Activation of Cl^- Channels by Human Chorionic Gonadotropin in Luteinized Granulosa Cells of the Human Ovary Modulates Progesterone Biosynthesis

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Chloride permeability pathways and progesterone (P4) secretion elicited by human chorionic gonadotropin (hCG) in human granulosa cells were studied by electrophysiological techniques and single-cell volume, membrane potential and Ca^{2+}_i measurements. Reduction in extracellular Cl^- and equimolar substitution by the membrane-impermeant anions glutamate or gluconate significantly increased hCG-stimulated P4 accumulation. A similar result was achieved by exposing the cells to hCG in the presence of a hypotonic extracellular solution. Conversely, P4 accumulation was drastically reduced in cells challenged with hCG exposed to a hypertonic solution. Furthermore, conventional Cl^- channel inhibitors abolished hCG-mediated P4 secretion. In contrast, 25-hydroxycholesterol-mediated P4 accumulation was unaffected by Cl^- channel blockers. In human granulosa cells, hCG triggered the activation of a tamoxifen-sensitive outwardly rectifying Cl^- current comparable to the volume-sensitive outwardly rectifying Cl^- current. Exposure of human granulosa cells to hCG induced a rapid 4,4′-diisothiocyanatostilbene-2,2-disulphonic acid-sensitive cell membrane depolarization that was paralleled with an approximately 20% decrease in cell volume. Treatment with hCG evoked oscillatory and nonoscillatory intracellular Ca^{2+} signals in human granulosa cells. Extracellular Ca^{2+} removal and 4,4′-diisothiocyanatostilbene-2,2-disulphonic acid abolished the nonoscillatory component while leaving the Ca^{2+} oscillations unaffected. It is concluded that human granulosa cells express functional the volume-sensitive outwardly rectifying Cl^- channels that are activated by hCG, which are critical for plasma membrane potential changes, Ca^{2+} influx, and P4 production.

AT THE MIDDLE OF the ovarian cycle, LH surge induces luteinization, resumption of meiosis, *cumulus ooforus* expansion, and follicular rupture. LH induces the expression of several steroidogenic genes in follicular mural granulosa cells. In addition, it has been shown that gonadotropin activates an extracellular Ca^{2+}-independent and 4,4′-diisothiocyanatostilbene-2,2-disulfonic acid (DIDS)-sensitive Cl^- conductance in steroidogenic cells in several species, but its physiological relevance has not been elucidated (1). It has been shown that at low gonadotropin concentrations steroidogenesis requires the activation of Cl^- channels independent from an increase in cAMP (2). On the other hand, it has been suggested that Cl^- efflux from steroidogenic cells participates in the synthesis of cAMP and thus, stimulates steroidogenesis (3). Acute production of steroids requires *de novo* synthesis as well as protein kinase A (PKA) and cGMP-dependent protein kinase-dependent phosphorylation of the cholesterol transferring steroidogenic acute regulatory (StAR) protein, involved in the transfer of cholesterol from the outer to the inner mitochondrial membrane (4, 5). It has been reported that reduction of extracellular Cl^- ions during the culture of rat Leydig cells markedly enhances LH-stimulated steroidogenesis by increasing StAR protein synthesis via a general increase in cAMP-dependent protein synthesis or by StAR phosphorylation (6, 7). Therefore, depolarization driven by the efflux of Cl^- ions (8–10) could be the primary event of the steroidogenic signal transduction pathway. This depolarization would in turn trigger the activation of voltage-dependent Ca^{2+} channels (11), causing an increase in intracellular Ca^{2+}. The observed increase in $[Ca^{2+}]_i$ may modulate the activity of Ca^{2+}-activated currents, such as Ca^{2+}-dependent Cl^- or K^+ channels and Ca^{2+}-dependent phosphorylation events and eventually steroidogenesis (12–14).

Volume-sensitive outwardly rectifying (VSOR) Cl^- channels have been described in many mammalian cells, including steroidogenic cells, such as Leydig cells and adrenocortical cells (1, 8). VSOR Cl^- channel currents induced by volume changes in different cell types have common biophysical and pharmacological properties, and share evident similarities with the gonadotropin-activated Cl^- current. Both VSOR Cl^- channel currents and gonadotropin-activated Cl^- currents exhibit a moderate outwardly rectifying behavior and a depolarization-dependent inactivation (15). In this report, we addressed the question of whether hCG

Abbreviations: DIDS, 4,4′-Diisothiocyanatostilbene-2,2-disulphonic acid; DMSO, dimethyl sulfoxide; hCG, human chorionic gonadotropin; NMDG, N-methyl-D-glucamine; P4, progesterone; PKA, protein kinase A; StAR, steroidogenic acute regulatory; VSOR, volume-sensitive outwardly rectifying.

could activate VSOR Cl^- currents in luteinized human granulosa cells and hence, modulate P4 synthesis. Our results indicate for the first time in luteinized human granulosa cells that VSOR Cl^- channel activation by hCG plays a relevant role in steroidogenesis by promoting Cl^- efflux, cell membrane depolarization, and ensuing Ca^{2+} influx and Ca^{2+}_i mobilization.

