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17β-Estradiol and Tamoxifen Regulate a Maxi-Chloride Channel from Human Placenta

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Abstract. Steroid hormones have been implicated in the modulation of several transport processes, including conductive chloride transport in epithelial cells. Micromolar concentrations of these hormones have been determined in blood of pregnant women. The purpose of this work was to explore the effects of 17β-Estradiol, a steroid hormone, on the biophysical properties of the Maxi chloride channel present in apical membranes from human placental syncytiotrophoblast.

Apical membrane chloride channels from human term placentas were reconstituted in giant liposomes suitable for electrophysiologic studies by the patch-clamp method.

Low micromolar concentrations of 17β-Estradiol inhibit the Maxi chloride channels in excised patches in a potential-dependent manner. The addition of 1 μM 17β-Estradiol to the bath solution decreased the total current in the patch from 100% control to 71% at −40 mV holding potential and the current was not affected by 17β-Estradiol at +40 mV. However, the presence of the hormone did not affect the single-channel conductance, therefore its effect must be due to modulation of its open probability (*Po*). Interestingly, 17α-Estradiol did not change the total current in the patch. Tamoxifen, an antiestrogen, also showed inhibition, but in a voltage-independent manner.

Our results suggest that the Maxi Cl^- channel from human term placenta may be regulated by direct interaction of both compounds with the channel. From a functional point of view, the control of these channels by steroid hormones may be of great importance in placental physiology and their regulation may help to unravel their possible role in transplacental transport.

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Introduction

Estrogen (17β-Estradiol; 17βE) and other steroid hormones have been shown to exert a variety of nongenomic effects. Some of these are to increase the calcium uptake in myometrial cells [13], and to increase the intracellular calcium release in granulosa cells [28]. Vasodilation by 17βE has also been demonstrated in peripheral vessels [27] and in coronary arteries [44] mediated by stimulation of gating of the Maxi-K^+ channel. Tamoxifen, an antiestrogen, frequently used in the treatment of breast cancer [22], has been reported to block the volume-regulated chloride channel in epithelial cells [46] and to activate the large-conductance chloride channel in fibroblasts [21]. Recent studies show that estrogen and antiestrogen modulate the open probability of the Maxi-K^+ channel. Open probability is increased by Tamoxifen [10] and 17βE [43] due to direct interaction with the β_1 subunit of the voltage-gated channel. Some data are consistent with an interaction of the steroid with a cell-surface receptor distinct from the classic nuclear receptor [21]; other data are consistent with direct interaction with a binding site available in the channel [43]. However, in general, the identification of the pathway mediating modulation of ionic channels by estrogen or antiestrogen is not yet clear.

In addition to other hormones, the placenta synthesizes 17βE, which participates in several functions, among them the regulation of uterine activity during pregnancy and at the time of parturition. An interesting point is that the production of estrogen increases throughout the gestational period [14]. Hormone levels in maternal plasma and placental tissues from pregnant women at term have high concentrations (micromolar order) of Estradiol, with

the placenta concentration of Estradiol being half of that of maternal plasma [23].

The human placenta has two complete cellular layers separating maternal and fetal blood: the syncytiotrophoblast and the fetal capillary endothelium. Because of its syncytial nature, the former is believed to constitute the major barrier for maternofetal exchange of small solutes [41, 42]. The transport functions in the human placenta have great importance for fetal growth and development. Transcellular transport, including conductive pathways, across the syncytiotrophoblast must involve movement across both its microvillous (maternal-facing) and basal (fetal-facing) plasma membranes. Chloride is the main anion of extracellular fluid in the fetus, as it is in the adult, but at all gestational ages the fetal Cl^- concentration is 5–6 mM higher than in maternal blood [3]. There are no maternal-fetal differences in either Na^+ or K^+ concentration.

Considerable interest has been focused on the chloride conductance in the apical membrane of epithelial cells, which mediates secretion under the control of hormonal stimuli [19, 45]. Recently Condliffe et al. demonstrated that physiological con- centrations of Estradiol decrease the Cl^- secretory capacity of the female distal colon. A receptor, a protein distinct from the nuclear estrogen receptor, in colonic epithelial cells mediates this effect [7]. There is growing interest in identifying the conductive pathways across trophoblast membranes from human placenta, particularly that of chloride conductance in apical membrane, as well as its regulation [5, 12, 35, 37].

Chloride channels are present in the cell membranes of most cells, and a large variety of chloride channels have been reported. One of them is a Maxi chloride channel that has been identified in secreting and absorbing epithelia [15, 17, 19, 29, 30] and in other cell types [2, 4, 18, 24, 31] including the apical membrane from human placenta [6, 37, 38]. This type of channel may play a role in complex cellular regulation involving inhibition through phosphorylation by protein kinase C and activation by a Ca^{2+}-dependent process. More recently, Sabirov et al. conclude that this channel serves as a pathway for swelling-induced ATP release in C127i cells [40].

We have reported [37, 38] a high-conductance chloride channel from human term placentas reconstituted in giant liposomes. These cell-sized liposomes, generated by the method of cycle dehydration and rehydration of lipid vesicles, are suitable for electrophysiologic studies by the patch-clamp method [36]. The apical chloride channel shows some similarities with "Maxi" chloride channels described in the different tissues mentioned above, and it is the same channel described in small fragments of villous tissue dissected from human placenta [6]. The channel is selective for anions over cations, has a large conductance (>200 pS), multiple subconductance states and voltage dependence, being open between -50 and $+50$ mV and closing at more extreme potentials. The channel was inhibited by DIDS (stilbene derivative 4,4′diisothiocyanostilbene-2,2′disulphonic acid) and was inhibited directly by arachidonic acid (AA) and other cis unsaturated fatty acids [37]. Our results also suggest that the apical membrane Maxi Cl^- channel is a possible electrophysiological counterpart of the cis unsaturated fatty acid-inhibited conductance pathway previously described in apical membrane of human placenta [5].

