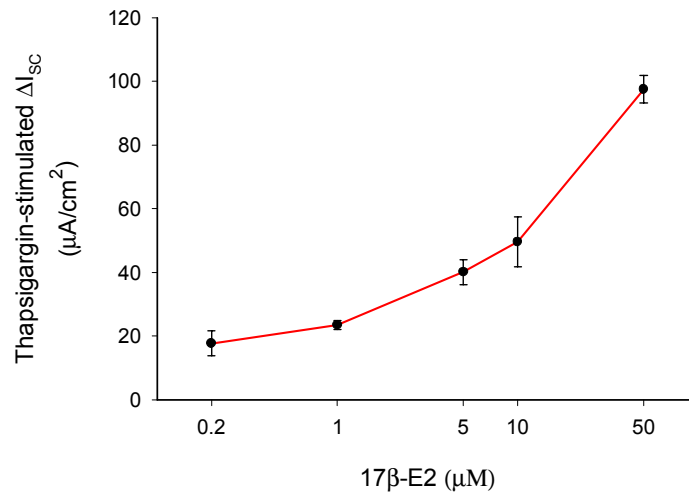
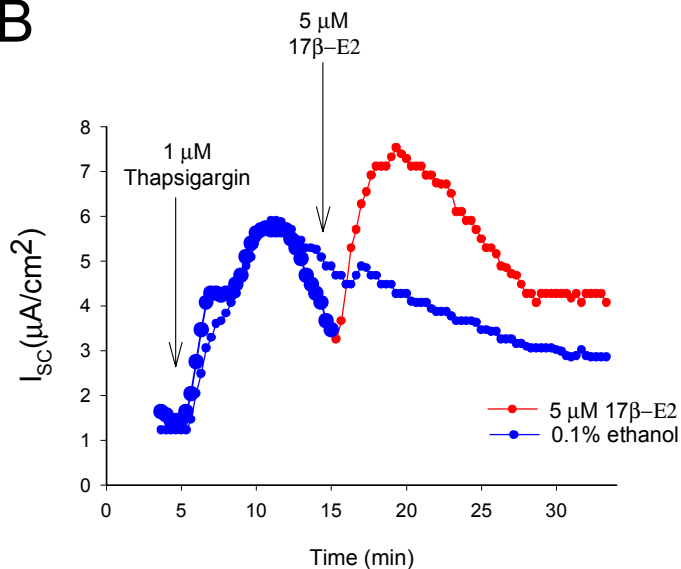
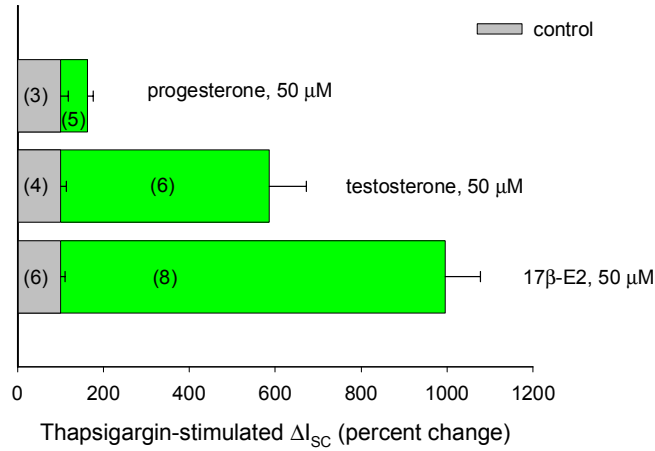
**A****B**

Figure 7 Estrogen Potentiation of  $Ca^{2+}$ -stimulated  $I_{SC}$  is Dose-dependent, and Very Rapid. A. T84 monolayers were mounted in Ussing chambers and voltage clamped. Increasing concentrations of 17  $\beta$ -estradiol (17 $\beta$ -E2) were added to each hemichamber 20 min prior to stimulation of  $Cl^-$  secretion by addition of 1  $\mu M$  thapsigargin to the apical hemichamber. Significant potentiation was observed at and above 5  $\mu M$  17 $\beta$ -E2. Values are the mean  $\pm$  SEM for 3 to 12 monolayers at each point. Thapsigargin-stimulated  $\Delta I_{SC}$  for vehicle control (0.1% ethanol) was  $20.42 \pm 1.90 \mu A/cm^2$  (n=12). B. T84 monolayers were mounted in Ussing chambers and voltage clamped.  $Cl^-$  secretion was stimulated with 1  $\mu M$  thapsigargin. After the peak  $I_{SC}$  response, 5  $\mu M$  17 $\beta$ -E2 or 0.1% ethanol (vehicle control) was added to both hemichambers. 17 $\beta$ -E2 caused a rapid and significant increase in  $I_{SC}$ . Shown is a representative experiment.

A



B

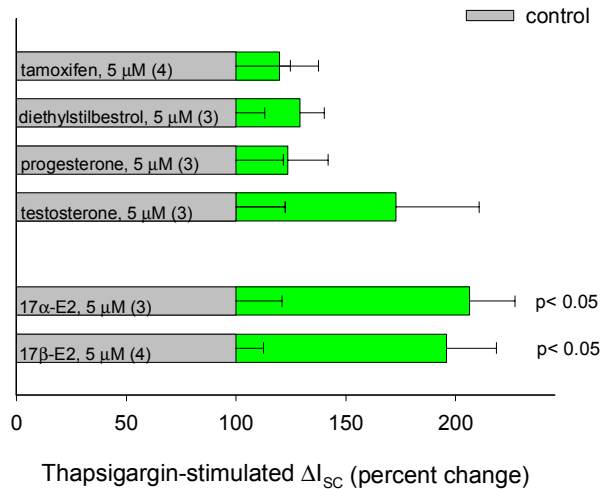


Figure 8 Estrogen Potentiation of  $\text{Ca}^{2+}$ -stimulated  $I_{SC}$  is Dose-dependent, Rapid, and Steroid Specific. T84 monolayers were mounted in Ussing chambers, voltage clamped, and treated with the indicated steroids (50  $\mu\text{M}$  in A, and 5  $\mu\text{M}$  in B) by addition to both hemichambers. After 20 min, 1  $\mu\text{M}$  thapsigargin was added to stimulate  $\text{Cl}^-$  secretion. Thapsigargin-stimulated  $I_{SC}$  is expressed as a percent of 0.1% ethanol controls (mean  $\pm$  SEM, number of monolayers in parentheses). 17  $\alpha$ -estradiol (17 $\alpha$ -E2) and 17  $\beta$ -estradiol (17 $\beta$ -E2) were equipotent and other steroids tested had no significant affect at 5  $\mu\text{M}$  steroid concentration.

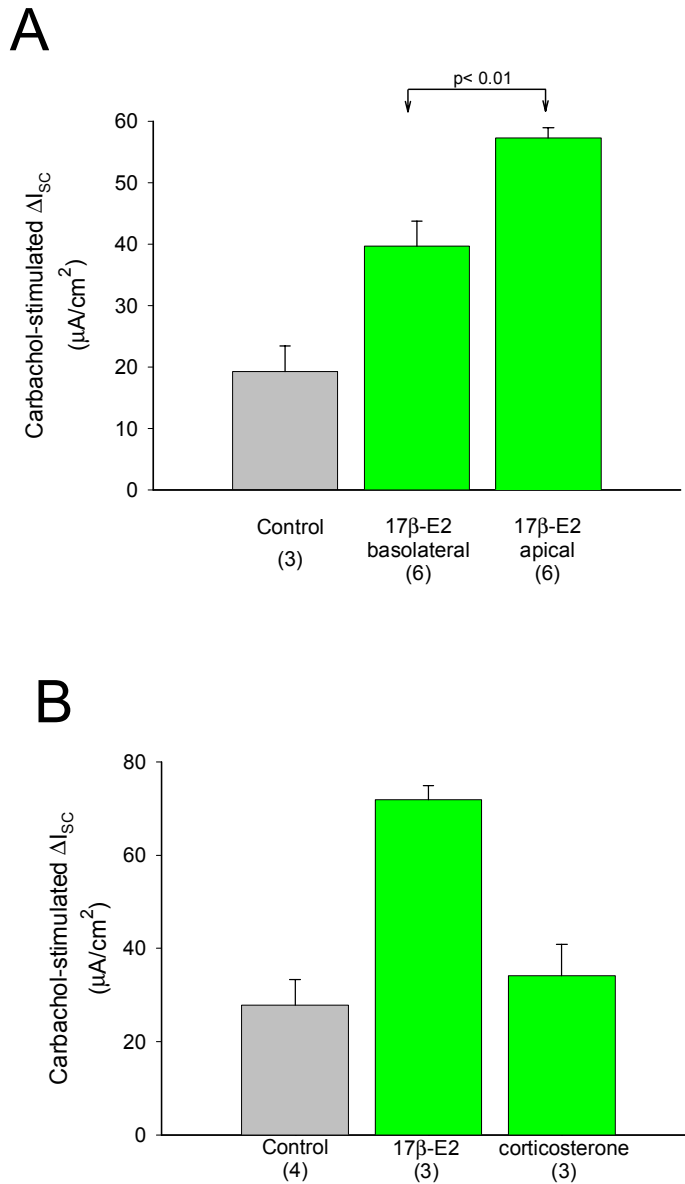


Figure 9 17  $\beta$ -estradiol (17 $\beta$ -E2) Potentiates Thapsigargin-and Carbachol-stimulated Cl<sup>-</sup> Secretion. A. T84 monolayers were mounted in Ussing chambers and voltage clamped. 17 $\beta$ -E2 (50  $\mu$ M) was added to the apical or basolateral hemichamber 20 min prior to stimulation of Cl<sup>-</sup> secretion with 100  $\mu$ M carbachol. Apical 17 $\beta$ -E2 caused a significantly greater potentiation of  $I_{sc}$  compared to basolateral 17  $\beta$ -E2. Values shown are the mean  $\pm$  SEM. The number of monolayers in each group is indicated in parentheses.

B. T84 monolayers were mounted in Ussing chambers, voltage clamped, and treated with 50 $\mu$ M 17 $\beta$ -E2 or corticosterone by addition to both hemichambers. After 20 min, 100  $\mu$ M carbachol was added to stimulate Cl<sup>-</sup> secretion. Corticosterone did not cause any potentiation. Values shown are the mean  $\pm$  SEM. The number of monolayers in each group is indicated in parentheses.

17  $\beta$ -estradiol Potentiation of Thapsigargin-and Carbachol Stimulated Chloride Secretion in T84 Monolayers Grown in Steroid Free Culture Media

The culture media used to grow T84 monolayers contains 5% newborn calf serum (NBCS) that has small amounts of estrogens and other steroids. Phenol red in the culture media also has weak estrogenic activity (Berthois and others 1986). To exclude the effects of steroids in the culture media, several experiments were conducted with T84 monolayers grown in phenol red-free DME/F12 supplemented with 15 mM HEPES, antibiotics, and charcoal-stripped (steroid-free) NBCS. Steroids were removed from NBCS (charcoal-stripped) by adding 2% weight/volume charcoal and heating to 55°C for 1 hour. The charcoal was removed by centrifugation, and the serum was sterilized by gentle filtration through a 0.22  $\mu$  Millipore filter. Phenol red-free bicarbonate-buffered Ringer solution was used in these experiments.

17  $\beta$ -estradiol potentiation was not enhanced in monolayers grown in steroid-free environment. 17  $\beta$ -estradiol at concentrations lower than 5  $\mu$ M did not cause significant potentiation of thapsigargin- or carbachol-stimulated  $\text{Cl}^-$  secretion in monolayers that were serum starved and/or grown in steroid-free culture media (Figure 10, 11). As cultures grown under serum-free conditions did not alter the response of T84 monolayers to 17  $\beta$ -estradiol, subsequent experiments used monolayers and cells cultured in standard culture medium and unstripped serum.