Materials and Methods

GC preparation and culture

Human GCs were isolated from follicular aspirates of women undergoing *in vitro* fertilization at IDIMI (Santiago, Chile). The use of these cells was approved by the local ethics committee and by signed informed consent of the patients. Primary cultures of human granulosa cells were prepared from cells collected from patients (n = 15) undergoing oocyte retrieval following standard follicular hyperstimulation, due to male factor infertility at the Departamento de Obstetricia y Ginecología, IDIMI, Hospital San Borja Arriarán, Universidad de Chile. Patients received a GnRH agonist (Lupron, Abbott Laboratories, Abbott Park, IL), for pituitary suppression and recombinant FSH (Puregon, Organon Lab, Oss, The Netherlands) and human menopausal gonadotropin (HMG, Ferring, Kiel, Germany) for follicular recruitment, followed by a single dose of human chorionic gonadotropin (hCG) (10,000 IU; Pregnyl Organon Lab) 36 h before oocyte retrieval. After isolation and purification, human granulosa cells were initially cultured on fibronectin-coated 100-mm tissue culture dishes in DMEM/F12 medium (1:1; DMEM/F12) supplemented with 10% heat-treated fetal calf serum (FCS), 20 nM insulin, 20 nM selenium, 20 nM apo-transferrin and antibiotics in an atmosphere of 95% air-5% CO_2 at 37 C for 24–72 h. Once granulosa cells reached 60–80% confluence, they were switched to a serum-free DMEM/F12, 0.1% BSA, 100 μg/ml transferrin and 20 nM selenium medium for 48 h before performing the experiments (16).

Chemicals and solutions

Drugs (all from Sigma-Aldrich, St. Louis, MO) were dissolved in dimethylsulfoxide (DMSO, Sigma-Aldrich) and diluted in the cell culture medium until reaching the desired concentration. Final DMSO concentration in experimental and control conditions was 0.1%.

In all experimental conditions in which the effect of a reduction in extracellular [Cl^-] was tested, Cl^- was substituted (equimolar) with glutamate or gluconate. The extracellular solution used for membrane potential, cell volume and Ca^{2+}_i measurements contained (in mM) 100 NaCl, 5 KCl, 0.5 $MgCl_2$, 2 $CaCl_2$, 70 sorbitol and 10 HEPES, osmolarity 288 ± 6 mosmol/liter.

Progesterone assay

Human granulosa cells were cultured in 24-well plates. On d 3, cells were subjected in triplicates to the different experimental maneuvers. After 1, 2, 3, 4, 12, and 24 h, supernatants were collected and P4 concentration was measured by RIA (17). Mean values were normalized to untreated control cells. Data are expressed as the average of normalized mean values.

Electrophysiological experiments

For electrophysiological experiments, cells were grown on 12-mm cover slips and directly mounted on the experimental chamber (RC-25; Warner Instruments, Hamden, CI) installed on the stage of an inverted microscope (Olympus IX70, New York, NY). Solution changes were done by a gravity-fed perfusion system and the solution level in the chamber was kept constant by a peristaltic pump. Bath and pipette solutions were designed to study Cl^- currents. The bath solution contained (mM): 5 NaCl, 95 NMDGCl, 2 $CaCl_2$, 1 $MgCl_2$, 100 sorbitol and 10 HEPES (pH 7.4), adjusted with Tris. Osmolarity was adjusted with sorbitol to 300 ± 5 mosmol/liter using an osmometer (Advanced Instruments, Norwood, MA). Pipette solution contained (mM): 5 NaCl, 133 CsCl, 1 $MgCl_2$, and 10 HEPES, pH 7.2, adjusted with Tris and an osmolarity of 295 ± 5 mosmol/liter. Patch-clamp pipettes were made from thin borosilicate (hard) glass capillary tubing with an outer diameter of 1.5 mm (Clark Electromedical, Hamden, CT), using a BB-CH puller (Mecanex, Geneva, Switzerland). The nystatin perforated-patch whole-cell configuration was used as described (18) and whole-cell currents were recorded with an Axopatch 200B (Molecular Devices Corp., Sunnyvale, CA) amplifier. Command voltage protocols and whole cells currents acquisition were controlled by pClamp 10.2 (Molecular Devices Corp.) via a laboratory interface (Digidata 1322A; Molecular Devices Corp.). The bath was grounded via an agar-KCl bridge. Nystatin (Sigma-Aldrich) stock solution was freshly made in DMSO at 50 mg/ml. Aliquots of stock solution were added to the pipette solution to obtain a final concentration of 165 μg/ml. Square pulses of 5 mV were used to monitor the electrical access to the cell. Usually, a stable access resistance (<15 MΩ) was achieved after 10–15 min. The acquisition rate was 1 kHz.