Presently it is known that the regulation of Maxi chloride channels by estrogen and antiestrogen represents a non-classical effect of these compounds in fibroblasts [21], in C1300 neuroblastoma cells [9] and in endothelial cells [26], among others. Considering this evidence, our aim in the current study was to investigate the effects of 17β-Estradiol, a steroid hormone, and Tamoxifen, an analog of 17β-Estradiol, on the biophysical properties of the Maxi chloride channels present in apical membranes from human placenta.

Materials and Methods

Preparation of Placental Apical Membrane

Human placental apical membrane vesicles were prepared by a method described by Glazier et al. [16] and modified by Riquelme et al. [38]. Placentas (700 to 800 gm) were obtained immediately after delivery from the San José Hospital and transported to the laboratory on ice. The subsequent preparation involved precipitation of non-microvillous membrane with magnesium ions, differential centrifugation and sucrose step gradient. All solutions were buffered with 20 mM Tris-HEPES, pH 7.4. A portion (2 to 3 ml) of the microvillous membrane-enriched preparation containing about 10 to 15 mg of protein was overlayed on the sucrose gradient. The band at the 37/45% sucrose interface was collected and diluted tenfold with 20 mM Tris-HEPES, pH 7.4, before centrifugation at $100{,}000 \times g$ for 60 minutes. The pellets were resuspended in 300 mM sucrose, 20 mM Tris-HEPES, pH 7.4, and stored at –150°C (liquid nitrogen). Vesicle purity was determined routinely by assaying for alkaline phosphatase activity, an apical membrane marker [38].

Reconstitution of the Apical Membrane into Giant Liposomes

Giant liposomes were prepared by submitting a mixture of the reconstituted brush border membrane vesicles and asolectin lipid vesicles to a partial dehydration/rehydration cycle, as reported by Riquelme et al. [36]. A membrane aliquot containing 100 to 150 μg of protein was mixed with 2 ml of a 13 mM (in terms of lipid phosphorus) suspension of the asolectin vesicles. After the partial dehydration/rehydration cycle, the diameter of the resulting giant multilamellar liposomes ranged from 5 to 100 μm.

Patch-Clamp Measurements

Aliquots (3 to 15 μl) of giant liposomes were deposited, mixed with 1 ml of buffer of choice for electrical recording (bath solution) into

