Estrogen Inhibition of Cystic Fibrosis Transmembrane Conductance Regulator-Mediated Chloride Secretion

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Accepted for publication June 12, 2000 This paper is available online at http://www.jpet.org

ABSTRACT

Cystic fibrosis (CF) is an autosomal genetic disease associated with impaired epithelial ion transport. Mutations in the CF gene alter the primary sequence of the CF transmembrane conductance regulator (CFTR). Several therapeutic modalities have been proposed for CF patients, including the phytoestrogen genistein. Experiments were completed in cellular and subcellular systems to evaluate the impact of naturally occurring and synthetic estrogens on epithelial ion transport, and specifically on the CF protein CFTR. 17β-Estradiol, a naturally occurring estrogen, caused a rapid and reversible inhibition of forskolin-stimulated chloride secretion across T84 epithelial cell monolayers with a Ki of 8 μM. In addition, 17α-estradiol, a stereoisomer that fails to bind and activate nuclear estrogen receptors was equipotent with 17β-estradiol, arguing against a genomic-mediated mechanism of action. Synthetic estrogens, including diethylstilbestrol and the antiestrogen tamoxifen likewise inhibited forskolin-stimulated ion transport. Aldosterone, dexamethasone, and cholesterol were without effect at the highest concentrations tested (≥1 mM). Studies indicated that diethylstilbestrol and other synthetic estrogens that inhibited anion secretion in intact monolayers likewise inhibited CFTR chloride channel activity with similar concentration dependencies in excised membrane patches. Experiments with radioactive photoactivatable estrogen derivatives demonstrated that these compounds bind directly to CFTR expressed in insect cells. Taken together, the data suggest that estrogens can interact directly with CFTR to alter anion transport.

Cystic fibrosis (CF) is the most common lethal genetic disease of Caucasians, affecting some 1:2500 live births in the United States (Welsh et al., 1995). The genetic basis of this autosomal recessive disease has been traced to a defect in the gene on chromosome 7 that encodes a CAMP-regulated chloride channel, the CF transmembrane conductance regulator (CFTR). Defective CAMP-mediated chloride secretion and increased apical membrane sodium absorption in CF patients results in abnormal airway surface liquid, defective mucociliary clearance, and bacterial infection (Pilewski and Frizzell, 1999). At the molecular level, there are several mechanisms whereby mutations in CFTR produce a loss of, or impaired, CAMP-dependent chloride conductance (Welsh and Smith, 1993). One potential strategy to treat CF patients has been to identify pharmacological agents that restore normal function to the mutant forms of CFTR.

One such group of agents that has received much attention is the flavones and isoflavones. The effects of flavones on CFTR-mediated ion transport were first reported by Nguyen et al. (1991), who showed that quercetin and kaempferol stimulated chloride secretion across T84 human colonic epithelial monolayers. Subsequently, the isoflavone genistein was shown to be an activator of wild-type (wt) CFTR both in vitro and in vivo in human subjects (Illek and Fischer, 1998; Schultz et al., 1999). Although it is often reported that genistein is a specific tyrosine kinase inhibitor (Akiyama et al., 1987), initial reports regarding genistein also dealt with estrogenic effects of this class of compounds (Schultz et al., 1999). Thus, genistein and other isoflavones are phyestrogens, naturally occurring compounds that can activate estrogen receptors. Indeed, isoflavones are weak estrogens and can function both as estrogen agonists and antagonists, depending on the hormonal milieu and target tissue. As such, genistein, one of two primary isoflavones in soybean, has attracted much attention from the research community be-

ABBREVIATIONS: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; Isc, short-circuit current; FBS, fetal bovine serum; PKA, protein kinase A; i, single-channel current; I, mean current; fc, corner frequency; PAGE, polyacrylamide gel electrophoresis; CPTcAMP, 8-chlorophenyl-thio-cAMP; DES, diethylstilbestrol; ENac, epithelial sodium channel; GABA, γ-aminobutyric acid; wt, wild-type.
cause of its potential antiestrogenic effects. Because genistein and other flavones and isoflavones are currently being proposed as therapeutic agents for the treatment of CF, and because such compounds display phytoestrogenic properties, it is important to understand the effects of estrogens and phytoestrogens on ion transport properties in normal and CF cells.

The major focus of attention regarding estrogens and CFTR has been directed toward genomic effects, where, for example, estrogens have been shown to regulate CFTR expression levels (Rochwerger and Buchwald, 1995). In addition, androgens and estrogens have been reported to differentially affect CFTR expression in developing fetal rat lung epithelium (Sweezey et al., 1997). Studies also have shown that estrogens are required to maintain the functional competence of the exocrine pancreas and are responsible for the cyclic changes in airway goblet cell number during the menstrual cycle (Tausig, 1984). Thus, estrogenic influences not only affect CFTR expression levels but also affect the physiology of two of the most severely affected organs in CF, namely, the airways and the pancreas. Moreover, it has been shown that pancreatic tissue from non-CF patients contains high levels of [3H]estradiol-binding activity, whereas no such binding activity was observed in CF pancreatic tissue (Grossman et al., 1987).