Membrane potential measurements

Human granulosa cells (d 4–7) cultured on 25 mm-glass cover slips were put into a recording chamber mounted on a confocal Zeiss LSM Pascal 5 system (Carl Zeiss, AG, Jena, Germany) and plasma membrane potential changes were monitored using $DiBAC_4$ (3) (Molecular Probes, Eugene, OR), as previously described (19). This anionic fluorescent dye is distributed across the plasma membrane depending on the membrane potential and following Nernst's equation (20). $DiBAC_4$ (3) (200 nM) was applied extracellularly for about 20 min to ensure dye distribution across the cell membrane. Changes in fluorescence intensity were monitored for 30 min by sampling every 10 sec using an excitation wavelength of 515 nm and an emission wavelength of at least 600 nm. Fluorescence data were transformed to mV using a calibration curve as described previously (20).

Cell volume measurements

Human granulosa cells (d 4–7) cultured on 25 mm-glass cover slips were put into a recording chamber mounted on a confocal Zeiss LSM Pascal 5 system (Carl Zeiss), and changes in cell water volume of individual cells were assessed by measuring variations in the concentration of the intracellularly trapped fluorescent dye calcein-AM (Molecular Probes), with an excitation wavelength of 488 nm and emission wavelength between 515 and 560 nm recorded with a band pass filter, as previously described (21, 22). Briefly, human granulosa cells were loaded with 5 μM calcein-AM for 5 min and then superfused with an isosmotic solution for 15 min before subjecting the cells to hypotonicity or treatments. Images were obtained at 10-sec intervals and the fluorescence of a 10-μm^2 area in the center of a cell was measured. Data are presented as V_t/V_0 values, where V_0 is the cell water

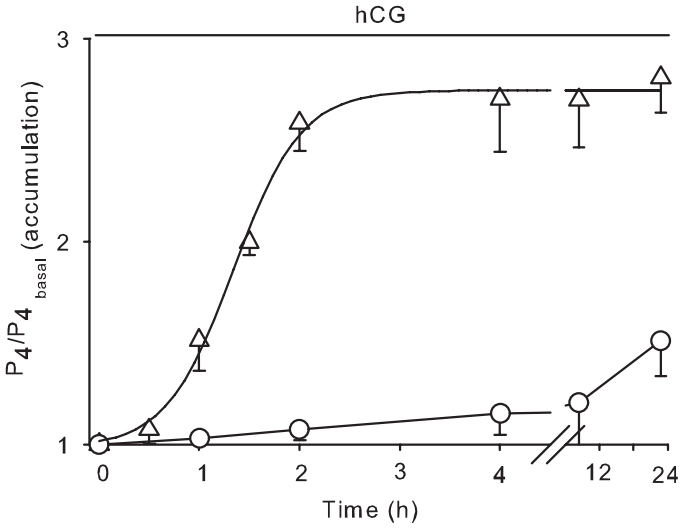

FIG. 1. Time course of P4 accumulation stimulated by hCG. *Triangles* represent P4 accumulation induced by hCG (10 IU/ml) normalized with respect to control (basal, nonstimulated) values. *Circles* indicate P4 accumulation induced by hCG in the presence of 10 μM cycloheximide (mean ± SEM, n = 15 independent determinations).