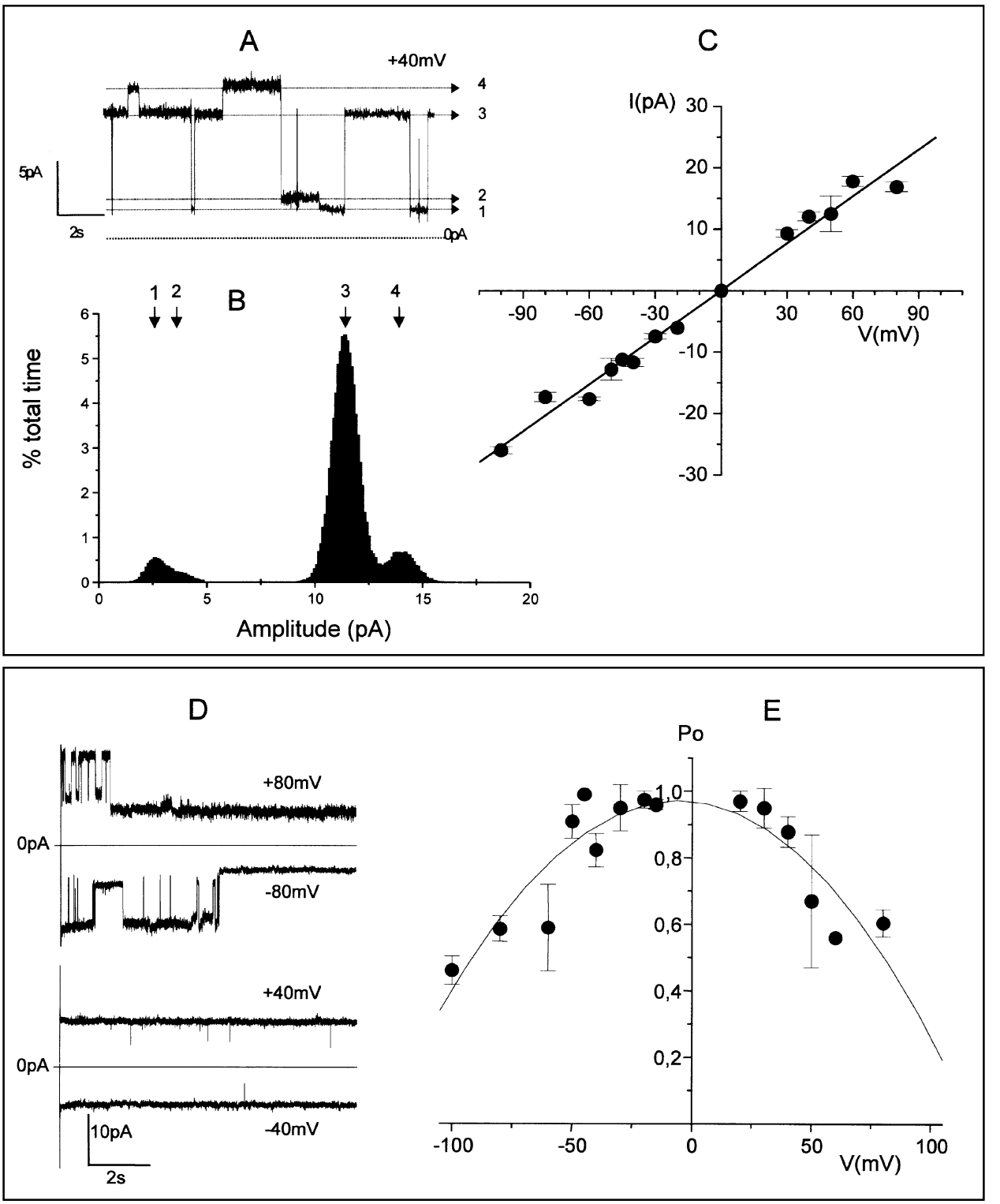
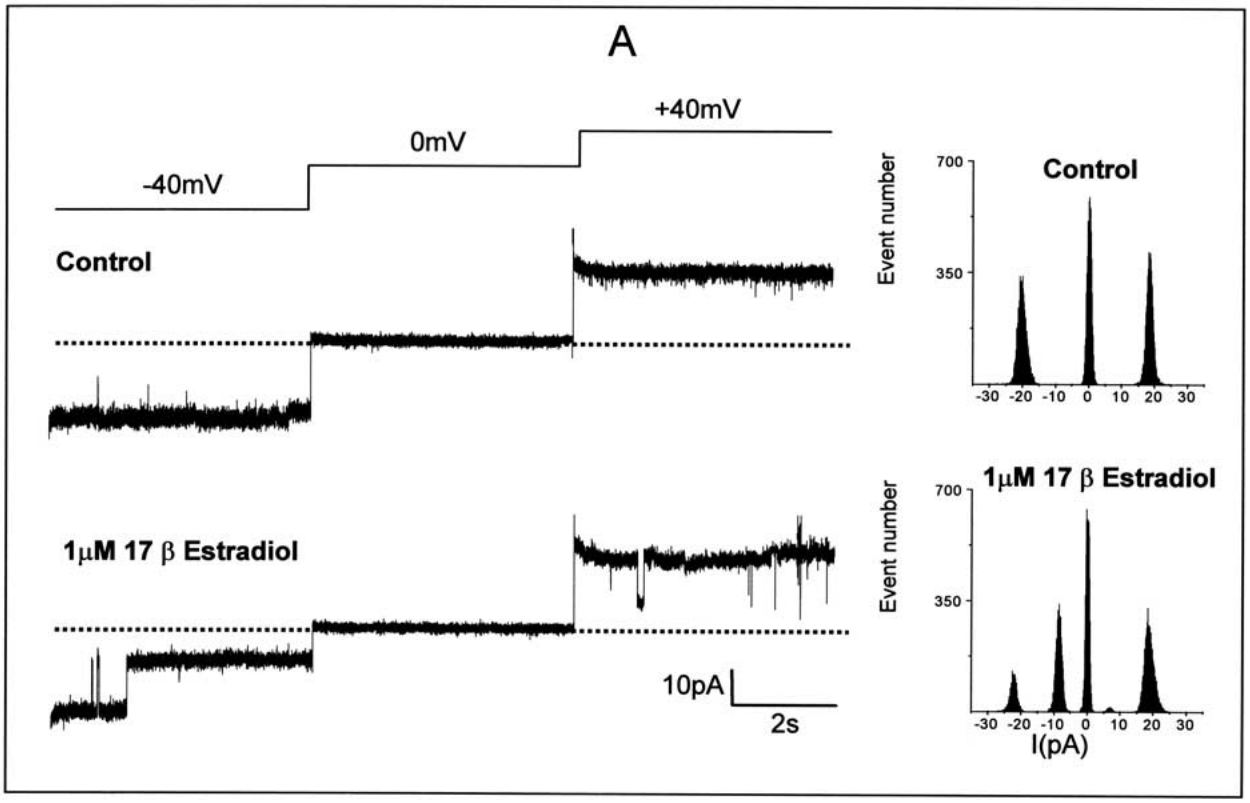


Fig. 1. Maxi apical Cl^- channel from human placenta. (***A*** and ***D***) Single-channel currents recorded in excised patches from apical membranes reconstituted into giant liposomes. Bath and pipette solutions contained (in mM) 140 NMDGCl, 2.6 $CaCl_2$, 1.3 $MgCl_2$, 10 Na-HEPES, at pH 7.4. Currents were recorded at +40 mV in ***A***. In ***D***, the patch was held at 0 mV and voltage steps (±40 and ±80 mV) were applied. (***B***) Current amplitude histogram corresponding to record showed in (***A***). Lines in the record and arrows in the histogram show primary states and substates. (***C***) Current-voltage (*I-V*) relation of the chloride channel for the primary state, obtained with standard solutions in both sides. The slope conductance is 255 ± 9 pS (mean ± SEM, $n = 26$). (***E***) Voltage dependence of the open probability (*Po*). Each point gives the mean of 3 to 16 patches; error bars represent SEM.