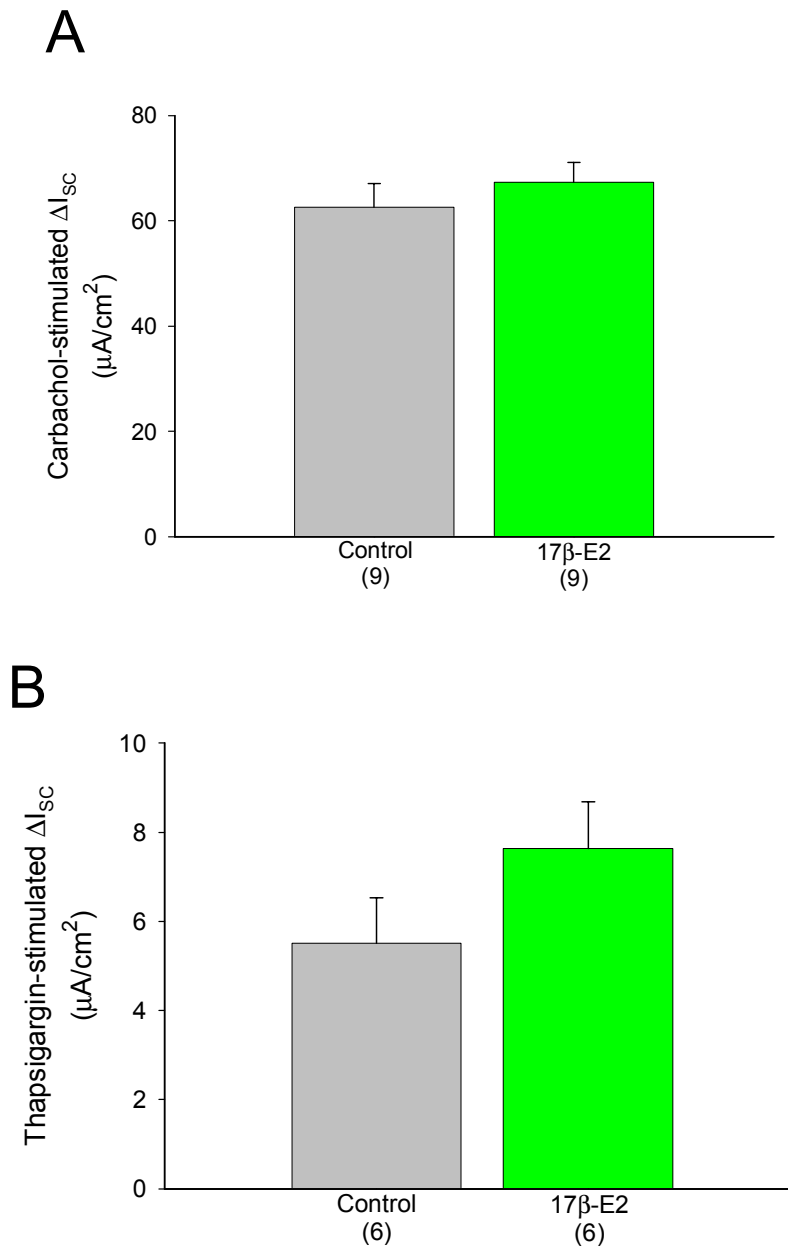


Figure 10 17  $\beta$ -estradiol (17 $\beta$ -E2) Potentiation of Thapsigargin-and Carbachol-stimulated Chloride Secretion is not Significant at Lower (1 $\mu$ M) 17 $\beta$ -E2 Concentrations in T84 Monolayers Grown in Steroid-free Media. T84 monolayers were grown in phenol red-free culture media and treated with charcoal-stripped (steroid-free) 5% NBCS for 3 days prior to experimentation. T84 monolayers were mounted in Ussing chambers, voltage clamped, and treated with a bilateral addition of 1  $\mu$ M 17 $\beta$ -E2, 20 min prior to stimulation with either 100  $\mu$ M carbachol (A) or 1  $\mu$ M thapsigargin (B). No significant potentiation was observed. Values shown are mean  $\pm$  SEM (n in parentheses).



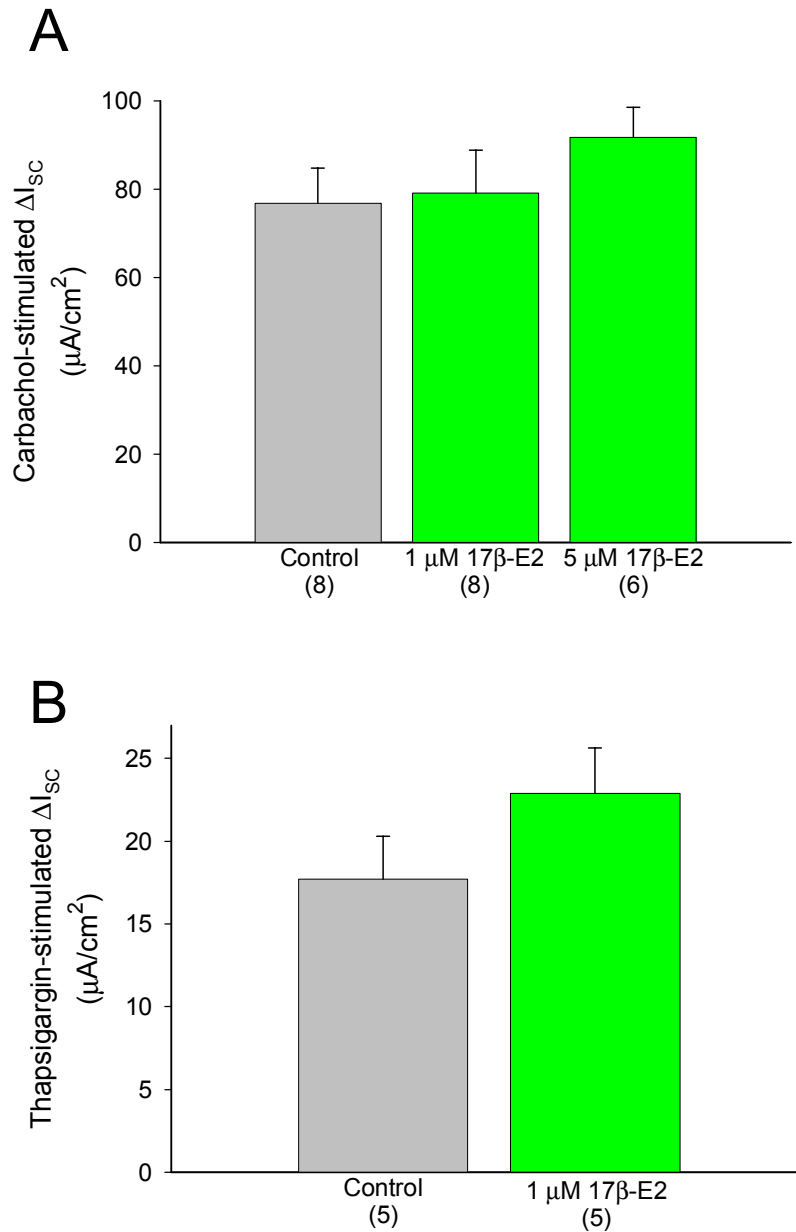


Figure 11 17  $\beta$ -estradiol (17 $\beta$ -E2) Potentiation of Thapsigargin-and Carbachol-Stimulated Chloride Secretion is not Significant at Lower 17 $\beta$ -E2 Concentrations in Serum-starved T84 Monolayers Grown in Steroid-free Media. T84 monolayers were grown in phenol red-free culture media and treated with charcoal-stripped (steroid-free) 5% NBCS for 3 days and charcoal-stripped (steroid-free) 0.5% NBCS for 24 hours prior to experimentation. T84 monolayers were mounted in Ussing chambers, voltage clamped, and treated with a bilateral addition of 17 $\beta$ -E2, 20 min prior to stimulation with either 100  $\mu M$  carbachol (A) or 1  $\mu M$  thapsigargin (B). No significant potentiation was observed. Values shown are mean  $\pm$  SEM (n in parentheses).

### Tamoxifen, does not Block 17 $\beta$ -estradiol Potentiation of $\text{Ca}^{2+}$ -stimulated $\text{Cl}^-$ Secretion

To study whether tamoxifen (a nonsteroidal triphenylethylene), a competitive inhibitor of estrogen binding to nuclear estrogen receptors, blocked the 17  $\beta$ -estradiol potentiation of  $\text{Cl}^-$  secretion, T84 monolayers were mounted in Ussing chambers, voltage clamped, and preincubated with 50  $\mu\text{M}$  tamoxifen for 30 min prior to addition of 5  $\mu\text{M}$  17  $\beta$ -estradiol. Twenty minutes after addition of 17  $\beta$ -estradiol,  $\text{Cl}^-$  secretion was stimulated with 1  $\mu\text{M}$  thapsigargin. Tamoxifen by itself did not have any effect on thapsigargin-stimulation. Tamoxifen did not block 17  $\beta$ -estradiol potentiation of thapsigargin-stimulated  $\text{Cl}^-$  secretion (Figure 12), instead it potentiated the 17  $\beta$ -estradiol response ( $p < 0.05$ ). The observation that tamoxifen did not block 17  $\beta$ -estradiol potentiation of thapsigargin-stimulated  $\text{Cl}^-$  secretion suggests that the classical nuclear estrogen receptors are not involved.

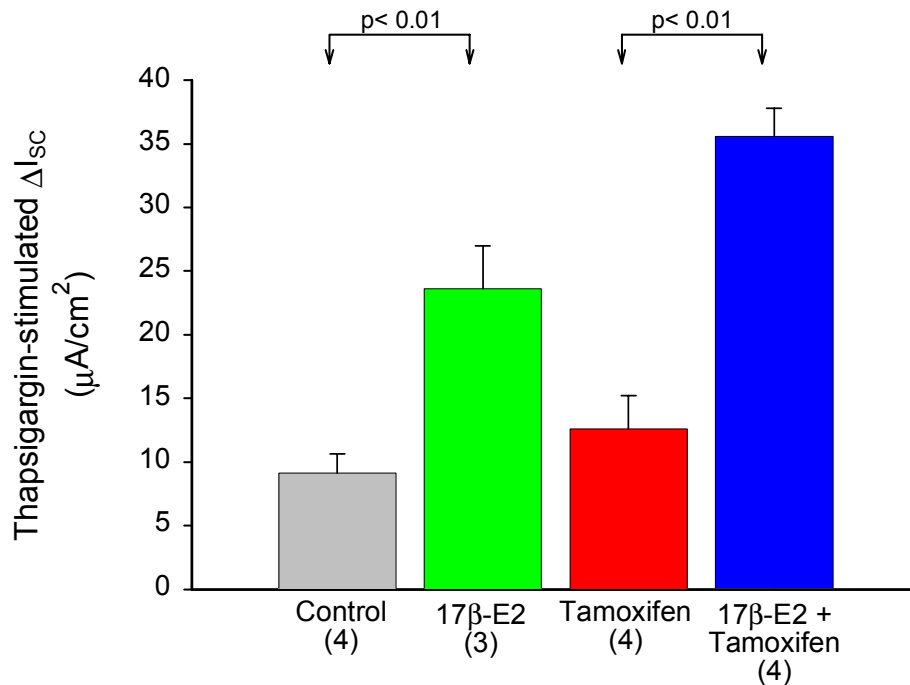


Figure 12 Tamoxifen does not Block 17  $\beta$ -estradiol (17 $\beta$ -E2) Potentiation of  $Ca^{2+}$ -stimulated  $Cl^-$  Secretion. T84 monolayers were mounted in Ussing chambers, voltage clamped and preincubated with 50  $\mu M$  tamoxifen (a nonsteroidal triphenylethylene antiestrogen) for 30 min before addition of 5  $\mu M$  17 $\beta$ -E2. After 20 min,  $Cl^-$  secretion was stimulated with 1  $\mu M$  thapsigargin. Values shown are the mean  $\pm$  SEM for the peak thapsigargin response (n in parentheses). Tamoxifen did not block 17 $\beta$ -E2 potentiation suggesting that the classical nuclear estrogen receptors are not involved in this action of 17 $\beta$ -E2.

17  $\beta$ -estradiol Potentiation of  $\text{Ca}^{2+}$ -stimulated  $\text{Cl}^-$  secretion Coincides with an Increase in Monolayer Electrical Conductance

T84 monolayers were mounted in Ussing chambers, voltage clamped, and treated with 17  $\beta$ -estradiol for 20 minutes prior to stimulation of  $\text{Cl}^-$  secretion with 1  $\mu\text{M}$  thapsigargin or 100  $\mu\text{M}$  carbachol. At 20 or 30 sec intervals, a biphasic pulse of 5 mV was applied and the resulting current deflection used to calculate monolayer conductance (G) by Ohm's law. 17  $\beta$ -estradiol treatment by itself did not cause any change in monolayer electrical conductance. Thapsigargin or carbachol stimulation caused a significant increase in monolayer conductance in 17  $\beta$ -estradiol treated monolayers compared to 0.1% ethanol controls. A significantly increased conductance in 17  $\beta$ -estradiol treated monolayers suggests that 17  $\beta$ -estradiol causes the opening of additional ion channels following thapsigargin or carbachol stimulation. Figure 13 shows original tracings from a typical experiment.