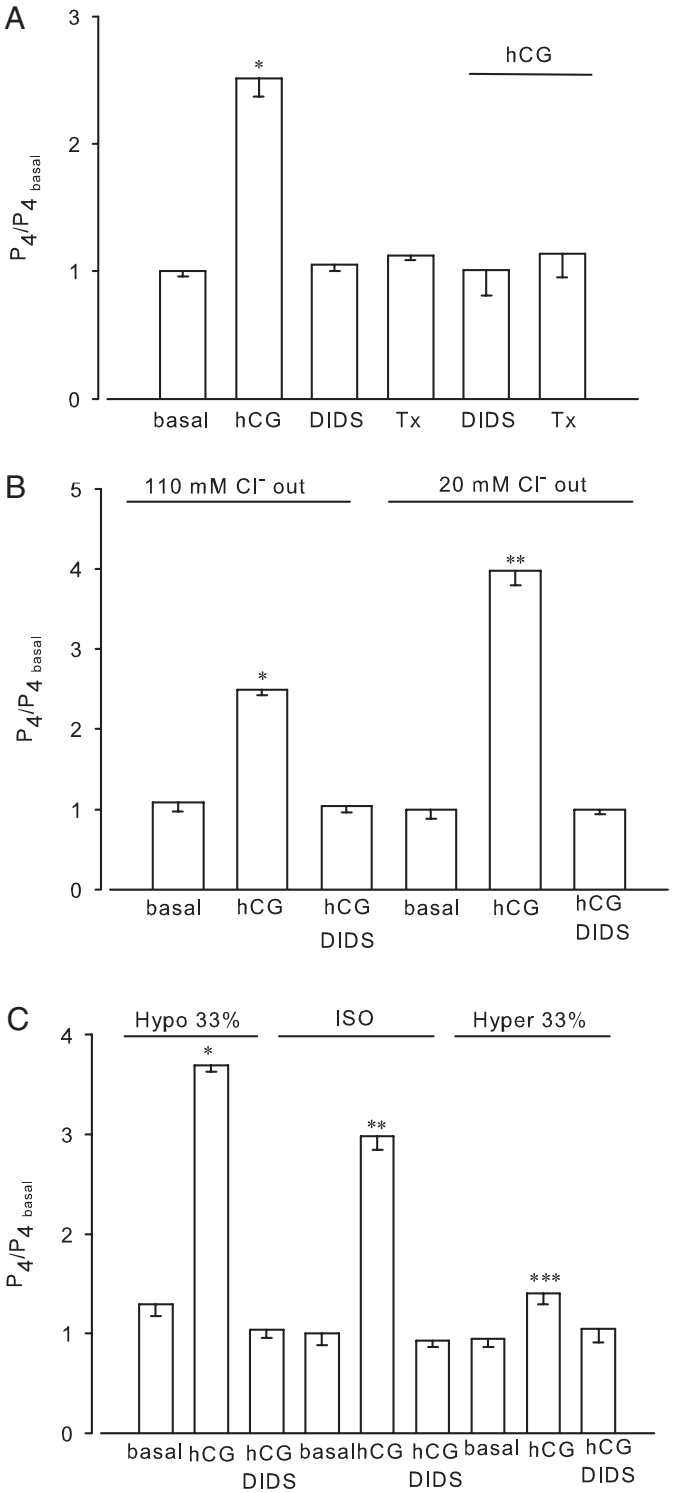

FIG. 2. Cl^- efflux is necessary for steroidogenesis induced by hCG. *Bars* represent P4 accumulation after 2 h of continuous stimulation with hCG, normalized with respect to control values (mean ± SEM; *, $P < 0.05$ between experimental and control conditions; ** and ***, $P < 0.05$ between experimental conditions). A, Human granulosa cells were stimulated with hCG in the presence or absence of DIDS (100 μM) or tamoxifen (Tx, 10 μM). B, Human granulosa cells were exposed to hCG for 2 h in a high-Cl^- (110 mM) or low-Cl^- (20 mM) extracellular medium in the presence or absence of DIDS (100 μM). These experiments were carried out by equimolar substitution of NaCl with Na-glutamate. C, Human granulosa cells were stimulated with hCG for 2 h in 200 (Hypo 33%), 300 (Iso), and 400 (Hyper 33%) mosm/liter extracellular solutions in the presence or absence of DIDS (100 μM).

volume in isosmotic solution at time 0 and V_t is the cell water volume at time t. V_t/V_0 was calculated from the fluorescence intensity ratio F_0/F_t.

Intracellular $Ca^{2+}{}_i$ measurements

Intracellular Ca^{2+} was measured by dual-wavelength emission ratiometric laser scanning confocal microscopy, using the Ca^{2+}-sensitive fluorescent dyes fluo-3 and fura-red, as described in (23, 24). Human granulosa cells (d 4–7) cultured on 25 mm-glass cover slips were loaded with fluo-3 and fura-red for 20–45 min at room temperature in the presence of 5 μM fluo-3 acetoxymethyl ester (fluo-3-AM) and 15 μM fura-red-AM, dissolved in pluronic acid/DMSO (Invitrogen, Carlsbad, CA). Cells were thoroughly washed and experiments were started after 30 min. Each cover slip was placed in a perfusion chamber mounted on a confocal Zeiss LSM Pascal 5 system (Carl Zeiss). Dyes were excited with the 488 nm line of an argon-krypton laser and emission was detected simultaneously at 515–530 nm (fluo-3) and long pass at least 670 nm (fura-red) with a ×40/1.4 NA oil immersion objective. Changes in $Ca^{2+}{}_i$ were measured in a field-of-view consisting of 10–30 cells. Fluo-3 and fura-red fluorescence emission intensity were acquired every 30 sec. Changes in $Ca^{2+}{}_i$ were inferred from changes in the relative fluorescence ratio, calculated by dividing R at each time point by R_0, the fluorescence ratio measured as the average fluorescence ratio 1–2 min before stimulation.

Data analysis

The increase in steroid production after hCG stimulation was defined by the ratio stimulated steroid concentration over basal steroid concentration, expressed as n-fold increase. Absolute basal values were 24.3 ± 8.5 ng/50000 live cells, whereas hCG-stimulated values at 2 h reached 62.7 ± 12.7 ng/50,000 live cells. At least four individuals were considered for each experimental condition. Experimental data are presented as mean ± SEM. Fisher's least significant difference procedure was used to compare multiple groups employing Statgraphics Plus 5.0 (GraphPad Software, Inc., San Diego, CA). Statistical significance was considered at $P < 0.05$. For data of membrane potential, cell volume and $Ca^{2+}{}_i$ measurements, one-sample t test was used to test for significance.

Results

Kinetics of progesterone accumulation in the media of cultured human granulosa cells

First, we explored the time course of hCG-stimulated P4 accumulation in the culture media. To that end, human granulosa cells were stimulated with hCG (10 IU/ml) present in the culture medium described above, and P4 accumulation in the extracellular medium was determined at different times (Fig. 1). As depicted, hCG stimulation induced a cycloheximide-inhibitable increase in P4 accumulation, well described by a sigmoid function. The maximal P4 accumulation (2.7 ± 0.15-fold increase) was obtained after 2 h of sustained hCG-stimulation, reaching thereafter a plateau.