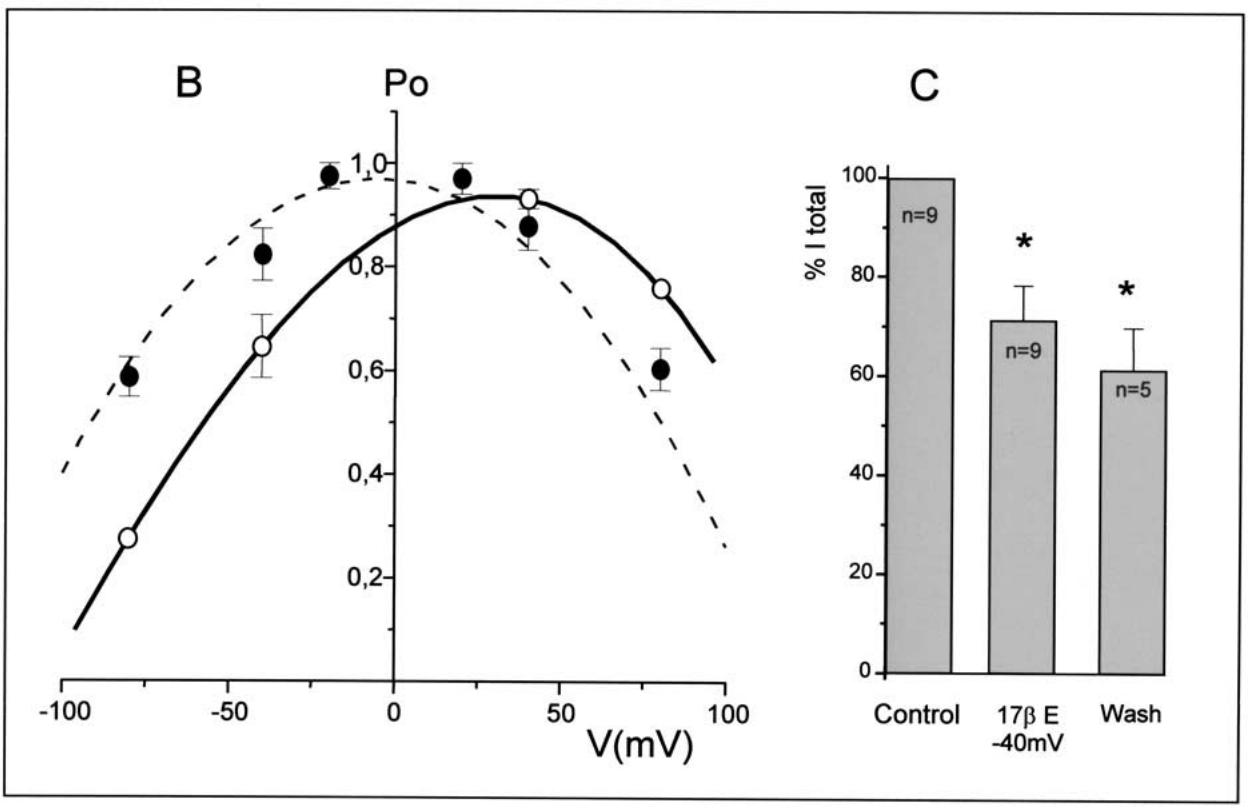


Fig. 2. Effects of 17β-Estradiol on Maxi apical Cl^- channel from human placenta. (***A***) Single-channel currents are shown at +40 and −40 mV, before and after addition of 1 μM 17βE. Representative all-points histograms for the indicated interventions at both potentials from this experiment are shown. Control histogram at −40 mV revealed a peak at −20 pA at any time in the absence of Estradiol and at −10 pA with Estradiol. (***B***). Voltage dependence of open probability in both conditions: control (filled circles) and in presence of 17βE (empty circles). Each point gives the mean ± SEM for $n = 8$ patches. (*C*) Irreversible effect of 17/*β*E shown as % of total patch current (%I total) in relation to control conditions.

3.5-cm Petri dishes, which could be mounted directly on the stage of an inverted microscope. Single-channel recordings were obtained by patch-clamp techniques as described by Hamill et al. [20]. Gigaseals were formed on giant liposomes with microelectrodes of 5 to 10 MOhm resistance. After sealing, withdrawal of the pipette from the liposome surface resulted in an excised patch. Current was recorded with an EPC-9 patch-clamp amplifier (Heka, Lambrecht/Pfalz, Germany) at a gain of 50 to 100 mV/pA and a filter setting of 10 kHz. The holding potential was applied to the interior of the patch pipette, and the bath was maintained at virtual ground. The bath was grounded via an agar bridge and the junction potential was compensated for when necessary. The signal was analyzed off-line by means of the TAC (Bruxton Corporation) and Pulse Fit (Heka, Lambrecht/Pfalz, Germany) software. All measurements were made at room temperature.

Solutions and Reagents

The pipette and bath solutions had the following composition (in mM): 140 N-methyl-D-glucamine chloride (NMDGCl), 2.6 calcium chloride, 1.3 magnesium chloride, and 10 sodium HEPES, pH 7.4. Tamoxifen and 17α-Estradiol were dissolved in alcohol and 17β-Estradiol in water or alcohol as a stock solution and were stored at −20°C before use. The final concentration was obtained by dilution of the stock to keep the alcohol at 0.1%. At this concentration, alcohol alone has no effect on single-channel activity [11]. The hormones and all reagents and chemicals were from Merck (Darmstadt, Germany) and Sigma (St. Louis, MO).

Statistics

Measures of statistical significance were obtained using either Student's *t*-test or the Wilcoxon rank test. A p value of less than 0.05 was considered significant.