Besides the classical genomic effects of steroid hormones, it is now apparent that several steroids interact with membrane receptors to cause rapid responses in various cell types (McEwen, 1991; Kelly and Wagner, 1999; Levin, 1999; Watson et al., 1999; Norfleet et al., 2000). In addition, steroids have been shown to influence the kinetic properties of both anion and cation channels. Thus, vitamin D₃ has been shown to be a potent modulator of osteosarcoma calcium channels (Caffrey and Farach-Carson, 1989; Farach-Carson et al., 1991), and estrogens modulate potassium channels by a cell membrane-delimited cGMP-mediated pathway (White et al., 1995) or by direct interaction with the channels themselves (Valverde et al., 1999). Anion channels are similarly modulated by steroids, affecting chloride currents in tissues as diverse as neural tissue (Gee et al., 1987), osteoblasts (Zanello and Norman, 1997), and aortic endothelial cells (Li et al., 2000). Horwitz and coworkers (Yang et al., 1989; Greenberger et al., 1990) also have demonstrated that progesterone binds P-glycoprotein and blocks P-glycoprotein-mediated drug transport. Moreover, many drugs that are transported by P-glycoprotein also block swelling-induced currents (Valverde et al., 1997). Cyclic AMP (cAMP) is a ubiquitous second messenger that is involved in a variety of physiological processes, including secretion, transport, and cell cycle control. The catalytic subunit of cAMP-dependent protein kinase A (PKA) was examined to an upper frequency of 140 Hz. Latter experiments in the series were acquired at a sampling rate of 800 Hz.

### Materials and Methods

**Cell Culture.** T84 cell monolayers (Dharmsathaphorn et al., 1984) were grown in Dulbecco’s modified Eagle’s medium-Ham’s F12 (1:1; Life Technologies, Inc., Gaithersburg, MD) containing 10% fetal bovine serum (FBS). The cells were incubated in a humidified atmosphere containing 5% CO₂ at 37°C. For measurements of short-circuit current (Isc), T84 cells were seeded on Costar Snapwell cell culture inserts (1.13 cm²), and the culture medium was changed every second day. L cells, a murine fibroblast cell line stably expressing wt-CFTR, were maintained as previously described (Yang et al., 1993). Briefly, cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were passaged twice weekly. For patch-clamp studies, cells were plated onto plastic coverslips coated with human placental collagen (collagen type VI; Sigma Chemical Co., St. Louis, MO) and channel activity was evaluated 2 to 4 days after plating. Si9 cells (Invitrogen, San Diego, CA), a cultured cell line from the insect Spodoptera frugiperda, were maintained in Grace’s medium at 27°C to which was added yeastolate and lactalbumin hydrolysate together with 10% FBS.

**Isc Measurements.** Costar Snapwell cell culture inserts were mounted in an Ussing chamber (Jim’s Instruments, Iowa City, IA) and the monolayers short-circuited continuously. Transepithelial resistance was measured periodically by applying a 56-mV pulse, and the resistance calculated from Ohm’s Law. Steroids and forskolin were added to both sides of the monolayers at the indicated concentrations. Amiloride was added only to the mucosal bathing solution. The rat colonic mucosa from dexamethasone-treated rats (male Sprague-Dawley; 4 mg/kg for 3 days) was prepared as previously described (Bridges et al., 1989). Rats were treated with dexamethasone to induce expression of greater amiloride-sensitive sodium-absorptive current. Changes in Isc were calculated as a difference between the sustained phase of the response and their respective baseline values.

**Patch-Clamp Recordings and Analysis.** Patch-clamp experiments were performed by using excised inside-out membrane patches from L cells expressing wt-CFTR. The data were acquired and analyzed as described previously (Venglarik et al., 1994) with minor modifications. All experiments were performed at 34–37°C unless otherwise stated, with membrane potentials held at ~80 mV (bath versus pipette) so that negative currents (shown as downward deflections) represent chloride channel openings. Cells were exposed to forskolin (2–5 μM) to endogenously phosphorylate CFTR before patch excision into a bath containing ATP. In some patches, the catalytic subunit of CAMP-dependent protein kinase A (PKA) was added to the bath after patch excision to ensure maximal CFTR channel activation. Unless otherwise noted, the 0.75-ml bath was refreshed at a rate of 4 bath volumes/min during the control and treatment periods. Recordings of up to 25 min in length were analyzed for each membrane patch. Single-channel current (i) was determined based on fits of multi-Gaussian functions to amplitude histograms of the current records without constraining the peak amplitudes to be equally spaced and thus further document that a homogenous population of channels was being evaluated. Mean channel amplitude during each treatment period (e.g., duration of exposure to a unique combination of compounds) was calculated as the average distance between peaks. Mean current (I) was determined by averaging all data points in the current record during the treatment period. Current records were visually examined for the duration of patch viability to determine the number of actively gating channels present in the patch (i.e., the maximum number of channels simultaneously open in conditions that maximize channel activity, e.g., ~0.3 mM ATP with PKA (Horn, 1991; Venglarik et al., 1994). Fluctuation analysis and estimation of the corner frequency (fc) was performed by using Bio-Patch software (version 3.30; Molecular Kinetics Inc., Pullman, WA) as previously described (Venglarik et al., 1994). Data were prepared for presentation by using SigmaPlot (version 4.0 for Windows; Jandel Scientific, San Rafael, CA). Values are presented as the mean ± S.E. unless otherwise noted.