Cl^- efflux is necessary for hCG-stimulated steroidogenesis

Several studies in mature rat Leydig cells and MA-10 mouse tumor Leydig cells have demonstrated that Cl^- ions and Cl^- channels play a relevant role in hormone release (1, 8, 25–27). Therefore, we studied whether hCG-stimulated P4 accumulation is sensitive to conventional inhibitors of Cl^- channels. As illustrated in Fig. 2A, DIDS, a general blocker of Cl^- channels as well as tamoxifen, a relatively specific blocker for VSOR Cl^- channels (28) significantly inhibited

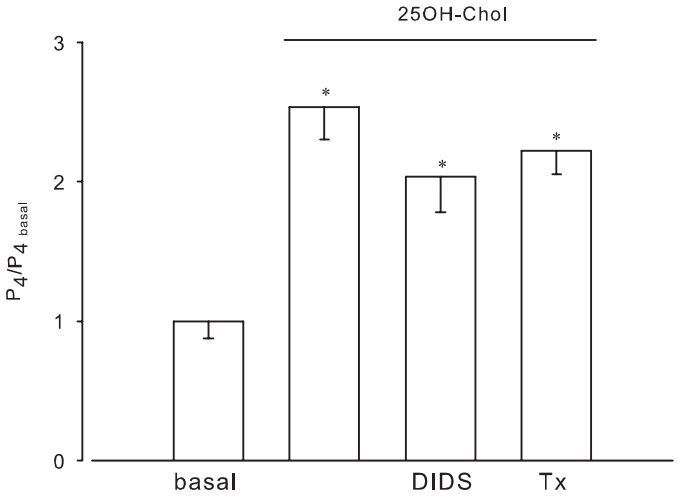

FIG. 3. Cl^- channel blockers modulate the limiting step of steroidogenesis. B, *Bars* represent P4 accumulation induced by 10 μM 25-hydroxycholesterol normalized with respect to control values, in the presence of the Cl^- channel inhibitors DIDS or tamoxifen (mean ± SEM, *, $P < 0.05$ between experimental and control values; n = 8).

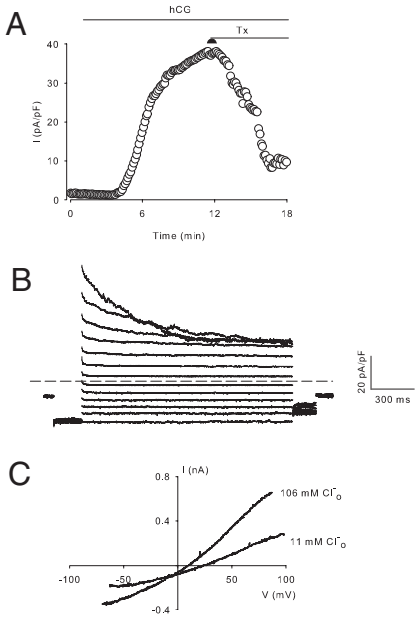

FIG. 4. hCG induces the activation of a current similar to VSOR Cl^- currents. A, Representative experiment showing the time course of current development evoked by 10 IU/ml hCG in isotonicity. Currents were measured at 80 mV at an interval of 7 sec and normalized by cell capacitance. Tamoxifen (Tx, 10 μM) during the time indicated. B, Representative steady-state nystatin-perforated whole-cell current traces triggered by a voltage step protocol from −100 to 100 mV in 20 mV increments from a holding potential of −30 mV. The test pulse (2000 msec) was preceded by a prepulse to −100 mV lasting 200 msec followed by a postpulse to −60 mV lasting 200 msec. The *dashed line* represents zero current. The currents were recorded at the time indicated by the *filled circle* in (A) (~10 min stimulation with hCG). C, Current-voltage relationship of Cl^- currents activated by hCG in low-Cl^- (11 mM) and high-Cl^- (106 mM) extracellular medium, using a 2.5-sec duration voltage ramp from −80 to 80 mV from a holding potential of 0 mV. These experiments were carried out by equimolar substitution of NaCl with Na- glutamate.

hCG-induced accumulation of P4 triggered by exposing human granulosa cells for 2 h to hCG, without affecting basal P4 levels. If efflux of Cl^- ions had an effect on P4 synthesis, increasing the driving force for Cl^- efflux might enhance P4 accumulation. As shown in Fig. 2B, a reduction in $[Cl^-]_o$ obtained by equimolar substitution with glutamate, a less-permeant anion, significantly increased P4 accumulation. Interestingly, maneuvers known to activate or inhibit VSOR Cl^- channels, such as hypotonicity and hypertonicity, considerably affected hCG-induced accumulation of P4, as depicted in Fig. 2C. Exposure of human granulosa cells to a hypotonic extracellular solution significantly enhanced hCG-mediated P4 accumulation, compared with the effect of the hormone in an isotonic extracellular solution. The effect of hypotonicity on hCG-induced P4 production was inhibited by DIDS. Conversely, extracellular hypertonicity strongly reduced hCG-mediated P4 accumulation.