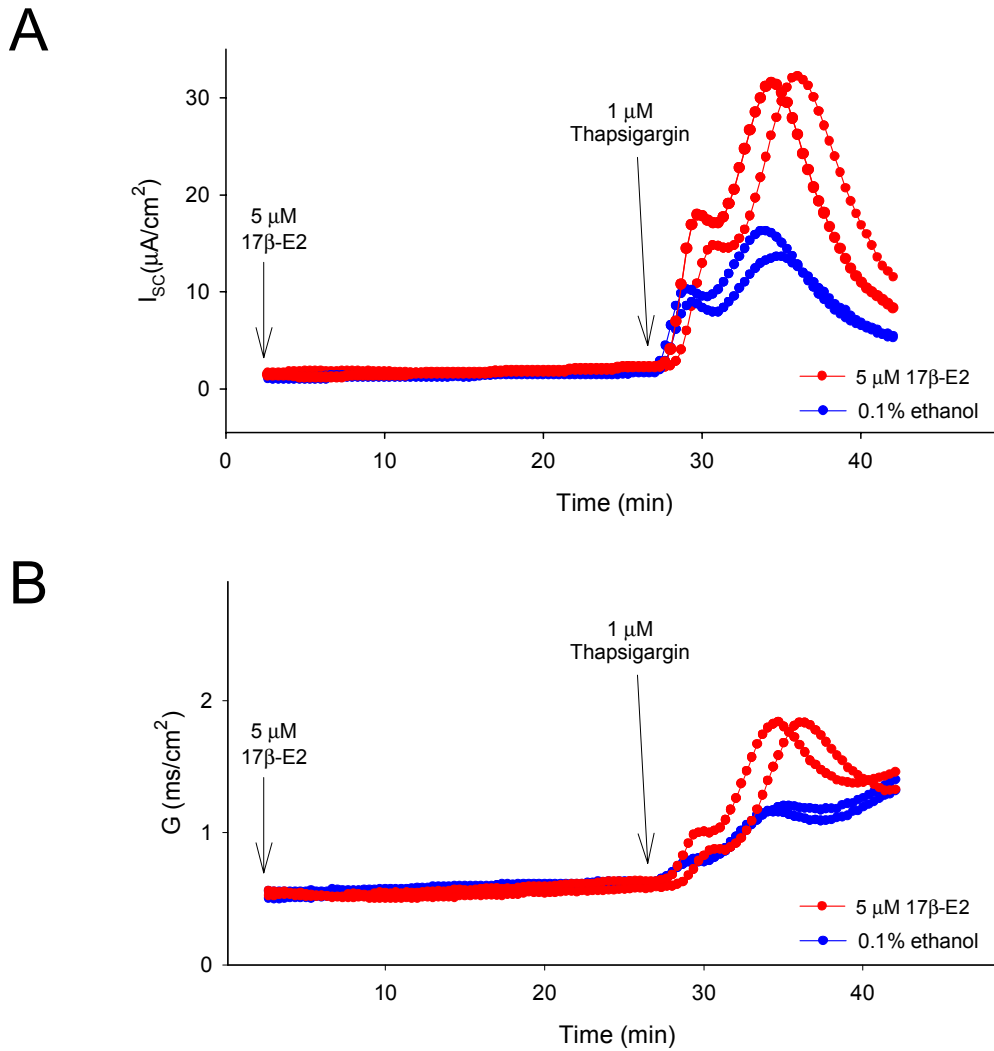


Figure 13 17  $\beta$ -estradiol (17 $\beta$ -E2) Potentiation of  $Ca^{2+}$ -stimulated  $Cl^-$  Secretion Coincides with an Increase in Monolayer Electrical Conductance. T84 monolayers were mounted in Ussing chambers, voltage clamped, and treated with 5  $\mu M$  17 $\beta$ -E2 for 20 min prior to stimulation of  $Cl^-$  secretion with 1  $\mu M$  thapsigargin. Shown are individual recordings of  $I_{sc}$  and monolayer conductance (G) from 2 monolayers in a typical experiment. The significantly increased conductance in 17 $\beta$ -E2-treated monolayers suggests that 17 $\beta$ -E2 causes the opening of additional ion channels following thapsigargin stimulation.

The Chloride Channel Blockers NPPB and DIDS do not Reduce 17  $\beta$ -estradiol Potentiation of Thapsigargin-or Carbachol-stimulated  $\text{Cl}^-$  Secretion

To test whether 17  $\beta$ -estradiol caused activation of additional apical  $\text{Cl}^-$  channels, T84 monolayers were treated with the  $\text{Cl}^-$  channel blockers DIDS or NPPB before addition of 17  $\beta$ -estradiol. DIDS, a disulfonic stilbene, does not affect CFTR when added to the extracellular side of the membrane, and NPPB, an arylaminobenzoate, is a partial CFTR blocker (Gray and others 1993; Schultz and others 1999). T84 monolayers were mounted in Ussing chambers, voltage clamped, and treated with either 50  $\mu\text{M}$  NPPB or 100  $\mu\text{M}$  DIDS (by addition to the apical hemichamber) for 30 minutes to block activity of  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels. Next, monolayers were treated with 17  $\beta$ -estradiol for 20 minutes prior to stimulation of  $\text{Cl}^-$  secretion with 1  $\mu\text{M}$  thapsigargin or 100  $\mu\text{M}$  carbachol. The results showed that 17  $\beta$ -estradiol potentiation of carbachol-stimulated  $\text{Cl}^-$  secretion was partially reduced in monolayers treated with NPPB, consistent with partial block of CFTR by NPPB (Gray and others 1993). However, in monolayers treated with DIDS 17  $\beta$ -estradiol potentiation was unaffected (Figure 14). There was no significant change in 17  $\beta$ -estradiol (5  $\mu\text{M}$  steroid concentration) potentiation of thapsigargin-stimulated  $\text{Cl}^-$  secretion in either NPPB or DIDS treated monolayers (Figure 15). These results suggest that 17  $\beta$ -estradiol potentiation of thapsigargin or carbachol-dependent  $\text{Cl}^-$  secretion does not involve activation of  $\text{Cl}^-$  channels other than CFTR in T84 cells.

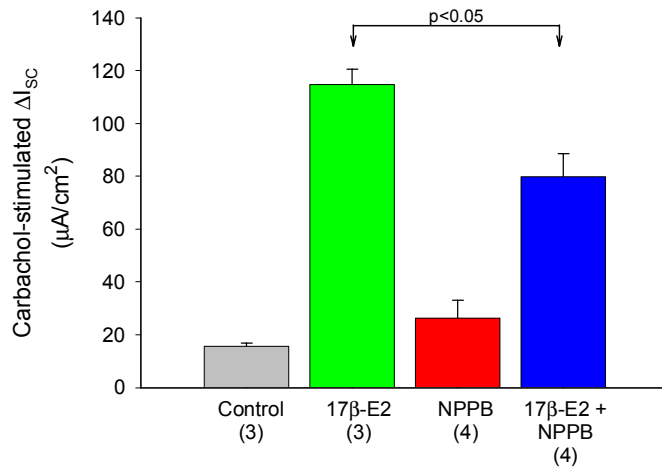
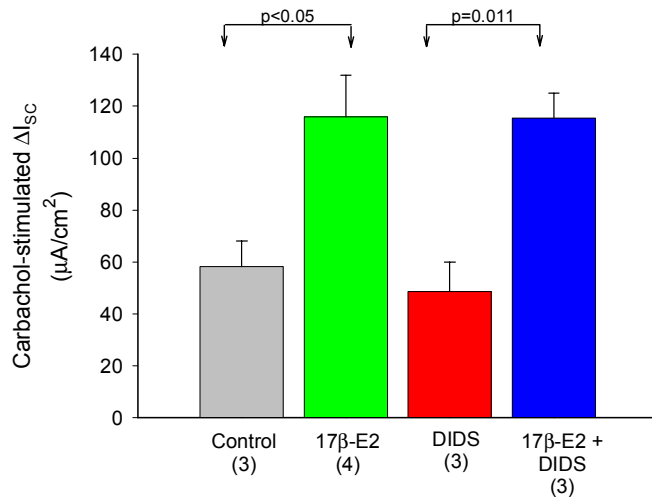
**A****B**

Figure 14 The Effect of Chloride Channel Blockers NPPB and DIDS on 17  $\beta$ -estradiol (17 $\beta$ -E2) Potentiation of Carbachol-stimulated Cl<sup>-</sup> Secretion. T84 monolayers were mounted in Ussing chambers, voltage clamped, and treated with either 50  $\mu$ M NPPB (A) or 100  $\mu$ M DIDS (B) for 30 min to block activity of Ca<sup>2+</sup>-activated Cl<sup>-</sup> channels. Next, monolayers were treated with 50  $\mu$ M 17 $\beta$ -E2 for 20 min prior to stimulation of Cl<sup>-</sup> secretion with 100  $\mu$ M carbachol. Values shown are the mean  $\pm$  SEM (n in parentheses) for the peak I<sub>sc</sub> response. These results show that 17 $\beta$ -E2 potentiation of carbachol-stimulated Cl<sup>-</sup> secretion is decreased in monolayers treated with NPPB, but not in monolayers treated with DIDS.

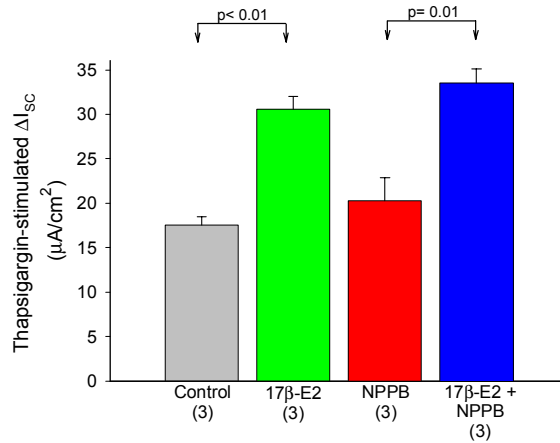
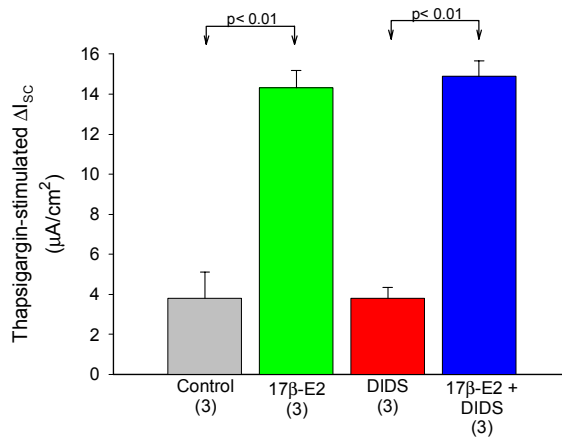
**A****B**

Figure 15 The Chloride Channel Blockers NPPB and DIDS do not Reduce 17  $\beta$ -estradiol Potentiation of Thapsigargin-stimulated  $Cl^-$  Secretion. T84 monolayers were mounted in Ussing chambers, voltage clamped, and treated with either 50  $\mu M$  NPPB (A) or 100  $\mu M$  DIDS (B) for 30 min to block activity of  $Ca^{2+}$ -activated  $Cl^-$  channels. Next, monolayers were treated with 5  $\mu M$  17 $\beta$ -E2 for 20 min prior to stimulation of  $Cl^-$  secretion with 1  $\mu M$  thapsigargin. Values shown are the mean  $\pm$  SEM (n in parentheses) for the peak  $I_{sc}$  response. These results suggest that 17 $\beta$ -E2 potentiation of thapsigargin-dependent  $Cl^-$  secretion does not involve activation of  $Cl^-$  channels other than CFTR in T84 cells.



17  $\beta$ -estradiol Potentiation of Thapsigargin-or Carbachol-stimulated  $\text{Cl}^-$  Secretion Requires Active CFTR  $\text{Cl}^-$  Channels

H89, an inhibitor of cAMP-dependent protein kinase A (PKA), was used to study the activity of the apical CFTR  $\text{Cl}^-$  channels in T84 monolayers. T84 monolayers were pretreated for 30 minutes with 30  $\mu\text{M}$  H89, voltage clamped, then treated with 50  $\mu\text{M}$  17  $\beta$ -estradiol (20 minutes), prior to stimulation of  $\text{Cl}^-$  secretion with 100  $\mu\text{M}$  carbachol. H89 treatment caused a small (statistically insignificant) decrease in carbachol-stimulated  $I_{\text{SC}}$  and 17  $\beta$ -estradiol potentiation of carbachol-stimulated  $I_{\text{SC}}$  (Figure 16). Treatment of monolayers with 30  $\mu\text{M}$  H89 for 30 minutes partially blocked cAMP dependent  $\text{Cl}^-$  secretion (forskolin-stimulated  $I_{\text{SC}}$  decreased from  $23.4 \pm .26 \mu\text{A}/\text{cm}^2$  to  $13.92 \pm 1.19 \mu\text{A}/\text{cm}^2$  (mean  $\pm$  SEM,  $n=4$ )), indicating that a much longer period of incubation with H89 is required for complete inhibition of PKA.

In subsequent experiments, monolayers were preincubated for 4 hours with 30  $\mu\text{M}$  H89 to study the effect of H89 on 17  $\beta$ -estradiol potentiation. T84 monolayers were preincubated for 4 hours with 30  $\mu\text{M}$  H89, mounted in Ussing chambers, voltage clamped and treated with 17  $\beta$ -estradiol for 20 minutes prior to stimulation with 1  $\mu\text{M}$  thapsigargin. H89 treatment blocked thapsigargin-stimulated  $I_{\text{SC}}$  in T84 monolayers grown in Millicell-PCF culture plate inserts, consistent with CFTR being the only apical  $\text{Cl}^-$  channel in T84 cells (Figure 17A). H89 treatment did not block thapsigargin-stimulated  $I_{\text{SC}}$  in monolayers grown in Millicell-HA inserts (Figure 17B). This was probably due to the difference in the culture plate inserts used. H89 treatment completely blocked 17  $\beta$ -estradiol potentiation of thapsigargin-stimulated  $I_{\text{SC}}$  in all monolayers (Figure 17). These observations suggest that the 17  $\beta$ -estradiol potentiation of  $\text{Ca}^{2+}$ -stimulated  $\text{Cl}^-$  secretion requires active CFTR  $\text{Cl}^-$  channels.