The analog recording and digital acquisition apparatus were as previously described (Venglarik et al., 1994; Schultz et al., 1995). Initially, digitized files were acquired at a sampling frequency of 400 Hz (low-pass 8 pole Bessel filter at 200 Hz; 902LPF, Frequency Devices, Haverhill, MA) and analyzed by using Bio-Patch software as previously described with the exception that power density spectra were examined to an upper frequency of 140 Hz. Latter experiments in the series were acquired at a sampling rate of 800 Hz.
(low-pass 8 pole Bessel filter at 300 Hz) and analyzed to an upper frequency of 200 Hz. For clarity of presentation, most data are plotted at a frequency of 200 Hz. Some records were acquired at sampling frequencies of up to 2 kHz (low-pass filter at 800 Hz) to determine whether additional Lorentzian components could be identified in this portion of the spectra, and for high-resolution presentation. For analysis, all data points for a treatment period were included in the construction of amplitude histograms. However, for presentation, data sets were restricted to 30-s duration so that the reader can directly compare the area under the fitted curves.

**Solutions for Patch-Clamp Experiments.** The pipette solution contained 140 mM N-methyl-d-glucamine-HCl, 1 mM CaCl$_2$, 2 mM MgCl$_2$, and 10 mM Bis-tris propane. The bathing solution contained 150 mM NaCl, 2 mM MgCl$_2$, 10 mM NaF, 0.5 mM EGTA, 0.26 mM CaCl$_2$, 0.3 mM ATP, and 10 mM Bis-tris propane. The pH of both the bath and pipette solutions was maintained between 7.33 and 7.37 in all experiments. Free Ca$^{2+}$ concentration in the bath was calculated to be 100 nM (Brooks and Stovey, 1992). Fluoride was included as a nonspecific inhibitor of any phosphatases that might be present at excision and can lead to channel inactivation (Tabcharani et al., 1991). We have previously evaluated wt-CFTR channel activity in the presence and absence of NaF and could identify no difference in kinetic behavior (Schultz et al., 1995). The disparity in this regard with the report by Berger et al. (1998) is at this time unresolved.

**Photoaffinity Labeling of CFTR.** The Sf9 S. frugiperda cell line was used for heterologous expression of the cloned human CFTR cDNA or the cloned $\beta$-galactosidase gene, by using the baculovirus Autographica californica as the infection vector (Larsen et al., 1996). Seventy-two hours postinfection, Sf9 cells were homogenized in sucrose-containing buffer and centrifuged (3000 g for 10 min) to yield a postnuclear supernatant. Samples were then subjected to centrifugation on a discontinuous sucrose density gradient to yield a fraction enriched in CFTR, which accumulated a 20% w/v sucrose, 40% w/v sucrose interface. Equilibrium-binding studies were performed as described (Nelson et al., 1992). Samples (500 $\mu$L) containing 100 ng of CFTR-enriched membrane protein, 30 nM [3H]protoioaryl azide (LY 110718) (14.8 Ci/mmol), or 30 nM [3H]hexestrol diazirine (30.76 Ci/mmol) were incubated at 4°C in the dark for 30 min in the absence or presence of 100-fold excess unlabeled compound. Samples were pipetted onto Parafilm and irradiated at 23°C in a UV cross-linker (Fisher Scientific). The energy settings for the cross-linker were factory calibrated at 2 J/cm$^2$ as described (Nelson et al., 1992). After cross-linking, samples were solubilized and subjected to immunoprecipitation as previously described (Gregory et al., 1990) by using a monoclonal antibody directed against CFTR (R&D Systems, Minneapolis, MN). Immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis (PAGE), the gel dried, and subjected to autoradiography. Gels were exposed to X-ray film (Kodak X-OMAT) for 3 to 4 weeks at ~80°C.

**Chemicals.** Disodium-ATP was obtained from Boehringer Mannheim (Indianapolis, IN). Forskolin was from Calbiochem (La Jolla CA). 8-Chlorophenyl-thio-cAMP (CPTcAMP) was from Sigma Chemical Co. The catalytic subunit of cAMP-dependent PKA was from Promega (Madison, WI). [3H]Protoioaryl azide and [3H]hexestrol diazirine were synthesized as previously described (Pinney et al., 1991; Pinney and Katzenellenbogen, 1991; Bergmann et al., 1994). All other chemicals were obtained from Sigma and were of reagent grade quality.