Results

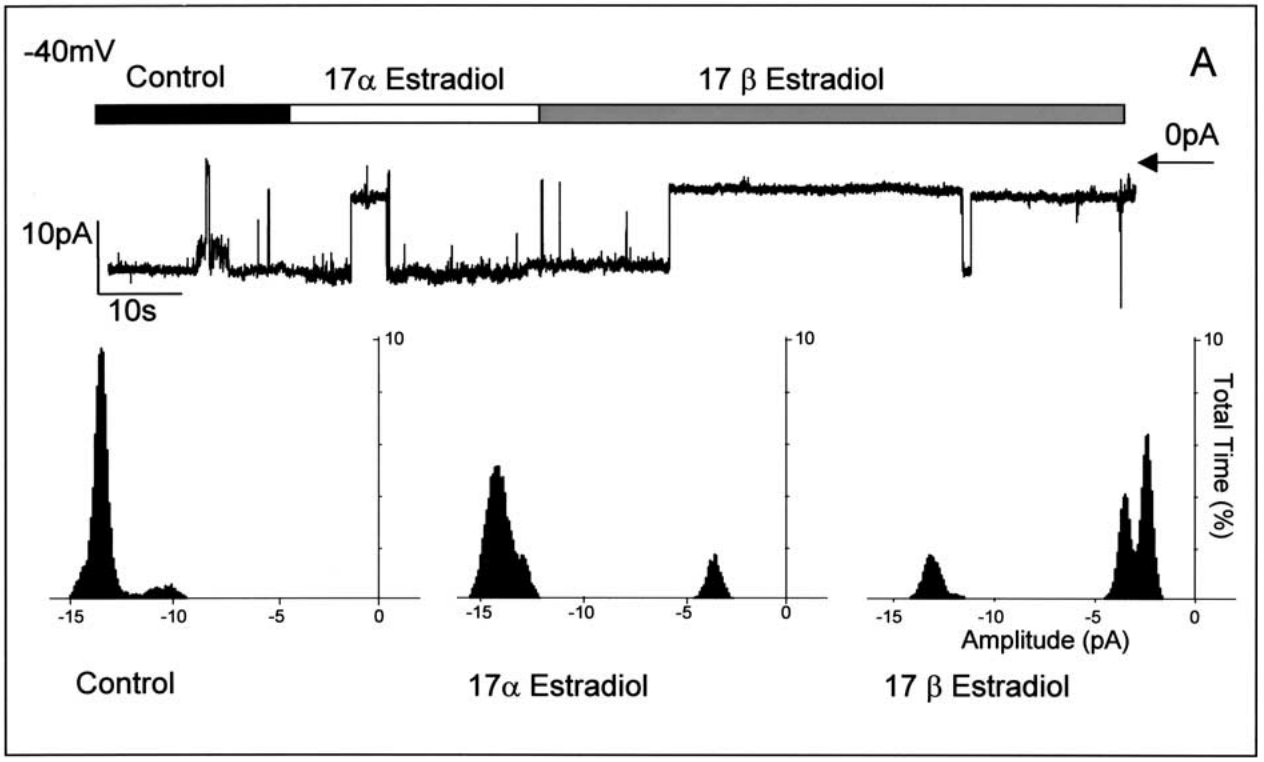
Maxi Chloride Channel from Apical Membrane Reconstituted in Giant Liposomes

The activity of large-conductance channels (over 200 pS) was detected in excised patches obtained from cell-size giant liposomes containing placental apical membrane. Various subconductance levels were seen, as described previously [38]. Single-channel currents for the chloride channel reconstituted from placental membranes are shown in Fig. 1*A* and *D* at the indicated holding potentials. The corresponding amplitude distribution histogram for record *A* is shown in Fig. 1*B*. Trace *A* shows a primary conductance state, but subconductances were also evident in the trace and the histogram. For the current-voltage relationship, only the dominant current level was used, ignoring any subconductance levels (Fig. 1*C*). This large conductance was observed in experiments carried out with bath and pipette solutions containing (in mM) 140 NMDGCl, 2.6 $CaCl_2$, 1.3 $MgCl_2$, 10 Na-HEPES, and pH 7.4. The slope conductance was 255 ± 9 pS (mean ± SE, n = 26). The open probability (*Po*) versus voltage relationship could be described by a bell-shaped curve (Fig. 1*E*). The channel was normally open at potentials between −50 and +50 mV, but higher voltage steps, in either a positive or negative direction, induced channel closure. These characteristics are similar to those reported before for the placental Maxi Cl^- channel [38].

Maxi Cl^- Channels from Apical Membrane Reconstituted into Giant Liposomes Are Inhibited Directly by 17β-Estradiol

There is some evidence about the effect of estrogen and antiestrogen on chloride channels. Tamoxifen blocks the volume-regulated chloride channels in epithelial cells [46] and activates the large-conductance chloride channels in fibroblast and neuroblastoma cells, the latter two being also affected by 17β-Estradiol [9, 21]. In addition, steroid hormones modulate a Maxi chloride channel in cultured porcine aortic endothelial cells [26]. In our present work, we investigated the effect of some of these agents on Maxi Cl^- channel activity from human placenta.