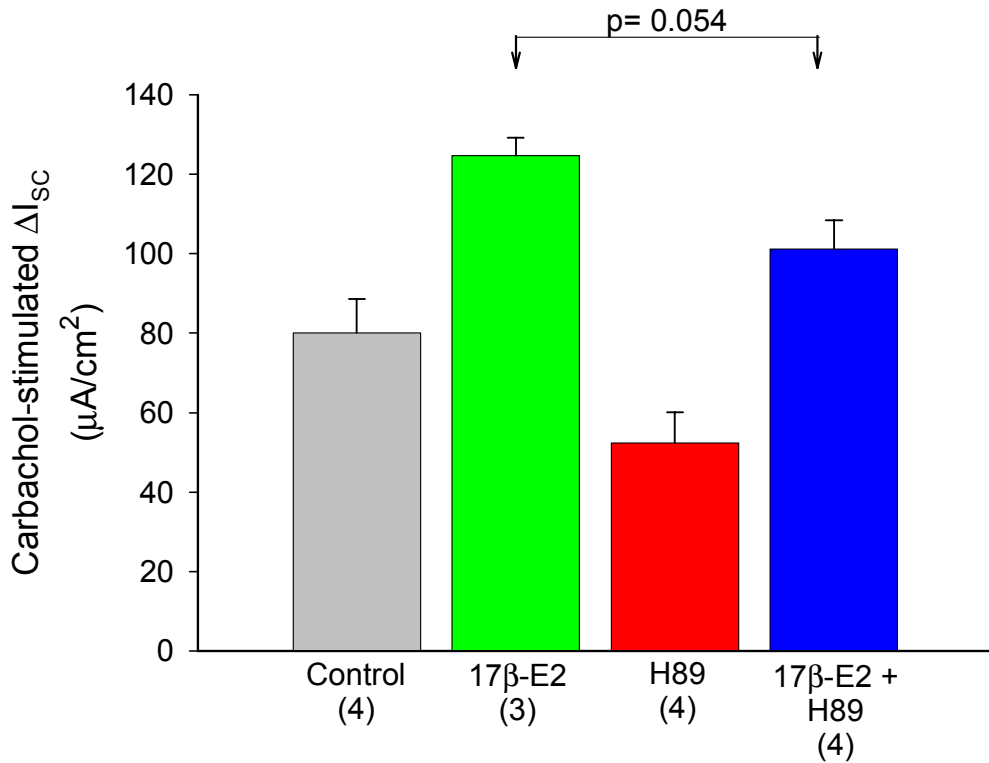


Figure 16 17  $\beta$ -estradiol Potentiation of Carbachol-stimulated  $Cl^-$  Secretion Requires Active CFTR  $Cl^-$  Channels. T84 monolayers were preincubated for 30 min with 30  $\mu M$  H89, an inhibitor of cAMP-dependent protein kinase A (PKA). After H89 treatment, monolayers were mounted in Ussing chambers, voltage clamped and 30  $\mu M$  H89 added to each hemichamber. Monolayers were treated with 50  $\mu M$  17  $\beta$ -estradiol (17 $\beta$ -E2) for 20 min prior to stimulation of  $Cl^-$  secretion with 100  $\mu M$  carbachol. Values shown are the peak carbachol response for each group (mean  $\pm$  SEM, n in parentheses). H89 treatment decreases carbachol-stimulated  $I_{sc}$  and 17 $\beta$ -E2 potentiation, consistent with CFTR being the only apical  $Cl^-$  channel in the T84 cell.

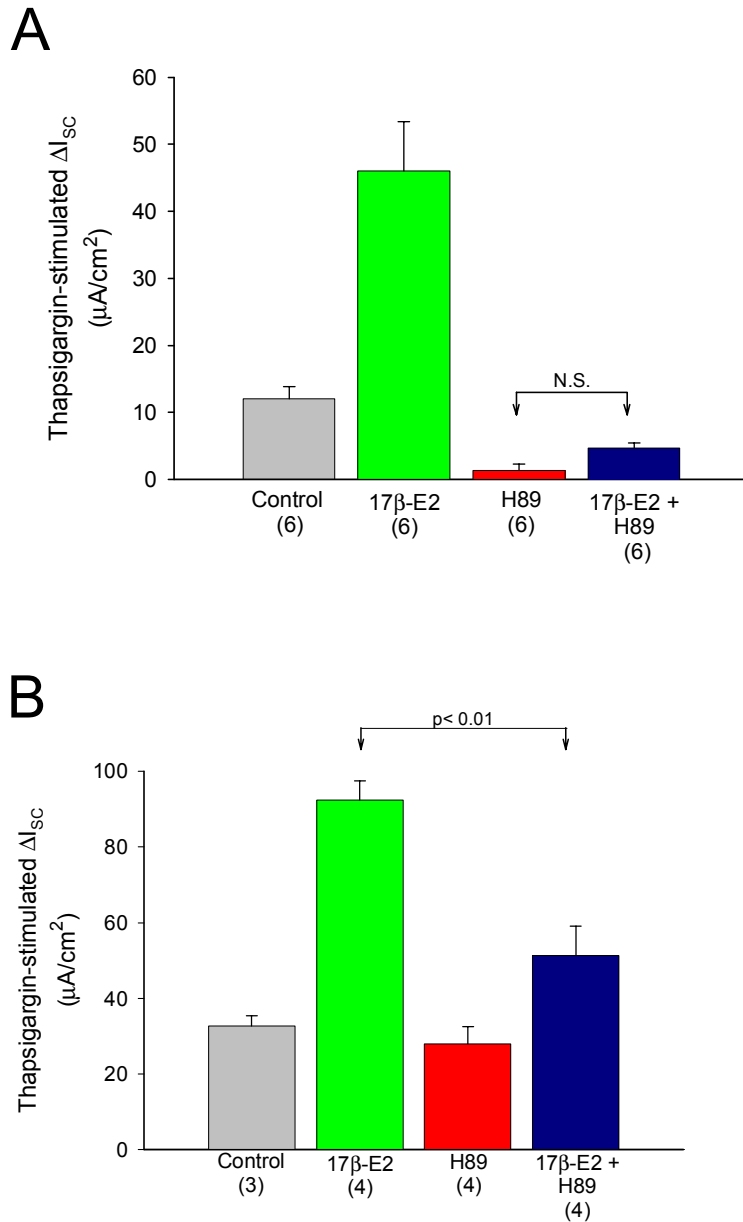


Figure 17 17  $\beta$ -estradiol Potentiation of Thapsigargin-stimulated Cl<sup>-</sup> Secretion Requires Active CFTR Cl<sup>-</sup> Channels. T84 monolayers were preincubated for 4 hours with 30  $\mu$ M H89, an inhibitor of cAMP-dependent protein kinase A (PKA). After H89 treatment, monolayers were mounted in Ussing chambers, voltage clamped and 30  $\mu$ M H89 added to each hemichamber. Monolayers were treated with 17 $\beta$ -E2 (5  $\mu$ M in panel A and 50  $\mu$ M in panel B) for 20 min prior to stimulation of Cl<sup>-</sup> secretion with 1  $\mu$ M thapsigargin. H89 treatment blocked thapsigargin-stimulated I<sub>sc</sub> (in panel A) and 17 $\beta$ -E2 potentiation (both in panel A and panel B), consistent with CFTR being the only apical Cl<sup>-</sup> channel in the T84 cell. Values shown are the peak response for each group (mean  $\pm$  SEM, n in parentheses).

17  $\beta$ -estradiol Does not Increase the Activity of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  Channels in the Basolateral Membrane of T84 Monolayers

A significantly increased conductance in 17  $\beta$ -estradiol treated monolayers suggested that 17  $\beta$ -estradiol caused the opening of additional ion channels following thapsigargin (or carbachol) stimulation (Figure 13). Also, the 17  $\beta$ -estradiol potentiation of thapsigargin- or carbachol-dependent  $\text{Cl}^-$  secretion did not involve activation of apical  $\text{Cl}^-$  channels other than CFTR in T84 cells (Figures 14,15). Experiments were conducted to determine whether 17  $\beta$ -estradiol increased the activity of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels in the basolateral membrane of T84 monolayers.

T84 monolayers were mounted in Ussing chambers and voltage clamped. The ringer solution bathing the monolayers was changed to impose a 140:5 mM apical to basolateral  $\text{K}^+$  gradient. The apical membrane was permeabilized with nystatin (350  $\mu\text{g}/\text{ml}$ ), and the basolateral spontaneous sodium current was blocked by addition of ouabain (50  $\mu\text{M}$ ). At this point, monolayers were incubated for 20 minutes with 5  $\mu\text{M}$  17  $\beta$ -estradiol, and stimulated with 1  $\mu\text{M}$  thapsigargin to activate basolateral  $\text{K}^+$  channels. 17  $\beta$ -estradiol did not change the thapsigargin-stimulated  $I_{\text{K}}$ , suggesting that activation of basolateral membrane  $\text{K}^+$  channels is not the target for 17  $\beta$ -estradiol action (Figure 18). Similar results have been obtained with T84 monolayers treated with 50  $\mu\text{M}$  17  $\beta$ -estradiol and stimulated with 100  $\mu\text{M}$  carbachol (Conner and Ecay, unpublished observations).

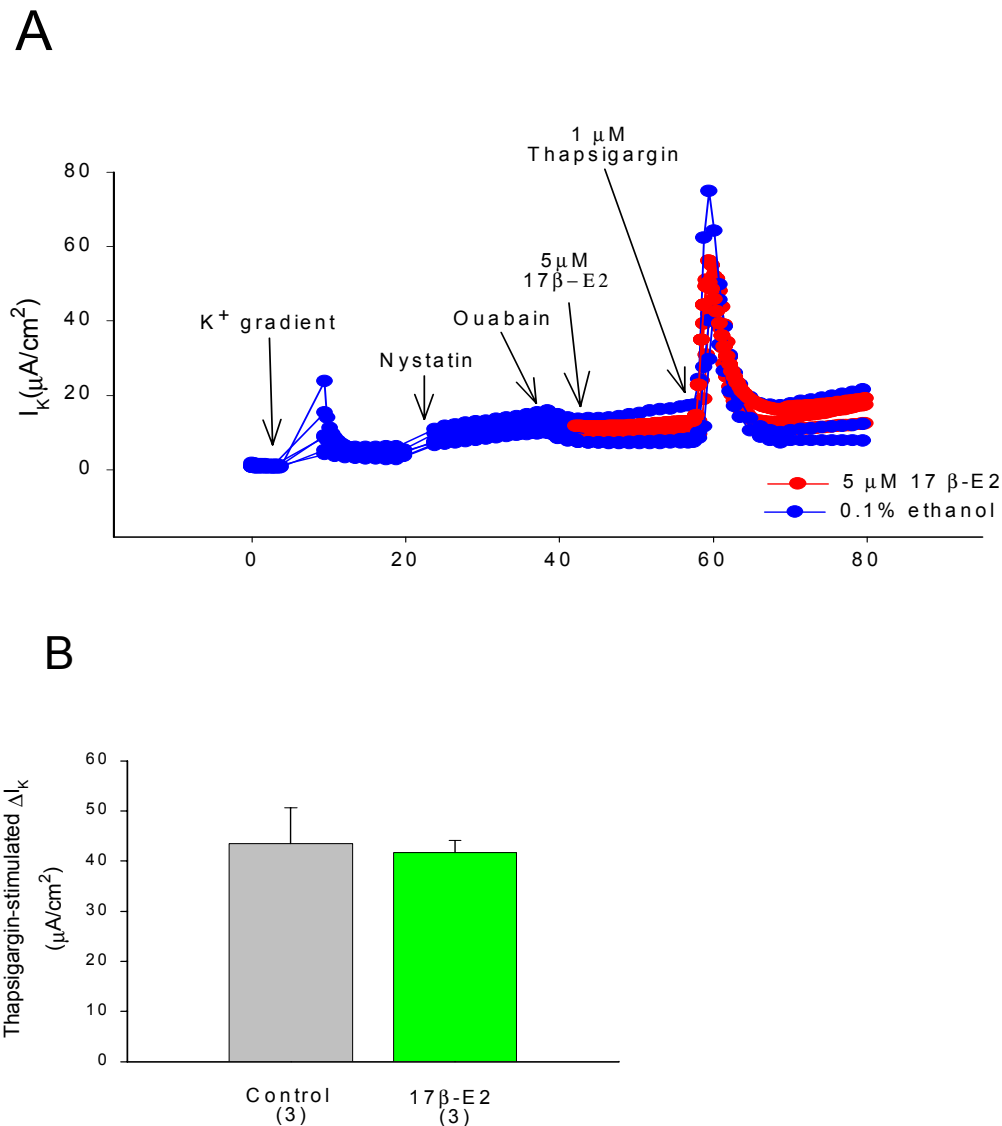


Figure 18 17  $\beta$ -estradiol does not increase the activity of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels in the basolateral membrane of T84 monolayers. T84 monolayers were mounted in Ussing chambers and voltage clamped. To measure basolateral membrane  $\text{K}^+$  channel activity the Ringer solution bathing monolayers was changed (at 0 min at A) to impose a 140:5 mM apical to basolateral  $\text{K}^+$  gradient. Next, the apical membrane was permeabilized to monovalent ions with nystatin (350  $\mu\text{g}/\text{ml}$ ) and ouabain (50  $\mu\text{M}$ ) was added to inhibit the spontaneous sodium current. 17  $\beta$ -estradiol (17 $\beta$ -E2) was added as indicated and after 20 min 1  $\mu\text{M}$  thapsigargin was added to activate basolateral  $\text{K}^+$  channels. Under these conditions, thapsigargin causes a rapid rise in potassium current ( $I_K$ ) that also decays rapidly. In the absence of a  $\text{K}^+$  gradient, there is no change in  $I_K$  after thapsigargin addition (not shown). Panel A presents individual current recordings from a typical experiment. Panel B shows the mean  $\pm$  SEM (n in parentheses) for the peak  $I_K$  responses. These results suggest that activation of basolateral membrane  $\text{K}^+$  channels is not the target for 17 $\beta$ -E2 action.