**Results**

**Synthetic Estrogens Inhibit CFTR Chloride Channel Activity.** Results presented in Fig. 1 demonstrate that diethylstilbestrol (DES), a synthetic estrogen, has a profound and reversible inhibitory effect on the channel activity of CFTR when recorded in the excised membrane patch configuration. Results are from a continuous recording of a membrane patch containing three CFTR chloride channels. After recording in control conditions, the continuous perfusion of the cytosolic bath was changed to include DES (30 $\mu$M). An immediate inhibition of channel activity was observed such that $I$ declined from 2.1 to 0.6 pA. This reduced level of CFTR chloride channel activity was maintained for more than a minute until the solution perfusing the cytosolic face was changed to wash out the DES. $I$ immediately returned nearly to pretreatment levels (1.8 pA). Further evidence presented in Fig. 1 suggests that the DES-induced reduction in CFTR-mediated chloride current is, in part, attributable to a short-lived block of actively gating channels. In the presence of DES, CFTR chloride channels are more likely to be in a nonconductive (i.e., blocked) state (Fig. 1, A and B). Within the resolution of the system, complete openings to the control channel amplitude were less likely to be observed in the presence of DES. The amplitude histogram (Fig. 1C) shows that DES reduced the precision with which single-channel amplitude could be determined. The computed standard deviation of the closed and first open level increased with DES exposure from 0.19 to 0.26 and from 0.20 to 0.25, respectively, and the apparent single-channel amplitude was reduced from 0.92 to 0.70 pA. In the presence of DES the second and third open levels could not be resolved. The broadening of histogram peaks and the reduction in amplitude are consistent with open-to-blocked and blocked-to-open transitions occurring at frequencies above the resolution of the recording device (Venglarik et al., 1994); an open or blocked dwell-time of <3.5 ms would be attenuated in the present conditions. Finally, fluctuation analysis (Fig. 1D) shows that power associated with low frequency (1–4 Hz) nucleotide-dependent gating is dramatically reduced and that power is increased at higher frequencies (30–50 Hz) when DES is present. The increase in power at higher frequencies is consistent with short-lived events that would not be well resolved in the time domain. Again, washout of the DES resulted in a complete reversal of the DES-induced effects (data not shown).

Results presented in Fig. 1 are typical of 18 such experiments with various concentrations of DES. At 100 $\mu$M, inhibition of channel activity was immediate and complete ($n = 4$). Over the treatment periods used in these experiments (2–3 min), membrane patches exposed to 100 $\mu$M became unstable and broke. Thus, attempts to achieve reversal with washout were inconclusive. Complete reversal on washout was observed for all other conditions tested. DES (30 $\mu$M) was associated with a 45 ± 13% reduction in $I$ and a 22 ± 4% reduction in $I$ (n = 7). As expected, 10 $\mu$M DES was associated with similar, although smaller, changes; 14 ± 7% reduction in $I$ and 6 ± 2% reduction in $I$ (n = 5). Fluctuation analysis indicated that power associated with low-frequency gating was diminished, and a DES-induced Lorentzian component with an f$\alpha$ in the 33- to 55-Hz range was observed at all concentrations tested.

Single experiments were conducted to evaluate the effects of other synthetic estrogens, protoioaryl azide and hexestrol diazirine, on CFTR channel activity in excised membrane patches. As shown in Figs. 2 and 3, protoioaryl azide and
hexestrol diazirine produced similar effects to those observed with DES. Presented in Fig. 2A are results from a continuous current record of a membrane patch excised from a CFTR transfected L cell. The concentration of DES present at the intracellular face of the membrane was as indicated. ● indicates the portion of the recording in A that is expanded for presentation in B. The dashed lines indicate the current level when all channels were closed. C, amplitude histograms constructed by using 30 s of continuous current record in each of the three conditions, respectively. D, power density spectra constructed from nine and five nonoverlapping data segments digitized at 1000 Hz after passing through a low-pass 8-pole Bessel filter (cutoff frequency 400 Hz). A multi-Lorentzian function was fitted to each data set. Parameters of each fitted line are as follows. Control: \( f_c_1 = 1.91 \text{ Hz}, S_0_1 = 0.19 \text{ pA}^2 \text{s}^{-1}, f_c_2 = 66 \text{ Hz}, S_0_2 = 3.8 \times 10^{-4} \text{ pA}^2 \text{s}^{-1}, \text{ offset} = 1.0 \times 10^{-4} \text{ pA}^2 \text{s}^{-1} \); 30 \( \mu \text{M} \) DES \( f_c_1 = 2.1 \text{ Hz}, S_0_1 = 0.034 \text{ pA}^2 \text{s}^{-1}, f_c_2 = 39 \text{ Hz}, S_0_2 = 9.0 \times 10^{-4} \text{ pA}^2 \text{s}^{-1}, \text{ offset} = 1.0 \times 10^{-4} \text{ pA}^2 \text{s}^{-1} \).

Fig. 1. DES-induced changes in wt-CFTR chloride channel activity. A, low-resolution (sampling rate 200 Hz) and B, high-resolution (sampling rate 2000 Hz) panels are excerpts from a continuous current record of a membrane patch excised from a CFTR transfected L cell. The concentration of DES present at the intracellular face of the membrane was as indicated. ● indicates the portion of the recording in A that is expanded for presentation in B. The dashed lines indicate the current level when all channels were closed. C, amplitude histograms constructed by using 30 s of continuous current record in each of the three conditions, respectively. D, power density spectra constructed from nine and five nonoverlapping data segments digitized at 1000 Hz after passing through a low-pass 8-pole Bessel filter (cutoff frequency 400 Hz). A multi-Lorentzian function was fitted to each data set. Parameters of each fitted line are as follows. Control: \( f_c_1 = 1.91 \text{ Hz}, S_0_1 = 0.19 \text{ pA}^2 \text{s}^{-1}, f_c_2 = 66 \text{ Hz}, S_0_2 = 3.8 \times 10^{-4} \text{ pA}^2 \text{s}^{-1}, \text{ offset} = 1.0 \times 10^{-4} \text{ pA}^2 \text{s}^{-1} \); 30 \( \mu \text{M} \) DES \( f_c_1 = 2.1 \text{ Hz}, S_0_1 = 0.034 \text{ pA}^2 \text{s}^{-1}, f_c_2 = 39 \text{ Hz}, S_0_2 = 9.0 \times 10^{-4} \text{ pA}^2 \text{s}^{-1}, \text{ offset} = 1.0 \times 10^{-4} \text{ pA}^2 \text{s}^{-1} \).

of reversal cannot be reported at this time. For experiments presented in Figs. 1 to 3, it should be noted that, in each case, the reduction in \( I \) was proportionally larger than the decrease in \( i \).