The addition of 1 μmolar 17β-Estradiol to the bath solution inhibits the Maxi chloride channels in a potential-dependent manner (n = 13) from 5 separate placentas (Fig. 2). Longer closures appeared in the presence of 17βE at negative potentials; however, at positive potential, this hormone does not have the same effect. Figure 2*A* shows channel recordings in both conditions and the corresponding histograms note that 17βE changes the peak of the negative current from −20 pA to −10 pA. This effectively reduces the total current in the patch—obtained as a ratio of the current with 17βE over the current in control condition ($I_{17\beta E}$ / $I_{control}$)—from 100% control to 71 ± 6% at −40 mV holding potential ($p < 0.01$, n = 9, Fig. 2*A,C*), without changing the unitary amplitude of the single channel. The effect of 17βE was irreversible; washing the patch with buffer containing free hormone did not recover the control activity (n = 5), as can be seen in Fig. 2*C*. The total current in the patch was not affected significantly by 17βE at +40 mV. The total current in the patch ($I_{17\beta E}$ / $I_{control}$) was slightly superior to control at this potential (109 ± 2%, n = 7). Figure 2*B* shows that the effect of 17β-Estradiol on *Po* was voltage dependent, decreasing substantially at negative potentials and increasing with depolarization compared with respective controls. The relationship for *Po* versus voltage maintains its bell-shaped form but the maximum values were shifted to more positive potentials. The addition of 1 μmolar 17β-Estradiol did not affect the single-channel conductance of the channel, i.e., 259 ± 4 pS in control condition and 245 ± 6 pS in the presence of the hormone (n = 5). Dose-dependent blockade of 17β-Estradiol applied to the bath solution at −40 mV is shown in Fig. 3*B*.



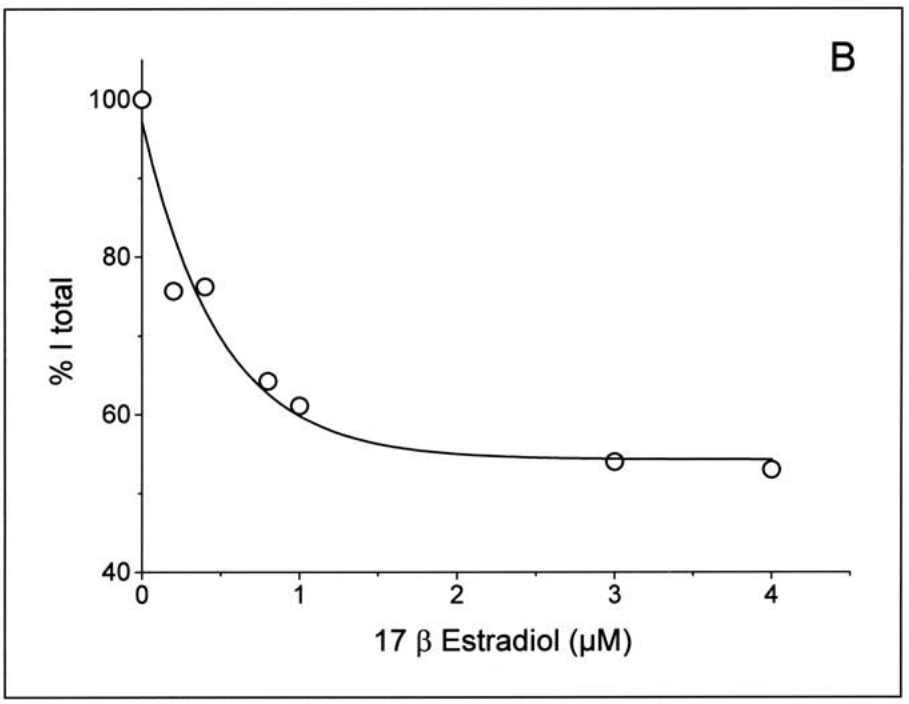


Fig. 3. The effect of 17β-Estradiol is stereospecific and dose-dependent. (***A***) Single-channel current from a single experiment and the corresponding histogram for indicated conditions are shown. The record shows the activity in control conditions, after addition of 1 μM 17αE and after the addition of 1 μM 17βE consecutively. The holding potential was −40 mV. Similar results were obtained in $n = 3$ patches. (***B***) Dose-response curve of the Maxi Cl^- channel to micromolar concentrations of 17βE. The graph represents the percentage of the total current under increasing concentrations of 17βE at a holding potential of −40 mV.