17  $\beta$ -estradiol Potentiation of  $\text{Ca}^{2+}$ -stimulated  $\text{Cl}^-$  Secretion is not Mediated through the PKC or MEK Pathways

T84 monolayers were mounted in Ussing chambers, voltage clamped, and preincubated with either bisindolylmaleimide (BIM), a protein kinase C (PKC) inhibitor, or PD 98059, a MAP kinase kinase (MEK) inhibitor. At this point the monolayers were treated with 17  $\beta$ -estradiol for 20 minutes prior to stimulation of  $\text{Cl}^-$  secretion with thapsigargin. BIM did not effect thapsigargin-stimulated  $I_{\text{SC}}$  or its potentiation by 17  $\beta$ -estradiol. PD 98059 caused a small and significant ( $p < 0.05$ ) potentiation of thapsigargin-stimulated  $I_{\text{SC}}$  but had no effect on 17  $\beta$ -estradiol potentiation. These observations suggest that the activation of PKC or MEK pathways are not involved in the 17  $\beta$ -estradiol effect (Figure 19).

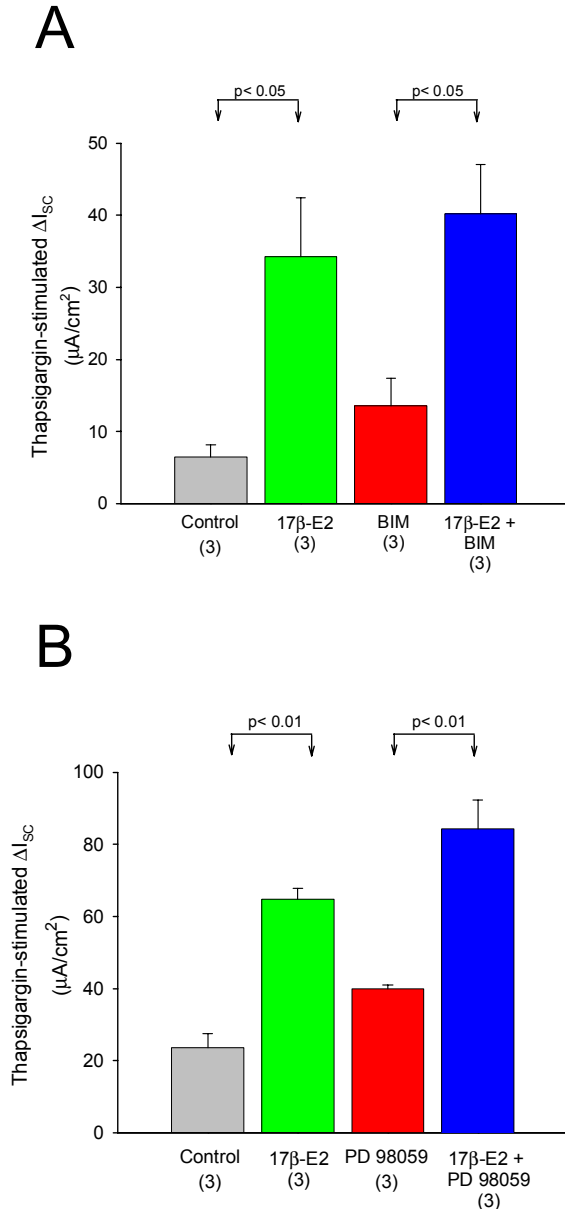


Figure 19 17  $\beta$ -estradiol Potentiation of Thapsigargin-stimulated  $Cl^-$  Secretion is not Mediated through the PKC or MEK Pathways. T84 monolayers were mounted in Ussing chambers, voltage clamped and preincubated for 30 min with either 1  $\mu M$  bisindolylmaleimide (BIM), a PKC inhibitor (A) or 20  $\mu M$  PD 98059, a MAP kinase kinase (MEK) inhibitor (B). Next, monolayers were treated with 5  $\mu M$  17  $\beta$ -estradiol (17 $\beta$ -E2) for 20 min prior to stimulation of  $Cl^-$  secretion with 1  $\mu M$  thapsigargin. Values shown are the mean  $\pm$  SEM (n in parentheses) for the peak thapsigargin responses. Neither BIM nor PD 98059 reduced thapsigargin-stimulated  $I_{sc}$  or its potentiation by 17 $\beta$ -E2 suggesting that activation of these pathways is not involved in the 17 $\beta$ -E2 effect.

17  $\beta$ -estradiol Potentiation of Thapsigargin-stimulated  $\text{Cl}^-$  Secretion is Dependent on the Presence of Extracellular  $\text{Ca}^{2+}$

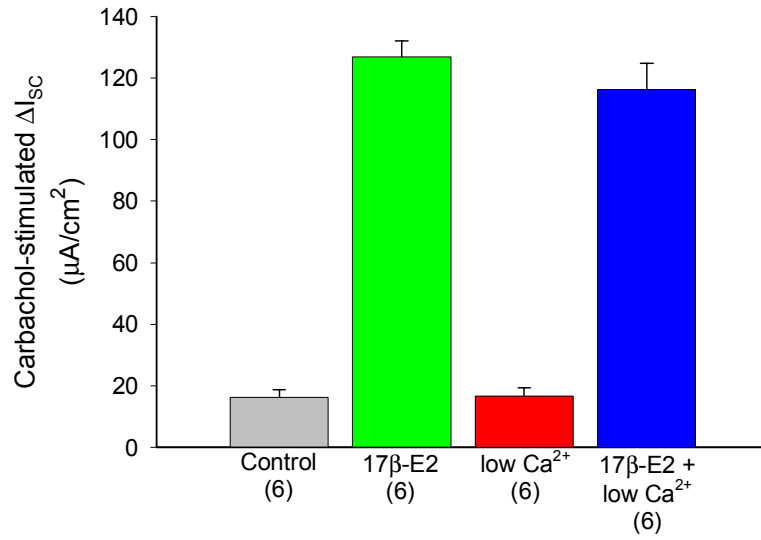
T84 monolayers were mounted in Ussing chambers, voltage clamped, and the Ringer solution bathing the monolayers was changed to Ringer solution containing low  $\text{Ca}^{2+}$  or zero  $\text{Ca}^{2+}$  Ringer. Next, monolayers were briefly treated with 17  $\beta$ -estradiol before stimulating  $\text{Cl}^-$  secretion with either thapsigargin or carbachol. 17  $\beta$ -estradiol potentiation of carbachol-stimulated  $I_{\text{SC}}$  did not change. However, 17  $\beta$ -estradiol potentiation of thapsigargin-stimulated  $\text{Cl}^-$  secretion was blunted in T84 monolayers bathed in low  $\text{Ca}^{2+}$  or zero  $\text{Ca}^{2+}$  Ringer (Figure 20).

Figure 20 17  $\beta$ -estradiol Potentiation of Thapsigargin-stimulated  $\text{Cl}^-$  Secretion is Dependent on the Presence of Extracellular  $\text{Ca}^{2+}$ . T84 monolayers were mounted in Ussing chambers, voltage clamped and the Ringer solution bathing the monolayers was changed to Ringer solution containing 100  $\mu\text{M}$   $\text{CaCl}_2$  (low  $\text{Ca}^{2+}$ ) in A, B, and C, and 0  $\mu\text{M}$  (zero  $\text{Ca}^{2+}$ ) in D. Next, monolayers were treated (for 20 min) with 50  $\mu\text{M}$  17  $\beta$ -estradiol (17 $\beta$ -E2) in A and B, and 5  $\mu\text{M}$  17 $\beta$ -E2 in C and D, prior to stimulation of  $\text{Cl}^-$  secretion with 100  $\mu\text{M}$  carbachol (A) or 1  $\mu\text{M}$  thapsigargin (B,C,D). No change in carbachol or thapsigargin-stimulated  $I_{\text{SC}}$  was observed in the presence of low  $\text{Ca}^{2+}$  or zero  $\text{Ca}^{2+}$  Ringer. 17 $\beta$ -E2 potentiation of carbachol-stimulated  $I_{\text{SC}}$  did not change in monolayers bathed in low  $\text{Ca}^{2+}$  Ringer, however 17 $\beta$ -E2 potentiation of thapsigargin-stimulated  $I_{\text{SC}}$  is blocked in monolayers bathed in low  $\text{Ca}^{2+}$  or zero  $\text{Ca}^{2+}$  Ringer. Values shown are the peak response for each group (mean  $\pm$  SEM, n in parentheses).

Figure 20 (continued)



**A**



**B**

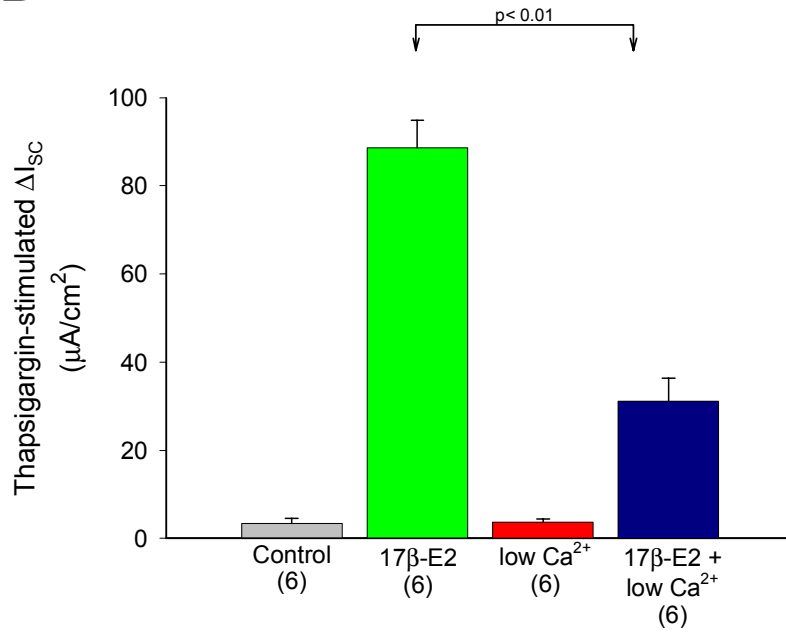
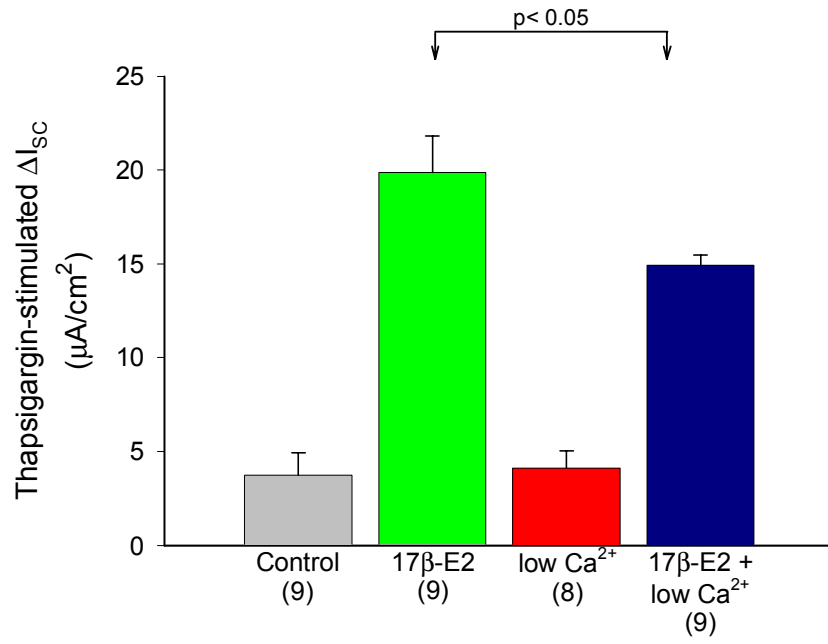
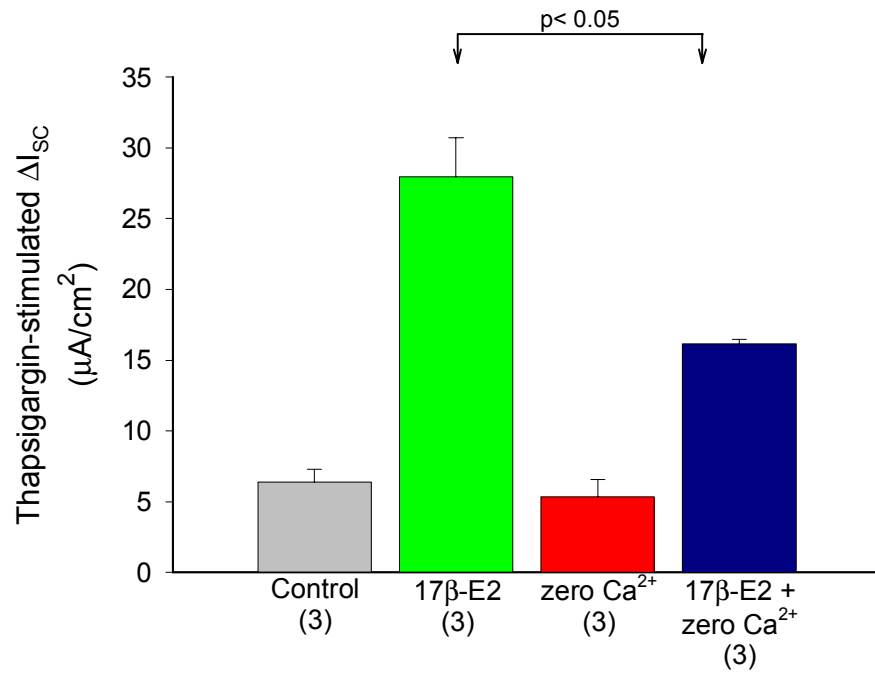