**Antiestrogens Bind Directly to CFTR.** Initial experiments to directly label CFTR in either L cells or T84 cells proved to be beyond current technical feasibility. Thus, the low copy number of CFTR expressed in mammalian cells, the affinity of CFTR for protioaryl azide and hexestrol diazirine, the efficiency of photo-cross-linking (maximally ~20% for these compounds on the nuclear estrogen receptor (Bergmann et al., 1994), and the use of a tritium label did not allow us to detect labeled CFTR in mammalian cells. However, labeling was detected by using high-CFTR expression systems. Sf9 cells, a cultured cell line from the insect *S. frugiperda*, were infected with the recombinant baculovirus A. *californica*, carrying either the \( \beta \)-galactosidase gene or the human CFTR gene. Because the spliced gene is activated by the virus’ own polyhedron promoter, the expression level
increases with time after infection with the recombinant virus and over the course of 3 to 5 days a very significant amount of the foreign protein is synthesized. For example, at a density of 10^6 cells/ml, the foreign protein can achieve a concentration of 1 mg/ml, corresponding to 50 to 75% of cellular protein (Summers and Smith, 1988).

Radiolabeling experiments were performed by using CFTR-enriched membrane vesicle fractions from Sf9 cells 72 h after infection with a human CFTR baculovirus construct. CFTR-enriched vesicles were incubated with either [3H]hexestrol diazirine (a Frank estrogen and a derivative of DES) or [3H]protoproyl azide (an antiestrogen derivative) to allow binding to CFTR. After irradiation to cross-link CFTR and [3H]protoproyl azide or [3H]hexestrol diazirine, membranes were lysed and subject to immunoprecipitation by using a monoclonal antibody against CFTR. Immunoprecipitations revealed labeling of a single major band at ~140 kDa for both [3H]protoproyl azide and [3H]hexestrol diazirine (Fig. 4), the mass expected for insect-derived CFTR, and comigrated with CFTR immunoprecipitated and phosphorylated with [γ-32P]ATP in the presence of the catalytic subunit of cAMP-dependent protein kinase (data not shown). Prolonged exposure of the gel did not reveal any further bands being labeled. Specificity of [3H]protoproyl azide and [3H]hexestrol diazirine labeling was determined by incubation of CFTR-enriched vesicles in the presence of a molar excess of unlabeled compounds before photoactivation and immunoprecipitation. Control experiments irradiating membrane fractions from either uninfected or β-galactosidase infected cells were unable to identify any specific labeling either in crude membrane fractions or fractions subject to immunoprecipitation by using an anti-CFTR antibody.

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**Fig. 2.** Protoproyl azide inhibits CFTR chloride channel gating in an excised membrane patch. A, continuous recording of an excised membrane patch containing >20 CFTR chloride channels. Addition of protoproyl azide to the static bath reduced I from 10.6 to 0.3 pA. B identifies the portion of the record expanded for presentation in B. B, expanded records and amplitude histograms of current records presented in A. Initially, i was 0.93 pA. In the absence of protoproyl azide, occasional events of 0.8 pA were observed. The dashed line indicates the current level when all channels are closed. C, amplitude histograms constructed by using 30 s of continuous current record in each of the conditions. Note that there is a 10-fold difference in the ordinate of the histograms such that the area under the curve is identical.

**Fig. 3.** Hexestrol diazirine inhibits CFTR chloride channel gating in an excised membrane patch. A, excerpts from a continuous recording of an excised membrane patch containing four CFTR chloride channels. Addition of hexestrol diazirine to the static bath reduced I from 1.0 to 0.15 pA. B identifies the portion of the record expanded for presentation in B. B, expanded records and amplitude histograms of the current record presented in A. Initially, i was 0.93 pA. In the presence of hexestrol diazirine events of varying amplitude, as indicated by the arrows, were observed. The dashed line indicates the current level when all channels are closed. C, amplitude histograms of the current record presented in A. Initially i was 0.97 pA. In the presence of hexestrol diazirine events of varying amplitudes were observed. The dashed line indicates the current level when all channels were closed.