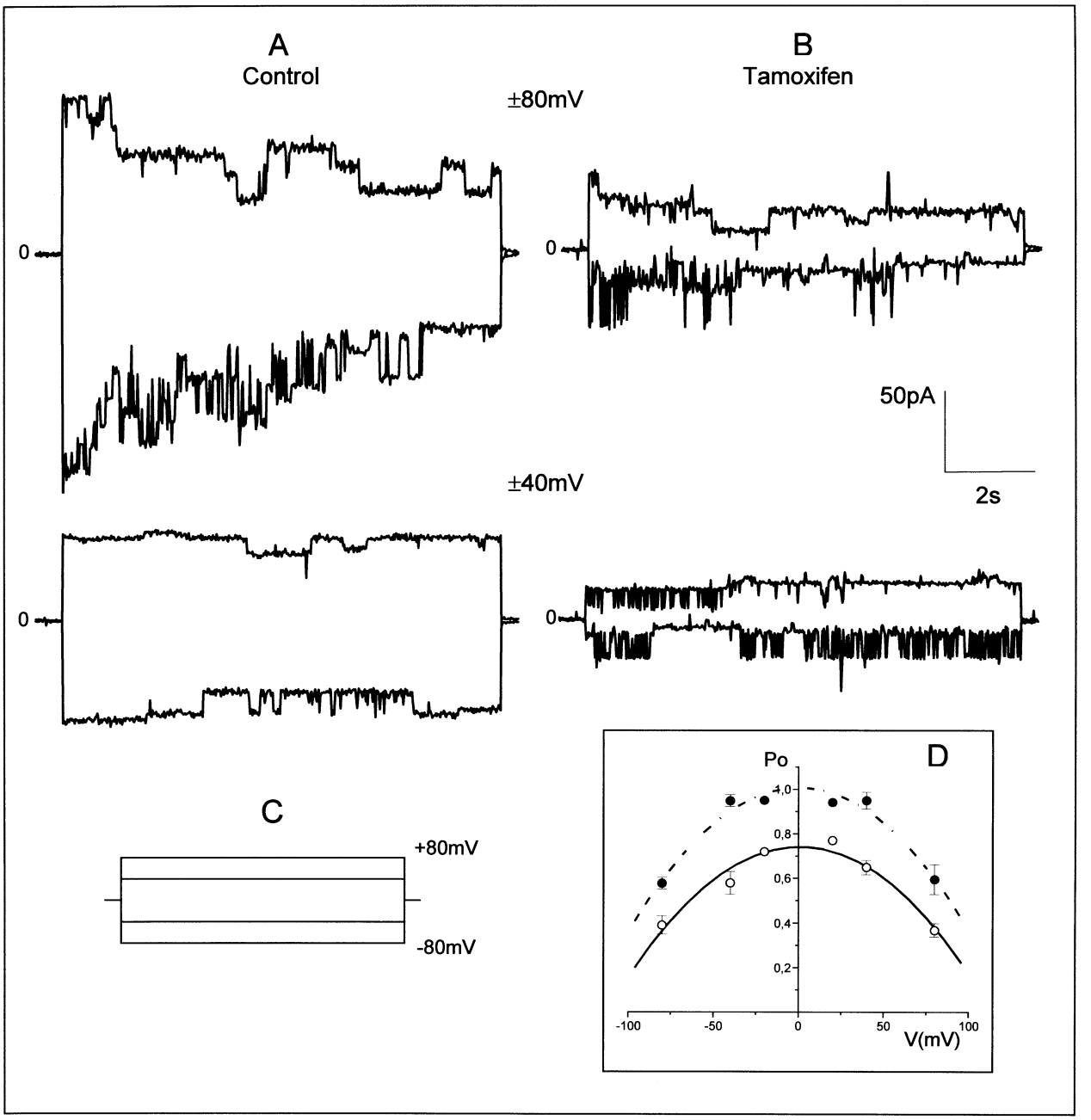


Fig. 4. Effect of Tamoxifen on channel activity. Representative recordings of a single Maxi Cl^- channel under control conditions (***A***) and with the patch perfused with a solution containing 15 μM of Tamoxifen (***B***). The patch was held at 0 mV and voltage steps (±80 mV and ±40 mV) were applied in both conditions (***C***). The zero-current level is indicated at the left of each trace. (***D***) Relationship between *Po* and voltage in the absence (empty circles) and presence of 15 μM Tamoxifen (filled circles). Data points are means ± SEM (n = 3–4) at each voltage.

The Effect of 17β-Estradiol is Stereospecific

The addition of 1 μM of the stereoisomer 17α-Estradiol (17αE) did not change the channel activity at +40 mV and at −40 mV. The total current in the patch, considering control as 100% ($I_{17\alpha E}$ / $I_{control}$) was 98.8 ±2.3% and 101 ±0.9% at +40 and −40 mV, respectively. Similar results were obtained at other potentials (*data not shown*). Figure 3*A* shows records and histograms at −40 mV under the following conditions: control, 17α-Estradiol and 17β-Estradiol applied consecutively in the same patch. Similar results were obtained in n = 3 independent experiments.

Tamoxifen, an Analog of 17β-Estradiol, Shows Strong Voltage-independent Inhibition

The addition of 15 μM Tamoxifen to the bath solution inhibits the Maxi chloride channels at negative and positive potentials. Tamoxifen inhibited chloride channels recorded at different holding potentials in an irreversible manner. Figure 4 shows currents elicited by voltage pulses (*C*) from 0 to ±80 mV (*A*) or 0 to ±40 mV (*B*), in the absence and presence of Tamoxifen for a seal with several chloride channels. The addition of 15 μM Tamoxifen to the bath markedly decreased channel activity (around 30%) at all voltages in the range between −80 mV and +80 mV. In the presence of Tamoxifen, the total current in the patch was effectively reduced ($I_{Tamoxifen}$ / $I_{control}$) from 100% control to 66 ±4% and 58 ±5% at ±40 mV holding potential ($n = 5$, $p < 0.05$), respectively, without changing the unitary amplitude of the single channel. Washing the patch with control solution did not recover the activity. The addition of 30 μM Tamoxifen to the bath at ±40 mV reduced the total current in the patch ($I_{Tamoxifen}$ / $I_{control}$) from 100% control to 30% and 21% at −40 and +40 mV holding potential, respectively. Figure 4*D* shows *Po* as a function of potential at 15 μM Tamoxifen and its respective control. There was no evidence for any voltage dependence inhibition in the range −80 to +80 mV. The single-channel conductance was not affected by Tamoxifen, the values were 259 ±4 pS in control condition and 245 ±6 pS in the presence of the Tamoxifen ($n = 4$).

Discussion

The present study demonstrates that physiological concentrations of 17β-Estradiol and micromolar concentrations of Tamoxifen inhibit, in excised patches, the Maxi chloride channels present in the apical membrane of human placental syncytiotrophoblast.