Figure 20 (continued)

C



D



### 17 $\beta$ -estradiol Decreases Intracellular $\text{Ca}^{2+}$ in Thapsigargin-stimulated T84 Cells

To study the effect of 17  $\beta$ -estradiol on intracellular  $\text{Ca}^{2+}$  in cells stimulated with thapsigargin, T84 cells suspended in HEPES saline buffer were loaded with fura-2 and treated with 17  $\beta$ -estradiol for 20 minutes. Next, the T84 cells were stimulated with 2  $\mu\text{M}$  thapsigargin. Intracellular  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ ) was determined by measuring fura fluorescence at 510 nm, using 340/380-nm dual wavelength excitation. A significant decrease in  $[\text{Ca}^{2+}]_i$  in cells pretreated with 17  $\beta$ -estradiol was observed (Figure 21). The thapsigargin-stimulated  $[\text{Ca}^{2+}]_i$  in 17  $\beta$ -estradiol-treated cells returned closer to basal level at the end of the experiment compared to untreated cells (Figure 21D).

T84 cells suspended in  $\text{Ca}^{2+}$  free HEPES saline buffer, briefly treated with 17  $\beta$ -estradiol also showed decrease in  $[\text{Ca}^{2+}]_i$  upon thapsigargin stimulation (Figure 22).

Figure 21 17  $\beta$ -estradiol Decreases Intracellular  $\text{Ca}^{2+}$  in Thapsigargin-stimulated T84 Cells. T84 cells suspended in HEPES saline buffer were loaded with fura-2 and briefly treated (20 min) with 17  $\beta$ -estradiol. Next, the T84 cells were stimulated with 2  $\mu\text{M}$  thapsigargin. Intracellular  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ ) was determined by measuring fura fluorescence at 510 nm, using 340/380-nm dual wavelength excitation. Panels A and C show significant decrease in  $[\text{Ca}^{2+}]_i$  in the presence of 100  $\mu\text{M}$  and 10  $\mu\text{M}$  17  $\beta$ -estradiol respectively. Panel B shows tracing from a representative experiment. Panel D shows the  $\Delta[\text{Ca}^{2+}]_i$  compared to basal concentration at the end of experiment in cells pretreated with 10  $\mu\text{M}$  17  $\beta$ -estradiol. The thapsigargin-stimulated  $[\text{Ca}^{2+}]_i$  in 17  $\beta$ -estradiol-treated cells returned closer to basal level at the end of the experiment compared to untreated cells.

Figure 21 (continued)

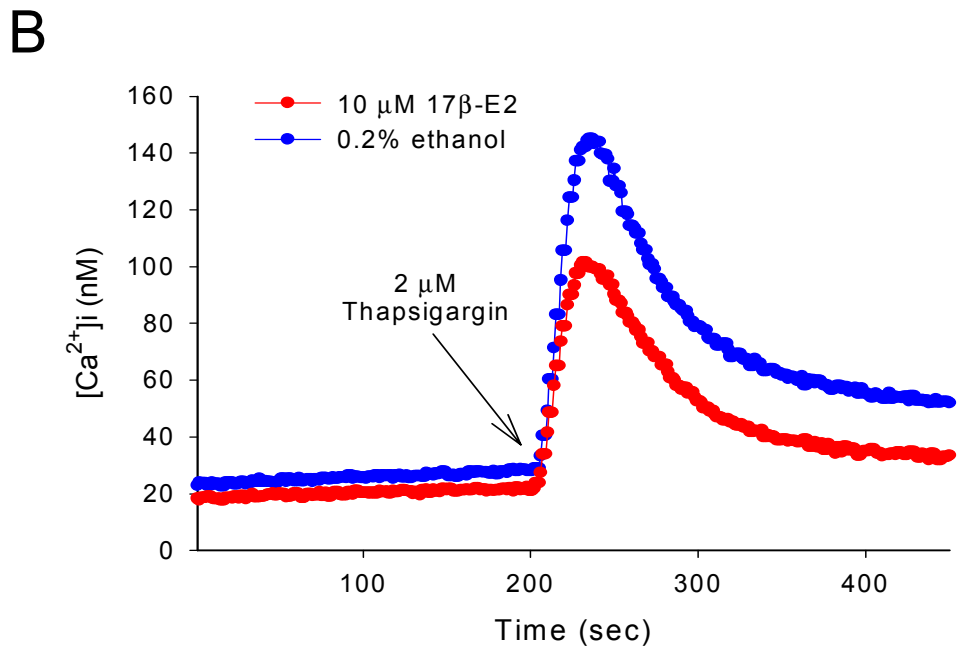
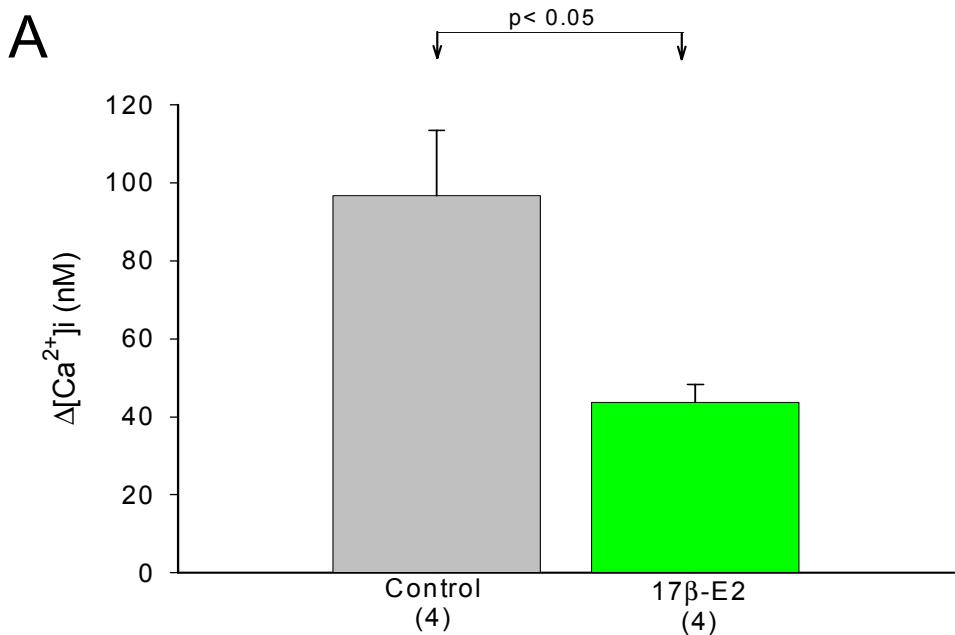
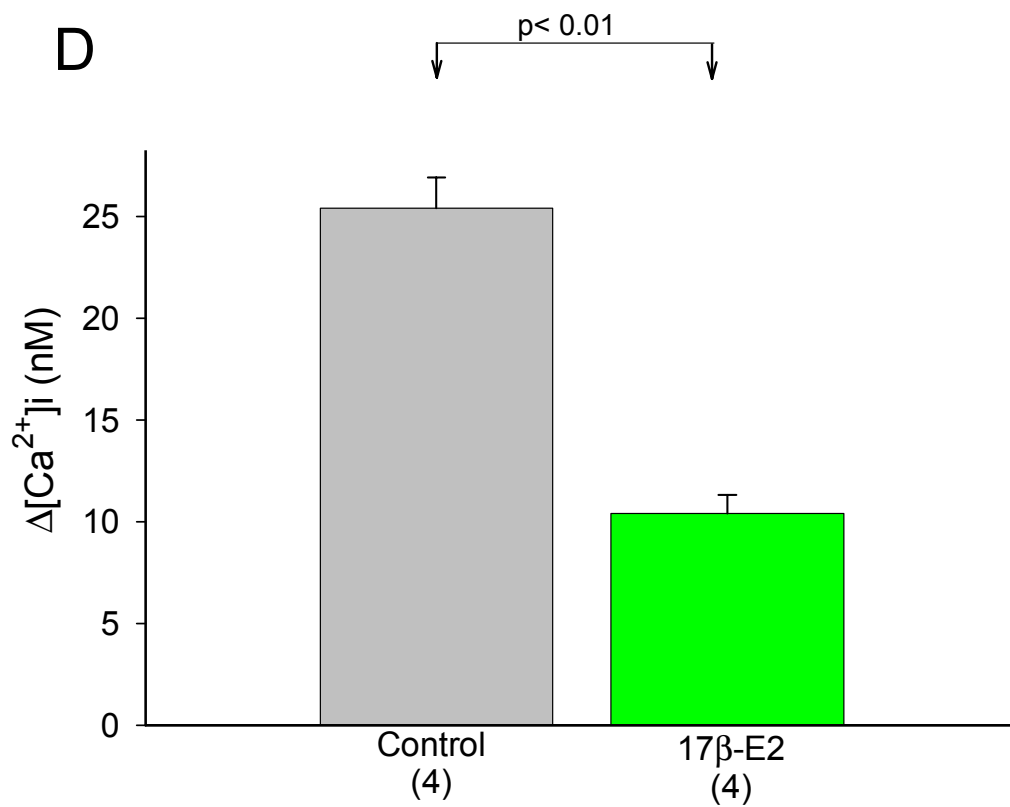
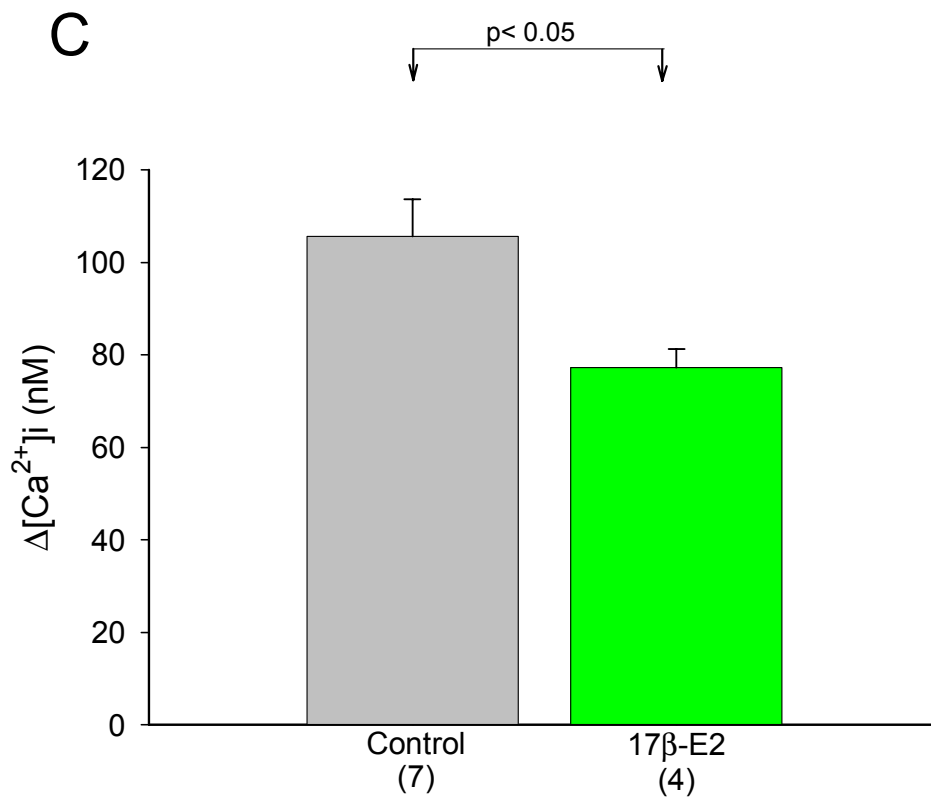


Figure 21 (continued)