**Fig. 4.** Hexestrol diazirine and protoproyl azide bind directly to CFTR. Sf9 insect cells were infected as described under Materials and Methods. After the generation of a CFTR-enriched membrane fraction, samples were incubated with photoactivatable radiolabeled ligands. Membrane fractions from Sf9 cells infected with wt-CFTR were exposed to either [3H]hexestrol diazirine in the presence (A) or absence (B) of excess unlabeled hexestrol diazirine, or [3H]protoproyl azide in the presence (C) or absence (D) of excess unlabeled protoproyl azide. After UV cross-linking samples were solubilized, immunoprecipitated, and resolved by SDS-PAGE. Gels were dried and subject to autoradiography at ~80°C for 3 to 4 weeks. CFTR labeling was observed as a band of 140 kDa. No specific labeling was observed when nonimmunoprecipitated membrane fractions were resolved by SDS-PAGE (data not shown) due to the low specific activity of the tritiated ligands.
Estrogens Inhibit Forskolin-Stimulated Transepithelial Chloride Secretion. Having shown a direct interaction between estrogens in vitro and in excised membrane patches, we determined whether such interactions modulated forskolin-stimulated transepithelial chloride secretion in vivo. The naturally occurring estrogen 17β-estradiol, which is able to bind nuclear estrogen receptors and exert genomic effects, caused a rapid, dose-dependent inhibition of forskolin-stimulated chloride secretion (Isc; Fig. 5A; Table 1). At maximally effective concentrations (concentrations above which no further inhibition in Isc was observed), 17β-estradiol induced inhibition of Isc was accompanied by a decrease in transepithelial conductance to prestimulated values, indicative of a blockade in a conductive pathway. Inhibition of forskolin-stimulated chloride secretion by 17β-estradiol also was readily reversible (Fig. 5B), thus after washout, forskolin-stimulated changes in Isc were indistinguishable from those obtained from cells previously unexposed to 17β-estradiol. In addition to inhibition of forskolin-stimulated chloride secretion, 17β-estradiol was equally effective at inhibiting

**Fig. 5.** Estrogens are potent inhibitors of cAMP-stimulated chloride secretion. A, stimulation of Isc across T84 monolayers by forskolin (2 μM) was inhibited by 17β-estradiol in a dose-dependent manner. B, after washout of 17β-estradiol, subsequent forskolin stimulation was identical with that before 17β-estradiol exposure. C, stimulation of Isc across T84 monolayers by CPTcAMP (100 μM) was inhibited by 17β-estradiol (50 μM). D, stimulation of Isc across T84 monolayers by forskolin also was inhibited by the 17β-estradiol 17α-estradiol (50 μM). E, stimulation of Isc across T84 monolayers by forskolin also was inhibited by the antiestrogen tamoxifen in a dose-dependent manner.
TABLE 1
Inhibition of chloride secretion across T84 monolayers by steroids and nonsteroids

Inhibition of forskolin-stimulated Isc was evaluated in the presence of increasing concentrations of steroids and nonsteroids, as described under Materials and Methods (see also Fig. 5). Each compound was tested in at least six experiments at four to five concentrations. $K_i$ was determined by fitting a Michaelis-Menten-type single binding-site saturation function to the data using a commercially available nonlinear least-squares curve-fitting routine (Sigma Plot 5.6; Jandel Scientific).

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<thead>
<tr>
<th>Steroid/Nonsteroid</th>
<th>$K_i$ (μM)</th>
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<tr>
<td>Diethylstilbesterol</td>
<td>4</td>
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<tr>
<td>Hexestrol</td>
<td>4</td>
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<tr>
<td>17β-Estradiol</td>
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<tr>
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<td>Nafoldine</td>
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<td>Vitamin D</td>
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CPTcAMP-stimulated chloride secretion, suggesting that the site of action of 17β-estradiol was not localized to adenylate cyclase (Fig. 5C). The 17β-estradiol stereoisomer 17α-estradiol also caused a rapid inhibition in forskolin-stimulated chloride secretion (Fig. 5D), which also was readily reversible on 17α-estradiol washout (data not shown). Interestingly, the $K_i$ for 17α-estradiol inhibition of forskolin-stimulated chloride secretion was identical with that of 17β-estradiol (Table 1). In addition to estrogen-dependent inhibition of forskolin-stimulated chloride secretion, tamoxifen (a triarylethylene antiestrogen) also caused a rapid, reversible, dose-dependent inhibition of forskolin-stimulated Isc (Fig. 5E; Table 1), although at a higher concentration than that observed for the estradiol stereoisomers.

To determine whether the inhibitory effects of 17α- and 17β-estradiol on forskolin-stimulated chloride secretion reflected merely a nonspecific effect of steroids (e.g., alterations in membrane fluidity), we tested a panel of steroids for their ability to inhibit stimulated secretion. Such studies revealed a rank potency order across different steroid classes (Table 1). Of the steroids tested, both synthetic frank estrogens (diethylstilbesterol and hexestrol) and naturally occurring estrogens (17α- and 17β-estradiol) were the most potent inhibitors of stimulated secretion. Progesterone and testosterone also inhibited chloride secretion, but at a higher concentration than that for estradiol (Table 1). In contrast, other steroid hormones such as aldosterone, a naturally occurring mineralocorticoid, and dexamethasone, a synthetic glucocorticoid, failed to inhibit chloride secretion even at millimolar concentrations (Table 1). Finally, the steroid precursor cholesterol also was ineffective at inhibiting CAMP-stimulated chloride secretion at concentrations up to 5 mM.

**Estrogens Fail to Modulate Amiloride-Sensitive Sodium Transport.** To determine whether estrogens exert a nonspecific effect on transepithelial ion transport mechanisms, we examined the effects of 17β-estradiol on the amiloride-sensitive epithelial sodium channel (ENaC) in freshly excised rat colonic epithelia. 17β-Estradiol alone (at a concentration ~10-fold above the $K_i$ for inhibition of chloride secretion) had no effect on unstimulated Isc across colonic epithelia (Fig. 6A). Application of amiloride (10 μM) in the continued presence of 17β-estradiol resulted in a rapid decrease in short-circuit current, consistent with inhibition of sodium channels. Subsequent exposure of cells to forskolin caused a slight sustained increase in Isc consistent with forskolin stimulation of chloride secretion, which was significantly reduced compared with nonestrogen-treated controls. Pretreatment of cells with amiloride in the absence of 17β-estradiol resulted in an inhibition of sodium transport that was indistinguishable from that observed in the presence of 17β-estradiol (Fig. 6B). Subsequent application of forskolin caused a marked rapid and sustained increase in chloride secretion (at a higher plateau than that observed for forskolin-stimulation after exposure to 17β-estradiol) that was rapidly inhibited by the application of 17β-estradiol. Such results recapitulating the data observed for the human colonic epithelial cell line T84.