The addition of 1 μM 17β-Estradiol to the bath solution decreased the total current in the patch at negative holding potentials. However, the presence of the hormone did not affect the single-channel conductance, therefore its effect must be due to modulation of its open probability (*Po*). The effect of 17β-Estradiol on *Po* was voltage dependent, with *Po* decreasing at negative potentials and increasing with depolarization compared with controls. The open probability (*Po*)-versus-voltage relationship in the presence of 17βE also could be described as a bell-shaped curve, but it was shifted to a more positive potential. It is notable that the stereoisomer 17α-Estradiol did not change the total current in the patch, indicating a specific interaction with the biological estrogen 17β-Estradiol.

Diverse mechanisms have been proposed to explain nongenomic pathways mediating acute modulation of membrane ion channels by estrogens. Some of these mechanisms are related to their interaction with binding sites, which generally determine a rapid effect as opposed to the long-term genomic effect. In some cases, the rapid effects represent the interaction of estrogen with a plasma-membrane target. One of these is the hormone action on the large-conductance chloride channel from aortic endothelial cells [26] and fibroblasts [21], which is consistent with an interaction of the steroid with a cell-surface receptor. The activation of this chloride channel by Tamoxifen was prevented by lower concentrations of 17βE. These authors assumed that this rapid effect of 17βE implies that these steroids influence the cell-surface receptor, a putative membrane estrogen receptor [34], which is distinct from the classic nuclear receptor [33]. Another possibility is that the rapid effect could be explained by the generation of intracellular signals [39]. In our case, in which the isolated membranes were reconstituted into giant liposomes, we have demonstrated that no intracellular signaling and/or interaction of the steroid with a cell-surface receptor is required for action of estrogen or antiestrogen on the apical chloride channel from human trophoblast. These observations suggest a direct interaction of estrogen with the channel. This result is consistent with results reported by Valverde et al. [43], who documented the direct interaction of 17βE with a voltage-gated channel subunit of Maxi-K^+ channel (BK).

In addition, our results show that Tamoxifen, a non-steroidal antiestrogen, produces voltage-independent inhibition of open probability, without changing single-chloride-channel conductance from human placenta. Tamoxifen is used to treat breast cancer and is being assessed as a prophylactic drug for those at high risk of developing tumors [22]. The pharmacological effects of Tamoxifen are believed to be mediated through the binding of Tamoxifen to the estrogen receptor, thereby antagonizing receptor function [1, 25]. However, several lines of evidence suggest that the action is not mediated via the estrogen receptor. Zhang et al. found that Tamoxifen and its derivatives are high-affinity blockers of specific chloride channels and this blockade appears to be independent of the interaction of Tamoxifen with the estrogen receptor [46]. Recently Dick et al. have reported in single-channel experiments that Tamoxifen increased the open probability and decreased the unitary conductance of Maxi-K^+ channel. The effect of Tamoxifen, in order to increase Maxi-K^+ channel *NPo* (N = number of channels in the patch), required the presence of the regulatory β_1 subunit. The activating effect of Tamoxifen was similar to that reported for 17βE on the Maxi-K^+ channel containing the β_1 subunit [43], with a similar ability to activate Maxi-K^+ channels in patches of excised membrane. Our results strongly suggest that Tamoxifen affects the chloride channel directly.

In conclusion, our electrophysiological studies show inhibition of the Maxi Cl^- channel from placenta by 17β-Estradiol and Tamoxifen. However, the biologically inactive isomer of 17β-Estradiol, 17α-Estradiol, did not affect these channels. In addition, because of the nature of the approach, i.e., a reconstituted system devoid of enzymes and metabolites, this inhibition is due to a direct interaction rather than mediation by a metabolic process.

From a functional point of view, the control of these channels by steroid hormones may be of great importance in placental physiology. The hormonal influences on cell growth and ion transport in fetal and adult tissues are under investigation in a number of laboratories. It is known that β-adrenergic agonists stimulate Cl^- secretory pathways in fetal and adult airway cells [32], and some of these studies show that steroid hormones associated with pregnancy can modulate the relative magnitude of baseline and β-adrenergic agonist-stimulated Cl^- secretion and Na^+ absorption [8, 45]. Zeitlin et al. [45] found that steroid hormones are modulators of Na^+ absorption and Cl^- secretion in tracheal epithelia from cells of fetal, pregnant, and non-pregnant animals. They discovered that cells of nonpregnant rabbits had a large Na^+ absorptive current and small Cl^- secretory current. In contrast, fetal cells had greater Cl^- secretion and much less Na^+ absorption; however, the situation is similar when steroid hormones are added to cell cultures from nonpregnant rabbits. Since cells from pregnant animals had ion transport characteristics of fetal cells, the authors assumed that the steroid hormone environment, present in high concentration during pregnancy in humans, influenced transport in both the maternal and the fetal circulation. Thus it could play a role in the hydration of the airways, both in the fetal state and in adulthood. However, the mechanism responsible for the regulation of Cl^- secretion and also for turning off fetal Cl^- secretion and for the massive reabsorption of fluid at birth remains elusive. To know the effect of theses hormones on conductive pathways from epithelial membranes, it is necessary to elucidate the molecular mechanism involved in secretory and absorbing processes regulated by hormones.

The results of our studies demonstrate that apical chloride channels from human placenta are modulated by steroid hormone (17βE) and Tamoxifen. The role of these channels in placenta is still unclear, however, the regulation of chloride channels could be important in the control of electrolyte and fluid transfer across the placenta in general. In particular, these results could help to understand the mechanism involved in the regulation of important functions like Cl^- secretion. In addition, if these channels contribute to setting the membrane potential, their regulation could have consequences for nutrient transport and delivery to the fetus. The regulation of these channels may help to unravel their possible role in transplacental transport in normal and pathologic placental tissue.

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