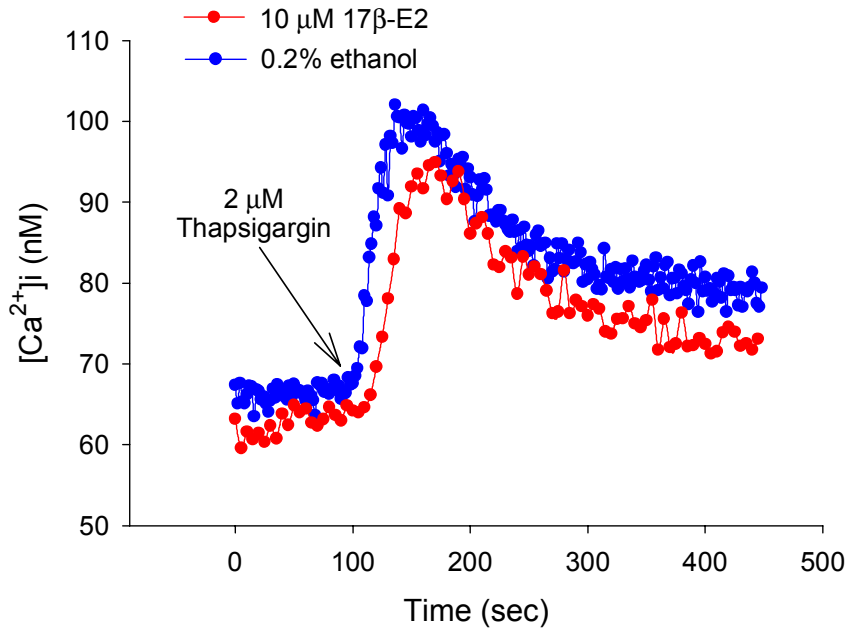
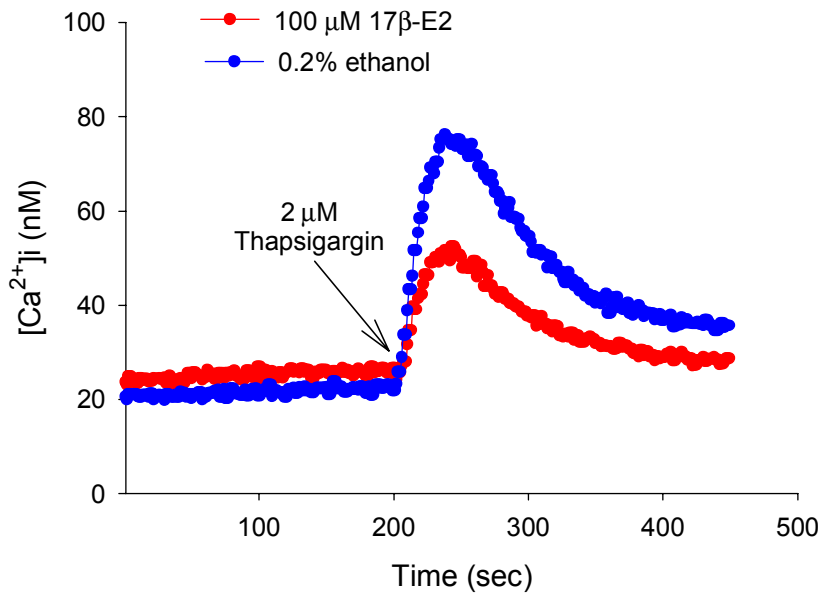
**A****B**

Figure 22 17  $\beta$ -estradiol Decreases Intracellular  $Ca^{2+}$  in Thapsigargin-stimulated T84 Cells. T84 cells were loaded with fura-2, suspended in  $Ca^{2+}$  free HEPES saline buffer and briefly treated (20 min) with 17  $\beta$ -estradiol. Next, the T84 cells were stimulated with 2  $\mu$ M thapsigargin. Intracellular  $Ca^{2+}$  ( $[Ca^{2+}]_i$ ) was determined by measuring fura fluorescence at 510 nm, using 340/380-nm dual wavelength excitation. Panels A and B (original tracings) show decrease in  $[Ca^{2+}]_i$  in the presence of 10  $\mu$ M and 100  $\mu$ M 17  $\beta$ -estradiol respectively.

## CHAPTER 4

### DISCUSSION

This study has demonstrated that steroids have two contrasting effects on epithelial chloride secretion. First, steroids inhibit cAMP-stimulated chloride secretion. Second, steroids potentiate  $\text{Ca}^{2+}$ -stimulated chloride secretion in T84 monolayers. Both these effects occur within minutes (or faster) suggesting that they are nongenomic.  $17\ \alpha$ -estradiol, a stereoisomer that does not activate any nuclear estrogen receptors, shows potency similar to  $17\ \beta$ -estradiol for inhibition and potentiation, confirming that these effects are nongenomic. Tamoxifen and the steroids corticosterone and d-aldosterone fail to inhibit cAMP-stimulated  $\text{Cl}^-$  secretion or potentiate  $\text{Ca}^{2+}$ -stimulated  $\text{Cl}^-$  secretion in T84 monolayers even at  $50\ \mu\text{M}$  concentrations, confirming that these nongenomic effects are not due to steroid induced changes in membrane fluidity. The inhibitory effects of sex steroids on  $\text{Cl}^-$  secretion is greater when applied to the apical side of the monolayers, consistent with steroids acting as blockers of the CFTR  $\text{Cl}^-$  channels (Singh and others 2000).  $17\ \alpha$ - and  $17\ \beta$ -estradiol caused minimum inhibition of forskolin-stimulated  $\text{Cl}^-$  secretion, but maximum potentiation of  $\text{Ca}^{2+}$ -stimulated  $\text{Cl}^-$  secretion. Diethylstilbestrol caused maximum inhibition of forskolin-stimulated  $\text{Cl}^-$  secretion, followed by progesterone and testosterone. At  $5\ \mu\text{M}$  steroid concentrations, diethylstilbestrol, progesterone, or testosterone do not have any significant effect on thapsigargin-stimulated  $\text{Cl}^-$  secretion.

My data suggest that the potentiation of  $\text{Ca}^{2+}$ -stimulated chloride secretion in T84 monolayers is an estrogen-specific effect. Amongst all the steroids tested,  $17\ \alpha$ - and  $17\ \beta$ -estradiol were the only steroids that consistently produced significant potentiation at a concentration as low as  $5\ \mu\text{M}$ . The steroids progesterone and testosterone produced potentiation

of thapsigargin-stimulated  $\text{Cl}^-$  secretion, but only at 50  $\mu\text{M}$  steroid concentrations. At 5  $\mu\text{M}$  steroid concentration, the inhibitory action of 17  $\beta$ -estradiol on cAMP-stimulated chloride secretion is minimal compared to its potentiating effect on  $\text{Ca}^{2+}$ -stimulated chloride secretion. Both the inhibitory and potentiating actions of 17  $\beta$ -estradiol occur simultaneously. 17  $\beta$ -estradiol causes a minimal inhibition of cAMP-stimulated chloride secretion by partially blocking CFTR  $\text{Cl}^-$  channels, but in the presence of increasing intracellular  $\text{Ca}^{2+}$  potentiates chloride secretion by stimulating the opening of additional conductive pathways. 17  $\beta$ -estradiol may potentiate  $\text{Ca}^{2+}$ -stimulated chloride secretion by opening store-operated calcium channels, increasing the sensitivity of CFTR activation to the rise in intracellular  $\text{Ca}^{2+}$  and triggering some additional signal transduction pathways.

Condliffe and others (2001) have reported that a physiological concentration of 17  $\beta$ -estradiol rapidly reduced the forskolin stimulation of  $\text{Cl}^-$  secretion in the distal colon of female rats. Additionally, in the rat distal colonic epithelium, a brief incubation of distal colonic epithelial sheets with 10-100 nM concentration of 17  $\beta$ -estradiol significantly reduced the  $\text{Cl}^-$  secretory responses to  $\text{Ca}^{2+}$  dependent secretagogue carbachol (Condliffe and others 2001). This study has demonstrated that supraphysiological concentration of 17  $\beta$ -estradiol inhibits cAMP-stimulated chloride secretion but enhances  $\text{Ca}^{2+}$ -stimulated chloride secretion in T84 monolayers. This difference is due to the model of  $\text{Cl}^-$  secretion used by Condliffe and others (2001). I have used the T84 colonic adenocarcinoma cell line (derived from a 72 year old man) to study colonic epithelial  $\text{Cl}^-$  secretion whereas Condliffe and others (2001) have used freshly dissected distal colonic epithelial sheets obtained from female Sprague-Dawley rats. Apart from direct effects of agonists on colonic epithelial cells, epithelial sheets are likely targets of indirect effects of enteric nerves or release of other factors from cells within lamina propria.



My observations with forskolin-stimulation are similar to those reported by Singh and others (2000), but there are subtle differences. Singh and others (2000) have observed that the synthetic estrogens (diethylstilbestrol and hexestrol) and the naturally occurring estrogens (17  $\alpha$ - and 17  $\beta$ -estradiol) were the most potent inhibitors of forskolin-stimulated  $\text{Cl}^-$  secretion followed by progesterone and testosterone. Tamoxifen also caused rapid, reversible, dose-dependent inhibition of forskolin-stimulated  $I_{\text{SC}}$  at a similar concentration as testosterone ( $K_i = 50 \mu\text{M}$ ). Contrary to these findings, my study has shown that diethylstilbestrol is the most potent inhibitor of  $\text{Cl}^-$  secretion, followed by progesterone, testosterone, and 17  $\alpha$ - and 17  $\beta$ -estradiol. Tamoxifen does not inhibit forskolin-stimulated  $\text{Cl}^-$  secretion in T84 monolayers.

There are several factors that may account for the differences in this study compared to that reported by Singh and others (2000). These differences are due to the differences in cell passage, culture media, and the culture plate inserts used. Singh and others (2000) have used culture media containing 10 % fetal bovine serum, they passaged their cells twice weekly, and their T84 cells were seeded in Costar Snapwell cell culture inserts (1.13  $\text{cm}^2$ ). I have used T84 culture media containing 5 % new born calf serum, passaged cells once a week, and the cells were seeded in Millicell-HA culture plate inserts (0.6  $\text{cm}^2$ ). In this study, cAMP-stimulated  $\text{Cl}^-$  secretion was induced by adding 10  $\mu\text{M}$  forskolin to the basolateral side of the monolayer. Singh and others (2000) stimulated  $\text{Cl}^-$  secretion by adding 10  $\mu\text{M}$  forskolin to both sides of the monolayer.

Carbachol acts by binding to a basolateral G protein-linked muscarinic receptor and causes subsequent hydrolysis of inositol phospholipids. Thapsigargin increases intracellular calcium by inhibiting  $\text{Ca}^{2+}$ -ATPase activity of the endoplasmic reticulum, independent of cell surface receptors and hydrolysis of inositol phospholipids. The carbachol-stimulated short-

circuit current ( $I_{SC}$ ) is more pronounced but considerably more transient compared to thapsigargin-stimulated  $I_{SC}$  because of negative regulation by mediators like epidermal growth factor, protein kinase C and inositol tetrakisphosphate (Barrett 1993; Barrett 1997). This explains why 17  $\beta$ -estradiol potentiation of carbachol-stimulated  $Cl^-$  secretion is only evident at higher concentrations (50  $\mu$ M or higher) of 17  $\beta$ -estradiol.

In most target organs, ER $\alpha$  is the predominant receptor (Lubahn and others 1993). However, the predominant estrogen receptor in the human colon and T84 cell line is ER $\beta$  (Campbell-Thompson and others 2001). A recent immunohistochemical study has confirmed that ER $\beta$  is highly expressed in superficial and crypt epithelium of normal colon in both males and females (Konstantinopoulos and others 2003). Tamoxifen binds to estrogen receptors and modulates their transcription function (Clarke and others 2001). Tamoxifen is referred to as a selective estrogen receptor modulator because it is known to act as agonist in some tissues but as antagonists in others (Grese and Dodge 1998; McDonnell 1999). Tamoxifen can show some agonistic activity with ER $\alpha$  but it is a pure antagonist for ER $\beta$  (McInerney and others 1998). Preincubation of T84 monolayers with tamoxifen does not cause reduction of 17  $\beta$ -estradiol potentiation of  $Ca^{2+}$ -stimulated  $Cl^-$  secretion indicating that the classical nuclear estrogen receptors are not involved. Future experiments should use pure estrogen receptor antagonists like ICI 164384 (Clarke and others 2001; Hall and others 2001) to confirm these findings.

The 17  $\beta$ -estradiol potentiation of  $Ca^{2+}$ -stimulated  $Cl^-$  secretion caused a significant increase in monolayer electrical conductance suggesting that 17  $\beta$ -estradiol opened additional ion channels following stimulation by carbachol or thapsigargin. DIDS, a disulfonic stilbene, is used to distinguish CFTR from other types of epithelial  $Cl^-$  channels. DIDS does not affect CFTR when added to the extracellular side of the membrane, but potently inhibits other types of  $Cl^-$

channels (Schultz and others 1999). The  $\text{Cl}^-$  channel inhibitor NPPB, an arylaminobenzoate, has been known to partially block single CFTR  $\text{Cl}^-$  channels in excised membrane patches (Zhang and others 2000), but it is not a specific inhibitor of the CFTR  $\text{Cl}^-$  channel (Schultz and others 1999). The 17  $\beta$ -estradiol potentiation of carbachol-stimulated  $\text{Cl}^-$  secretion was significantly decreased in monolayers treated with NPPB, suggesting blocking of CFTR  $\text{Cl}^-$  channels by NPPB, but no change in 17  $\beta$ -estradiol potentiation was observed in monolayers treated with DIDS. 17  $\beta$ -estradiol potentiation of thapsigargin-stimulated  $\text{Cl}^-$  secretion does not change when T84 monolayers are treated either with NPPB or DIDS. These observations prove that 17  $\beta$ -estradiol potentiation of  $\text{Ca}^{2+}$ -stimulated  $\text{Cl}^-$  secretion does not involve activation of  $\text{Cl}^-$  channels other than CFTR in T84 cells.