![Fig. 6](https://jpet.aspetjournals.org/)

**Fig. 6.** Estrogens fail to inhibit absorptive sodium currents. **A.** amiloride-sensitive Isc was not inhibited by the continuous presence of 17β-estradiol (60 μM), but did inhibit the forskolin-stimulated current. **B.** After inhibition of amiloride-sensitive currents, forskolin stimulated chloride currents could again be blocked on exposure to 17β-estradiol (60 μM).
Discussion

Because the discovery of intracellular steroid hormone receptors in the 1960s, steroid hormones have been recognized as producing their major long-term effects on cell structure and function by acting on the expression of specific genes. Indeed, estrogenic steroids have been shown to regulate the expression of epithelial-specific cationic and anionic channels (Rochweger and Buchwald, 1993; Sweeze et al., 1997, 1998). Yet, the concept that steroid hormones can exert effects by interacting with plasma membrane “receptors” has gained momentum only during the last few years (McEwen, 1991; Kelly and Wagner, 1999; Levin, 1999; Watson et al., 1999; Norfleet et al., 2000). Of particular interest are the observations that steroid hormones can modulate the activities of plasma membrane ion channels. For example, Maxi-K channels are activated by the binding of membrane-impermeant estradiol to the β-subunit (Valverde et al., 1999), clearly precluding an intracellular site of action. Furthermore, flux through the γ-aminobutyric acid (GABA)β/benzo-diazepine receptor-chloride channel complex is enhanced in the presence of pregnane steroid (Gee et al., 1987; Turner et al., 1989). In contrast, progesterone markedly inhibits glycine-gated chloride currents (Wu et al., 1990). Our studies indicate that estrogenic steroids are inhibitors of CFTR-mediated chloride secretion across polarized human epithelial cells.

The immediate onset and rapid reversibility of the inhibitory effects of estrogens on chloride transport, observed both in excised membrane patches and in epithelial monolayers, argues against a genomic effect for these compounds, and suggests a direct interaction with a plasma receptor. Moreover, nuclear estrogen receptors exhibit a stereoselectivity with respect to carbon 17, with 17β-estradiol being the dominant effector compared with 17α-estradiol (Anstead et al., 1997). In contrast, 17α-estradiol and 17β-estradiol are equipotent in inhibiting CFTR-mediated chloride transport. These data suggest a plasma membrane receptor as the site of action of these steroids. In the present studies, only stereoisomers at carbon position 17 were evaluated because this provides the major discrimination for nuclear receptors. The results showing equipotency of 17α- and 17β-estradiol at inhibiting chloride secretion suggest that the 17 position is not directly involved in the binding interaction between estradiol and its receptor as far as pertains to inhibiting chloride secretion. It is therefore likely that stereoisomers at other positions on the steroid nucleus will discriminate between efficacious and nonefficacious steroids.

Although estrogens have been reported to directly influence intracellular cAMP levels by interacting with adenylate cyclase (Aronica et al., 1994), this is unlikely to be the mechanism by which estrogens inhibit forskolin-stimulated chloride secretion because the estrogens tested also were able to inhibit cPT-cAMP-stimulated chloride secretion. Early studies also indicated that steroid effects could be related to their lipophilicity. For example, the efficacy of natural and synthetic progesterational steroids as anesthetics has been known for many years (Holzbauer, 1976). However, it is unlikely that changes in membrane fluidity could account for our observation that estrogenic steroids inhibit CFTR-mediated chloride secretion because other steroids (aldosterone and dexamethasone) were unable to inhibit chloride secretion, yet are likely to have similar effects on membrane fluidity as 17α- and 17β-estradiol. Moreover, the steroid precursor cholesterol, at concentrations as high as 5 mM, was unable to influence CFTR-mediated chloride secretion. These observations therefore argue against a nonspecific effect on membrane fluidity as the basis for estrogen-dependent inhibition of CFTR-mediated chloride secretion, but rather for a specific interaction with a plasma membrane receptor.

To evaluate whether estrogens specifically affected only chloride secretory pathways or whether other ion transport pathways were affected, the ability of estrogens to acutely modulate amiloride-sensitive sodium currents was assessed. In contrast to the immediate and rapidly reversible effects of estrogens on chloride secretion across T84 cell monolayers, no modulation of amiloride-sensitive sodium currents was observed, suggesting that the effects of estrogens on forskolin-stimulated chloride secretion were specific for that ion transport pathway. Estrogens have recently been reported to increase ENaC (Sweeze et al., 1997), however, effects were only observed after extended periods of incubation (≥5 days) of epithelial cells in estrogens; a phenomenon accounted for by increases in ENaC mRNA. Thus, our results demonstrate that estrogens selectively inhibit CFTR-mediated chloride secretion, and non-ENaC-mediated sodium absorption.