25-Hydroxycholesterol is a cholesterol analog that translocates to the inner mitochondrial membrane independently of StAR activity, and thus, it allows separating the cholesterol translocation step from cholesterol processing. We tested therefore whether 25-hydroxycholesterol-induced P4 accumulation was sensitive to Cl^- channels blockers. As shown in Fig. 3, 25-hydroxycholesterol-induced P4 accumulation was unaffected by DIDS and tamoxifen, indicating that these inhibitors do not act by inhibiting cholesterol processing, but exert their action on StAR-dependent cholesterol translocation.

hCG induces VSOR Cl^--like currents in human granulosa cells

Based on the effect of extracellular anisotonicity on P4 accumulation, we explored whether exposure of human granulosa cells to hCG would result in the activation of a VSOR-type Cl^- currents. As depicted in Fig. 4A, upon hCG exposure in an isotonic extracellular solution, cells responded by activating a current (measured at 80 mV) that reached steady-state at approximately 12 min and was effectively blocked by the VSOR Cl^- channel inhibitor tamoxifen. This current elicited by hCG (monitored at steady-state) exhibited similar properties to the swelling-induced Cl^- current described in epithelial cells (29, 30), as shown in Fig. 4B. The hCG-induced Cl^- current showed an outwardly rectifying behavior with a reversal potential of 8 ± 1.1 mV (n = 5) (E_{Cl} of 6.9 mV). A reduction in $[Cl]_o$ and equimolar substitution with glutamate led to a decrease in both outward and inward currents and a shift of the reversal potential toward more positive values (n = 5), as previously reported for VSOR Cl^- currents (30) (Fig. 4C).

hCG-induced VSOR Cl^- current$^-$-dependent membrane depolarization and cell shrinkage

It has been reported that bovine adrenocortical cells respond to acute exposure of ACTH with membrane depolarization mediated by the activation of a Cl^- current (31). Therefore, we addressed the question whether acute exposure of human granulosa cells to hCG and subsequent activation of VSOR Cl^- channels would result in membrane depolarization and, as expected for VSOR Cl^- current acti-

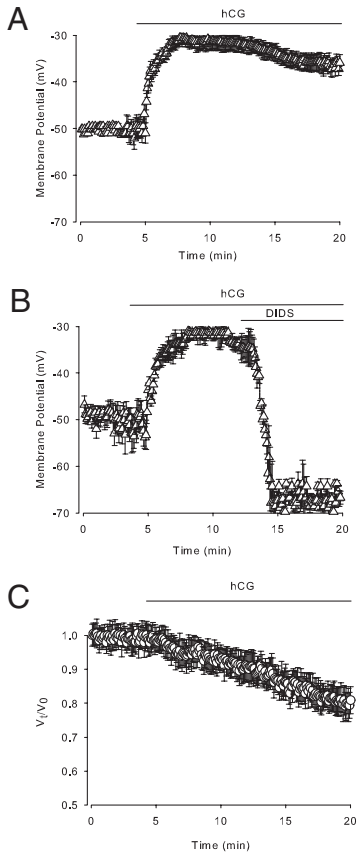

FIG. 5. hCG-induced membrane depolarization and cell shrinkage. Human granulosa cells were loaded with 5 μM calcein-AM and with 200 nM $DiBAC_4$ (3) for 30 min at 37 C. A, Time course of membrane depolarization induced by 10 IU/ml hCG. B, Effect of DIDS (100 μM) on membrane depolarization mediated by hCG. C, Time course of relative cellular volume induced by 10 IU/ml hCG. Each symbol represents the mean ± SEM of over 30 cells/field in n = 6 experiments.

vation, in cell shrinkage. To that end, we performed simultaneous measurements of membrane potential and cellular volume from human granulosa cells stimulated with hCG. As shown in Fig. 5A, hCG caused a rapid depolarization from -50 ± 3 to -29 ± 2 mV, close to the expected value for E_{Cl}. Blockade of VSOR Cl^- channels with DIDS (or tamoxifen, not shown) rapidly repolarized the cells to a value close to the expected equilibrium potential for K^+ (Fig. 5B). The hCG-induced depolarization was paralleled with a monotonic decrease in cellular volume of approximately 20% (n = 7), as depicted in Fig. 5C, suggesting that both resting membrane potential and hCG-induced membrane depolarization are dependent upon Cl^- conductance.

hCG-induced intracellular Ca^{2+} mobilization

Although it is well established that hCG effect is mediated primarily via the adenylate cyclase signaling pathway (14, 32, 33), the role of Ca^{2+} in hCG action is less studied. Therefore, we explored the effect of hCG on Ca^{2+}_i signals in primary cultures of human granulosa cells. As depicted in Fig. 6A, cells treated with 10 IU/ml hCG responded by increasing Ca^{2+}_i. The response could be arranged in four groups: cells showing an oscillatory pattern; a plateau behavior; cells exhibiting both components; and nonresponding cells. These data are summarized in Table 1. Next, we examined the effect of extracellular Ca^{2+} and DIDS on hCG-evoked Ca^{2+}_i signals. As shown in Table 1, removal of extracellular Ca^{2+} or exposure to DIDS significantly increased the percentage of cells exhibiting an oscillatory pattern while decreasing the percentage of cells displaying the plateau and mixed behavior, suggesting that these patterns are dependent on extracellular Ca^{2+} and activation of DIDS-sensitive Cl^- channels. Furthermore, hCG-stimulated P4 accumulation was drastically reduced under nominally free extracellular Ca^{2+} conditions (Fig. 6B).