The opening and closing of the CFTR  $\text{Cl}^-$  channel is regulated by the balance of kinase and phosphatase activity within the cell and by cellular ATP levels. Activation of cAMP-dependent protein kinase (PKA) causes phosphorylation of multiple serine residues within the regulatory domain of CFTR (Figure 23). Once the regulatory domain is phosphorylated, gating of the CFTR channel is regulated by a cycle of ATP hydrolysis at the nucleotide-binding domains. Protein phosphatases dephosphorylate the regulatory domain to return the CFTR channel to its quiescent state (Sheppard and Welsh 1999).

H89, a specific and potent inhibitor of PKA, completely blocked 17  $\beta$ -estradiol potentiation of  $\text{Ca}^{2+}$ -stimulated chloride secretion, proving that the 17  $\beta$ -estradiol potentiation of  $\text{Ca}^{2+}$ -stimulated  $\text{Cl}^-$  secretion requires active CFTR  $\text{Cl}^-$  channels. Future experiments should be designed to measure the cAMP levels in 17  $\beta$ -estradiol treated T84 monolayers to determine whether 17  $\beta$ -estradiol causes an increase in cAMP in T84 cells.

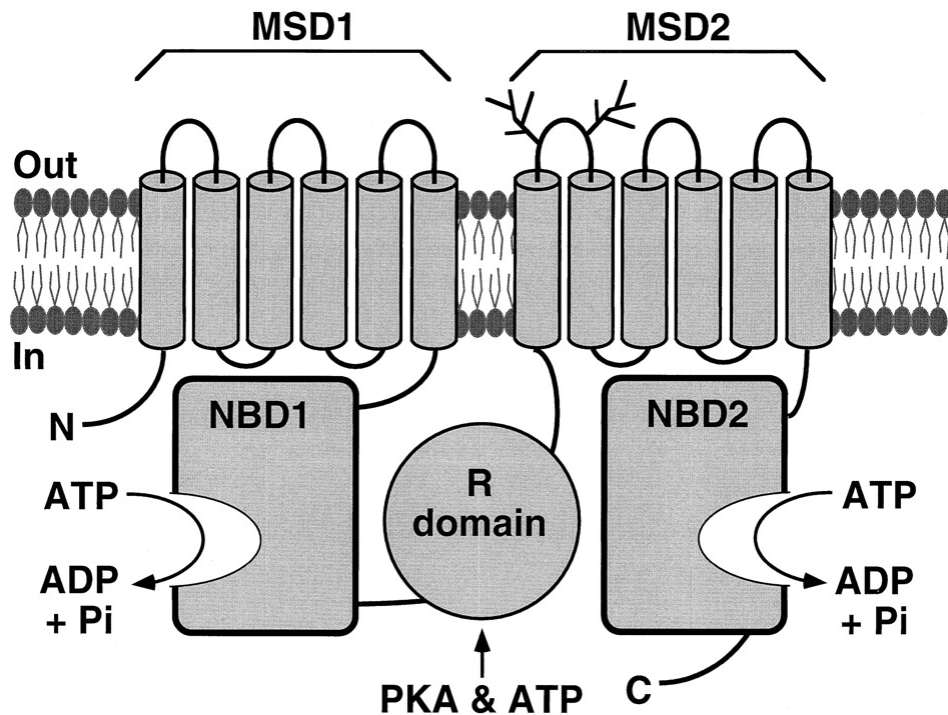


Figure 23 Model Showing Proposed Domain Structure of Cystic Fibrosis Transmembrane Regulator (CFTR). MSD, membrane-spanning domain; NBD, nucleotide-binding domain; R, regulatory domain; PKA, cAMP-dependent protein kinase (Sheppard and Welsh 1999).

To test whether 17  $\beta$ -estradiol increased the activity of  $\text{Ca}^{2+}$ -activated  $\text{K}^{+}$  channels in the basolateral membrane of T84 monolayers, the apical membrane was permeabilized with nystatin, and the spontaneous sodium current blocked by the addition of ouabain. Under these conditions, 17  $\beta$ -estradiol did not increase the thapsigargin-stimulated  $I_{\text{K}}$ , indicating that the activation of basolateral membrane  $\text{K}^{+}$  is not the target for the nongenomic action of 17  $\beta$ -estradiol. Future

experiments should be designed to study the apical CFTR activity by permeabilization of the basolateral membrane.

There are many references in the literature to suggest that 17  $\beta$ -estradiol can stimulate the activity of PKA, PKC, and/or MAP kinase pathways. In the rat brain 17  $\beta$ -estradiol acts on estrogen receptors locally within the hippocampus through a nongenomic mechanism to activate PKA and PKC, to phosphorylate the serotonin-1A receptors and uncouple them from their G proteins (Mize and Alper 2002). In intracellular recordings from female guinea pig hypothalamic slices it has been shown that 17  $\beta$ -estradiol rapidly activates both PKA and PKC to cause heterologous desensitization of mu-opioids and GABA(B) receptors, which has the potential to alter synaptic transmission in many regions of the central nervous system (Kelly and others 1999). 17  $\beta$ -estradiol increases oviductal protein phosphorylation in rats by a nongenomic action mediated by PKA and PKC (Orihuela and Croxatto 2001). In the human hepatoma HepG2 cell line 17  $\beta$ -estradiol has been found to rapidly activate PKC $\alpha$  translocation and ERK-2/mitogen-activated protein kinase phosphorylation (Marino and others 2002). The estrogen receptor antagonist ICI 182780 and the MEK inhibitor PD 98059 prevented 17  $\beta$ -estradiol-mediated-neuroprotection indicating that activation of the MAP kinase pathway is an important part of the neuroprotective mechanism (Mize and others 2003). In primary cultured rabbit kidney proximal tubule cells 17  $\beta$ -estradiol-BSA conjugate rapidly stimulates Ca<sup>2+</sup> uptake, which is mediated by cAMP and PKC pathways (Han and others 2000). 17  $\beta$ -estradiol causes a dose-dependent increase in PKC $\alpha$  in resting zone and growth zone chondrocytes in female rats (Sylvia and others 1998). In a human endometrial cell line (RL95-2) 17  $\beta$ -estradiol stimulates capacitance Ca<sup>2+</sup> entry through store-operated calcium channels via a PKC-sensitive pathway (Perret and others 2001). In the female rat distal colonic crypts the 17  $\beta$ -estradiol induced increase in intracellular

Ca<sup>2+</sup> involves activation of PKC $\delta$  (Doolan and Harvey 2003), and inhibition of forskolin-induced Cl<sup>-</sup> secretion is dependent on PKC activation (Condliffe and others 2001). Using the PKC inhibitor BIM (bisindolylmaleimide) and the MEK inhibitor PD 98059, this study has shown that the 17  $\beta$ -estradiol potentiation of Ca<sup>2+</sup>-stimulated Cl<sup>-</sup> secretion is not mediated through the PKC or MEK pathways.

The idea that calcium might enter cells through a capacitative store-operated mechanism was first introduced by Jim Putney almost two decades ago (Putney 1986). The hypothesis was that calcium entry is regulated by the state of filling of the calcium stores. By analogy with a capacitor in an electrical circuit, the calcium stores prevent entry when they are charged up but immediately begin to promote entry as soon as stored calcium is discharged. This capacitative entry mechanism is present in many excitable and nonexcitable cells (Putney 1986; Putney 1990; Berridge 1995; Putney 2003). Capacitative calcium entry can be switched on by a great variety of stimuli such as the calcium-mobilizing second messenger inositol 1,4,5-trisphosphate (Petersen and Berridge 1994; DeLisle and others 1995; Birnbaumer and others 2000; Raeymaekers and others 2002), the calcium ionophore ionomycin (Morgan and Jacob 1994), and inhibitors of the endoplasmic reticulum pumps such as thapsigargin (Petersen and Berridge 1994; Denys and others 2004). There is no consensus on precisely how store-operated calcium entry is controlled in electrically nonexcitable cells (Elliott 2001; Smani and others 2004).

It has been reported that in T84 monolayers, a capacitative store-operated calcium pathway is located in the basolateral membrane (Kerstan and others 1999). In the human endometrial cell line (RL95-2) 17  $\beta$ -estradiol-induced Ca<sup>2+</sup> influx is significantly increased by the depletion of intracellular stores by thapsigargin and decreased by chelerythrine chloride, an inhibitor of protein kinase C. These data indicate a nongenomic action of 17  $\beta$ -estradiol to

stimulate capacitative  $\text{Ca}^{2+}$  entry through store-operated calcium channels (Perret and others 2001). This study shows that the 17  $\beta$ -estradiol potentiation of thapsigargin-stimulated  $\text{Cl}^-$  secretion is dependent on the presence of extracellular  $\text{Ca}^{2+}$ . 17  $\beta$ -estradiol potentiation of thapsigargin-stimulated  $\text{I}_{\text{SC}}$  is blocked in T84 monolayers bathed in low  $\text{Ca}^{2+}$  or zero  $\text{Ca}^{2+}$  Ringer. This suggests that 17  $\beta$ -estradiol opens up additional store-operated calcium channels and increases  $\text{Ca}^{2+}$  entry in thapsigargin-stimulated monolayers by a rapid nongenomic mechanism. Thapsigargin increases intracellular calcium by inhibiting  $\text{Ca}^{2+}$ -ATPase activity of the endoplasmic reticulum. 17  $\beta$ -estradiol might act by releasing additional  $\text{Ca}^{2+}$  from intracellular stores in the presence of thapsigargin, providing the stimulus for opening of store-operated calcium channels. Elevation in intracellular  $\text{Ca}^{2+}$  opens additional basolateral potassium channels, providing additional driving force for  $\text{Cl}^-$  exit through constitutively open CFTR channels in the apical membrane. However, 17  $\beta$ -estradiol potentiation of carbachol-stimulated  $\text{I}_{\text{SC}}$  did not change in monolayers bathed in low  $\text{Ca}^{2+}$ . This is probably due to the difference in the mechanism by which carbachol raises intracellular  $\text{Ca}^{2+}$ . Carbachol binds to a basolateral G protein-linked muscarinic receptor, and yields calcium-mobilizing messenger  $\text{Ins}(1,4,5)\text{P}_3$  via activation of phospholipase C and phosphatidylinositol hydrolysis. In 17  $\beta$ -estradiol treated monolayers, carbachol might not be producing enough  $\text{Ins}(1,4,5)\text{P}_3$  to cause sufficient depletion of intracellular  $\text{Ca}^{2+}$  stores necessary to stimulate the opening of store-operated calcium channels.

Contrary to expectations, T84 cell suspensions treated with 17  $\beta$ -estradiol showed significant lower peak  $[\text{Ca}^{2+}]_i$  upon thapsigargin-stimulation compared to controls. To enhance cell viability, T84 cultures were trypsinized the day before each experiment and maintained overnight in suspension. Preincubation of T84 cells with 17  $\beta$ -estradiol alone (unless stimulated

with thapsigargin) did not change  $[Ca^{2+}]_i$ . The thapsigargin-stimulated  $[Ca^{2+}]_i$  in 17  $\beta$ -estradiol-treated cells returned closer to basal level at the end of the experiment compared to untreated cells.

Cell polarity, a distinguishing feature of epithelial cells, is a prerequisite to the transepithelial transport of solutes (McRoberts and Barrett 1989). T84 cells, when grown on semipermeable supports develop tight junctions, form polarized monolayers of cells with distinct apical and basolateral membranes. In suspension, these cells lose their polarity, their tight junctions are disrupted; they form rounded cells, and hence behave differently from T84 cells grown in a monolayer. My experiments with T84 cell suspensions suggest that 17  $\beta$ -estradiol causes different and distinct nongenomic effects in cell suspension. These differences are due to loss of polarity in suspension and subsequent rearrangement of the cytoskeleton. Remnants of trypsin in cell suspension might make the cells fragile and affect the rapid responses of 17  $\beta$ -estradiol. Future experiments should be designed to measure  $[Ca^{2+}]_i$  in 17  $\beta$ -estradiol treated T84 monolayers.

17  $\beta$ -estradiol inhibits cAMP-stimulated chloride secretion by acting as a CFTR channel blocker and binding directly to CFTR (Singh and others 2000), but potentiates  $Ca^{2+}$ -stimulated chloride secretion by enhancing the calcium sensitivity of CFTR activation. 17  $\beta$ -estradiol increases monolayer electrical conductance but does not increase the activity of calcium-activated potassium channels or any calcium-activated apical chloride channels. 17  $\beta$ -estradiol potentiation is not mediated via the PKC or MEK pathways and does not require the classical nuclear estrogen receptors. 17  $\beta$ -estradiol might act by opening additional basolateral store-operated calcium channels or opening additional apical CFTR channels. Future experiments should use 17  $\beta$ -estradiol conjugated to bovine serum albumin to determine the role of any



unidentified membrane receptor in 17  $\beta$ -estradiol potentiation. Better understanding of the nongenomic actions of estrogens on epithelial  $\text{Cl}^-$  secretion will open new perspectives in the pharmacological treatment of pathological conditions like cystic fibrosis and secretory diarrhea.

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