The conclusion of a direct functional effect of estrogens on CFTR-mediated ion transport is greatly strengthened by observations in excised membrane patches. Indeed, the present results show that DES, a compound that reversibly inhibits CFTR-mediated chloride secretion in T84 monolayers, also reversibly inhibits CFTR channel activity in excised membrane patches. Concordant inhibitions were observed with two other estrogen compounds. Furthermore, the results indicate that each of the compounds tested interacts with the actively gating state of CFTR to reduce the apparent single channel amplitude by the introduction of a short-lived block. However, this mechanism alone does not fully account for the observed reduction in anion current carried by CFTR. If the only effect of estrogens was to introduce a short-lived channel block, then the apparent single channel amplitude would decrease in proportion to the off rate of the estrogen. Such a result suggests one of two possibilities. One possibility is that estrogen has a site of action in addition to the open state of the CFTR channel. Interaction with a closed state that prolongs the nonconductive lifetime would manifest itself as a dramatic drop in open probability and infrequent transitions to a conductive state. Alternatively, estrogens might interact with the open state to introduce an extended blocked state in addition to the short-lived block. Again, the open probability would be expected to dramatically drop, and transitions to the open state would be limited by the off rate of the estrogen. Substantial additional experimentation is required to conclusively discriminate between these two possibilities. Regardless, results from studies conducted with excised membrane patches provide evidence in both the time and frequency...
domain that estrogen compounds introduce at least two additional kinetic states of CFTR: 1) a short-lived blocked state within an open burst that accounts for the reduction in apparent single channel amplitude, the broadening of histogram peaks, and an increase in power at higher frequencies in the spectrum; and 2) a long-lived nonconductive state that accounts for the substantial reduction in open probability. Whether a single estrogenic-binding site can account for both short- and long-lived events remains to be determined.

CFTR is expression is low in natively expressing cells, and relatively low even in heterologous expression systems. Initial attempts to radiolabel CFTR in L-cells proved to be technically unfeasible due to the low level of CFTR expression, the efficiency of photocross-linking (maximally ~20% for these compounds on the nuclear estrogen receptor; Bergmann et al., 1994) and the use of a tritium label did not allow us to detect labeled CFTR in mammalian cells. However, labeling was detected by using high-CFTR expression systems. Radiolabeling experiments performed by using CFTR-enriched membrane fractions from baculovirus-infected SI9 cells revealed a direct interaction of the estrogen hexestrol diazirine and the antiestrogen protoxylic azide with CFTR. In addition to being precipitated with monoclonal antibodies directed against CFTR, radiolabeled bands resolved by SDS-PAGE ran with an apparent molecular mass of ~140 kDa. This is consistent with the mass of CFTR obtained from insect cells, which are unable to perform the terminal glycosylation steps observed in mammalian cells. Because both hexestrol diazirine and protoxylic azide are membrane-transported molecules, it was not possible to evaluate whether these compounds interacted with extracellular, transmembrane, or cytosolic domains of CFTR.

It should be noted that high concentrations of estrogens were required to show an effect on CFTR gating and short-circuit current. In is therefore unlikely that estrogens themselves act to modulate CFTR activity under physiological conditions. Rather, we would speculate that an estrogen metabolite of much higher affinity would be the physiologically relevant species. Although speculative, such as concept has precedent in the literature. For example, studies on progesterone-mediated opening of GABA-activated chloride channels and vitamin D-mediated activation of osteoblast calcium channels have revealed that the actual compounds mediating the effects are metabolites of the parent compounds (Majewska et al., 1986; Paul and Purdy, 1992). Moreover, the channel-expressing cells metabolize the parent steroids, releasing the metabolites as paracrine and autocrine hormones that then act on the channels with 1,000- to 10,000-fold higher affinities ($K_D \sim 0.1-10$ nM) than the parent compounds ($K_D \sim 0.1-10$ $\mu$M). In addition, both positive- and negative-acting metabolites of the steroid progesterone (i.e., openers and blockers) have now been identified for the GABA-activated chloride channel (Deutsch et al., 1992).

Studies by Devor et al. (1996) have highlighted the importance of potassium channels in maintaining the driving force for chloride secretion through apically localized CFTR. In light of this, it is interesting to note that 17β-estradiol has been shown to directly block mink potassium channels, although is without effect on either Kv1.1 or Kir2.1 potassium channels (Waldegger et al., 1996). Thus, it is possible that some of the inhibition of chloride secretory current by steroids in short-circuit current analyses of T74 cell monolayers may in part be due to inhibition of basolateral potassium channels. However, there is at present no evidence that hIK1, the potassium channel involved in regulated chloride secretion from T84 monolayers is directly affected by steroids. In addition, the observation that steroids directly bind to CFTR, and block CFTR channel activity in excised membrane patches argues that steroid inhibition of chloride secretion is due at least in part, if not entirely, to direct blockade of CFTR.

Acknowledgments

We acknowledge the technical assistance of Hoa Trummell and Mai Hyun in tissue culture, Jeffrey Jones in Ussing chamber experiments, and Kip Smith and John Clark in the radiolabeling experiments.

References


Nelson DA, Aguilar-Bryan L and Bryan J (1992) Specificity of photolabelling of β-cell...
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