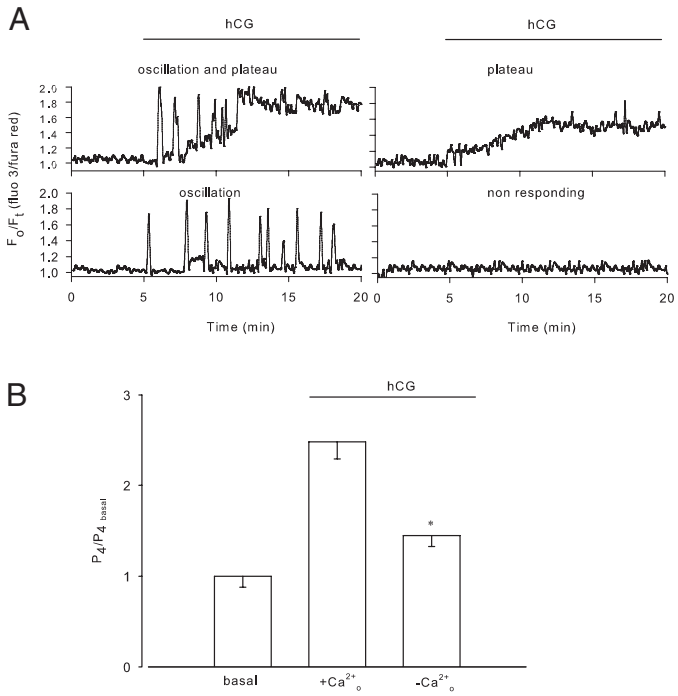

FIG. 6. hCG-induced Ca^{2+}_i signals. Human granulosa cells were loaded with fluo3/fura red for 20–45 min at room temperature. A, Representative traces of single cell microfluorometric experiments showing the time course of the different patterns of Ca^{2+}_i changes evoked by 10 IU/ml hCG. B, *Bars* represent P4 accumulation induced by 10 IU/ml hCG normalized with respect to control values, in the absence of external Ca^{2+} (mean ± SEM; *, $P < 0.05$ between experimental and control values; n = 4).

Discussion

In this work, we demonstrate that acute exposure of human granulosa cells to hCG leads to the activation a Cl^- current exhibiting features compatible with volume-sensitive outwardly rectifying Cl^- currents. Furthermore, we provide evidence that this Cl^- current is responsible, at least in part, for hCG-mediated membrane depolarization, cellular shrinkage and Ca^{2+} influx, as well as critical for hCG-induced steroidogenesis. Although previous studies have shown that Cl^- efflux profoundly affects steroidogenesis and Cl^- currents have been reported in several endocrine cells (8, 15, 34), there is little information available for human granulosa cells.

In vitro stimulation of human granulosa cells with hCG

TABLE 1. Ca_i^{2+} in human granulosa cells stimulated with 10 IU/ml hCG (% ± SEM)[a]

	Control	Nominally free Ca^{2+}	DIDS
Oscillation	8 ± 1	72 ± 4	71 ± 1
Plateau	25 ± 3	3 ± 2	5 ± 3
Oscillation and plateau	52 ± 3	8 ± 2	5 ± 2
Nonresponding	15 ± 2	17 ± 3	19 ± 2

[a] Total number of cells studied: 402; $n = 3$ independent experiments.

induces the accumulation of P4 following a sigmoidal behavior that reaches plateau after 2 h of continuous exposure to hCG. The effect on P4 accumulation of hCG was mimicked by exposing human granulosa cells to 1 mM dibutyryl-c-AMP for 2 h. This cell-permeable cAMP analog induced a 2.3 ± 0.14-fold increase in P4 accumulation (not shown). These results are in line with previous observations (27), showing that not only the activation of StAR by PKA-dependent phosphorylation is necessary for acute steroidogenesis, as the process also requires synthesis of new proteins, demonstrated by the effect of the protein translation inhibitor cycloheximide. Although we did not investigate the effect on StAR mRNA transcription in our system, it has been previously reported that P4 accumulation requires *de novo* StAR mRNA transcription and protein translation (35). Furthermore, the percentage of cells expressing the steroidogenic marker StAR protein increased after 2 h treatment with hCG from 43% to 98%, as judged by immunofluorescence experiments using a rabbit polyclonal antihuman StAR antibody, as previously described (36) (not shown). In view of these results, experiments on P4 accumulation were performed using a 2-h stimulation protocol with hCG.

Steroidogenesis induced by gonadotropins requires the activation of Cl^--selective ion channels. These anion currents, of unknown molecular identity, are strikingly similar to VSOR Cl^- currents recorded in multiple cell types of different species (37). Regardless of the extensive functional characterization, the molecular identity of the channel(s) accounting for VSOR Cl^- currents remains unresolved, even though a number of molecules have been proposed: ClC-2 (38, 39), ClC-3 (40); P-glycoprotein (41, 42), PI_{Cln} (43, 44), phospholemman (45), and bestrophin-1 (46).

Here we show that maneuvers that stimulate Cl^- efflux, such as extracellular replacement of Cl^- by a less-permeant anion or exposure of cells to hypoosmotic solutions enhance hCG-induced P4 accumulation. On the other hand, inhibition of Cl^- efflux by Cl^- channel blockers (47) or extracellular hypertonicity significantly reduces P4 accumulation. These data confirm previous observations obtained in various steroidogenic tissues from insects, birds, amphibians and mammals and for human granulosa cells (2, 26, 27). Interestingly, blockade of Cl^- efflux by DIDS or tamoxifen does not affect 25-hydroxycholesterol-induced steroidogenesis, suggesting that the effect of these drugs is located upstream to the rate-limiting reaction of acute steroidogenesis, *i.e.*, StAR-mediated mitochondrial membrane cholesterol translocation.

Using electrophysiological techniques, we demonstrate that hCG elicits the activation of a Cl^- current that is indistinguishable from the hypotonicity-induced Cl^- current, and thus attributable to VSOR Cl^- channels (1, 15, 25, 48–50). The hCG-induced Cl^- current was blocked by DIDS and tamoxifen, which also affected P4 accumulation.

It has been previously reported that modification of the membrane potential affects steroidogenesis in mammalian GCs (51, 52). Furthermore, K_{ATP} channels and BK-type K^+ channels have been shown to modulate steroidogenesis. In addition, K_{ATP} channels also participate in establishing the resting membrane potential (19). Using a fluorescence-based technique, we show that unstimulated human granulosa cells exhibit a resting membrane potential of approximately −50 mV. Under the experimental conditions used, stimulation by hCG drives the membrane potential to approximately −30 mV, a value in agreement with the equilibrium potential for Cl^-. In fact, −30 mV implies a $[Cl^-]_i$ of 33 mM, which is similar to the $[Cl^-]_i$ determined in intact capacitated human spematozoa (53). In agreement with this result, VSOR Cl^- channel inhibitors hyperpolarize the cells beyond the value of unstimulated cell resting membrane potential, approaching the expected equilibrium potential for K^+. However, depolarization induced by high extracellular K^+ does not induce hCG-mediated P4 accumulation (not shown), suggesting that anion efflux and perhaps cell shrinkage are necessary conditions for P4 synthesis. The observed hCG-mediated cell depolarization and VSOR Cl^- channel activation was paralleled by a 20% reduction in cellular volume, an additional indication supporting the notion that hCG activates VSOR Cl^- channels.

Intracellular Ca^{2+} mobilization upon activation of gonadotropin receptors with hCG in human primary granulosa cell cultures has been previously reported (33) as well as in human embryonic kidney 293 cells transfected with the human (33) or rat (54) receptor. Here we show that hCG evokes a complex $Ca^{2+}{}_i$ response pattern characterized by low frequency oscillations and/or a sustained increase. We found that 52% of the cells displayed an oscillatory-plateau response and only an 8% responded with oscillations alone. However, upon extracellular Ca^{2+} removal or exposure to DIDS, a high percentage (~70%) of cells only exhibited an oscillatory response, indicating that the sustained (plateau) phase is strongly dependent on Ca^{2+} influx presumably explained by Cl^- eflux-dependent membrane depolarization and subsequent activation of voltage-dependent Ca^{2+} channels. Interestingly, at variance with our results, 72% of human embryonic kidney 293 cells transfected with the rat receptor responded with Ca^{2+} oscillations that were partially dependent on extracellular Ca^{2+} (54).

Furthermore, we found that P4 accumulation is significantly impaired in the absence of extracellular Ca^{2+}, suggesting that $Ca^{2+}{}_i$ mobilization participates in P4 biosynthesis perhaps by amplifying the cAMP response due to a stimulatory effect of $Ca^{2+}{}_i$ on adenylyl cyclase activity.

In summary, our results suggest that hCG induces membrane depolarization through VSOR Cl^- channel-mediated Cl^- efflux, a necessary though insufficient step for steroidogenesis in primary cultures of human granulosa cells. A plausible explanation of our results is that hCG-

mediated depolarization, due to Cl^- efflux, is sufficient to drive the activation of voltage-dependent T- and L-type Ca^{2+} channels (11), allowing in turn Ca^{2+} influx, $Ca^{2+}{}_i$ mobilization, and subsequent modulation of StAR expression in a conventional PKA- and protein kinase C-dependent manner to promote P4 synthesis.

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