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Volume-sensitive Cl^- Current in Bovine Adrenocortical Cells: Comparison with the ACTH-induced Cl^- Current

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Abstract. In a previous study performed on zona fasciculata (ZF) cells isolated from calf adrenal glands, we identified an ACTH-induced Cl^- current involved in cell membrane depolarization. In the present work, we describe a volume-sensitive Cl^- current and compare it with the ACTH-activated Cl^- current. Experiments were performed using the whole-cell patch-clamp recording method, video microscopy and cortisol-secretion measurements. In current-clamp experiments, hypotonic solutions induced a membrane depolarization to −22 mV. This depolarization, correlated with an increase in the membrane conductance, was sensitive to different Cl^- channel inhibitors. In voltage-clamp experiments, hypotonic solution induced a membrane current that slowly decayed and reversed at −21 mV. This ionic current displayed no time dependence and showed a slight outward rectification. It was blocked to variable extent by different conventional Cl^--channel inhibitors. Under hypotonic conditions, membrane depolarizations were preceded by an increase in cell volume that was not detected under ACTH stimulation. It was concluded that hypotonic solution induced cell swelling, which activated a Cl^- current involved in membrane depolarization. Although cell volume change was not observed in the presence of ACTH, biophysical properties and pharmacological profile of the volume-sensitive Cl^- current present obvious similarities with the ACTH-activated Cl^- current. As compared to ACTH, hypotonic solutions failed to trigger cortisol production that was weakly stimulated in the presence of high-K^+ solution. This shows that in ZF cells, membrane depolarization is not a sufficient condition to fully activate secretory activities.

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Introduction

Chloride currents activated by cell swelling have been described in many mammalian cells including secretory cells, such as chromaffin cells [10], insulin-secreting cells [19] or Leydig cells [31]. Although some variations have been reported [28], Cl^- current induced by volume changes in different cell types share common biophysical and pharmacological properties. Usually, these membrane currents display a slight rectification in the outward direction. They do not exhibit time dependence except for high depolarizing voltage steps where these currents can inactivate in a time-dependent manner. They are inhibited to a variable extent by a wide variety of organic compounds such as 9-AC, 1,9 dideoxy-forskolin, DIDS, SITS, NPPB, niflumic acid or tamoxifen, but none of these compounds is very selective for this class of Cl^- channels.

The mechanisms by which cell volume changes activate these Cl^- channels are unknown. However, channel activities that require the presence of ATP inside the cell would be modulated or activated by different metabolic pathways, as for instance, tyrosine phosphorylation steps [36, 45] or signaling cascades involving small GTPases [30].

Molecular identity of volume-regulated Cl^- channels is always subject to controversy. Several proteins have been proposed as possible candidates, namely, C1C-3 [17, 46] a member of the C1C family [18], the protein pICln [20] and the MDR1-P-glycoprotein [40]. The different aspects of this unresolved question have been well discussed by Nilius and

Droogmans [27]. Functionally, volume-sensitive Cl^- channels can be involved in numerous physiological processes, such as cell volume regulation, modulation of the membrane potential, transport of osmolytes, cell proliferation, and so forth (for review, *see* Nilius et al. [28]).

In the present study, we show that zona fasciculata (ZF) cells isolated from bovine adrenal glands are sensitive to hypotonic solutions. In the presence of such solutions, these cells respond by a detectable change in cell volume correlated with a membrane depolarization caused by the activation of a Cl^- current. We have previously described that ACTH, the most potent activator of cortisol secretion in ZF cells, activated a cAMP-dependent Cl^- current that participated in the membrane depolarization of this cell type [13]. In this work, in addition to characterization of the volume-sensitive Cl^- current, our purpose was to compare it with the ACTH-induced Cl^- current. Such a comparison was mainly grounded on cell volume measurements under hypotonic or ACTH stimulation and discussed from basic biophysical and pharmacological properties of these two membrane currents. Lastly, determination of cortisol secretion was performed under hypotonic conditions and compared with that measured during ACTH exposure.

Materials and Methods

Cell Preparation

Adrenal glands were collected from a local slaughterhouse where they were removed from calves 4–6 months old. Isolated ZF cells were prepared as described by Bilbaut et al. [4]. Glands, freed of fat, were sliced with a Stadie-Riggs microtome, delivering slices about 0.5 mm thick. Only the second slice was used for enzymatic dispersion by sequential treatment with trypsin (Sigma, St. Louis, MO) at 0.125% in an equal volume of HAM F12/DMEM containing gentamycin (20 μg/l), penicillin-streptomycin (100 U/ml), L-glutamine (5 mM) and $NaHCO_3$, (14 mM) buffered with HEPES (15 mM) at pH 7.4. Dispersed cells were harvested, washed and cultured for one week in a medium containing HAM F12/DMEM (1:1), L-glutamine (5 mM), penicillin-streptomycin (100 U/ml), $NaHCO_3$ (14 mM), insulin (10 μg/ml), transferrin (10 μg/ml), and vitamin C (10^{-4} M), supplemented for 24 h with fetal calf serum (1%). For electrophysiological experiments, isolated cells were seeded at low density (4,000 to 6,000 cells/cm^2) in 35 mm Petri dishes, whereas for secretion measurements, they were cultured at high density (50,000 to 65,000 cells/cm^2) in 12-well dishes. For volume measurement, freshly isolated cells were seeded at high density in 35 mm Petri dishes in which the bottom was previously coated with a thin layer of Sylgard (Dow Corning, Midland, MI). Culture dishes were placed in a humidified incubator at 37°C and 5% CO_2 in air.

Solutions and Drug Preparation

The standard bath solution had the following composition (in mM): NaCl 135, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 2, glucose 10, buffered with HEPES 10 at pH 7.2 with NaOH. Isotonic Na^+-deficient solution was prepared by replacing 50 mM NaCl by 100 mM mannitol. Cell swelling was triggered with low-Na^+ solutions. As compared to the standard solution, these hypotonic solutions were deficient by 60 (18.5%) and 100 mOsm (31%). Osmolarity of standard and hypotonic solutions was controlled before use with a freezing-point depression osmometer. High-K^+ solutions (40 mM) were prepared by exchanging 35 mM NaCl by an equivalent amount of KCl from the standard solution. Low-Cl^- solution was obtained by replacing 135 mM Cl^- with methanesulfonate ions. For perforated recordings, the ionic composition of the pipette filling solution was (in mM): K aspartate 110, KCl 20, NaCl 10, $MgCl_2$ 2, EGTA 5, buffered with HEPES 10 at pH 7.2 by NaOH. Partition of the cell membrane was performed using the polyene antibiotic amphotericin B (Sigma) at a concentration of 240 μg/ml as described by Rae et al. [33]. For broken-membrane recordings, the ionic composition of the pipette filling solution was (in mM): K aspartate 50, KCl 60, NaCl 10, $MgCl_2$ 2, ATP (Mg) 2, EGTA 5, buffered with HEPES 10 at pH 7.2 by NaOH. Osmolarity of this solution was reduced by 40 mOsm as compared to external control solution.

Chloride current inhibitors, diphenylamine-2-carboxylic acid (DPC), 9-anthracene-9-carboxylic acid (9-AC), 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS) and tamoxifen were prepared just before use at different concentrations, as indicated in the text. All these drugs were obtained from Sigma and were solubilized in DMSO (DPC, 9-AC, NPPB, tamoxifen), or physiological saline (DIDS, SITS). The membrane properties of isolated cells were not affected by the final DMSO concentration, which was $\leq 0.1\%$. Human synthetic ACTH, fragment 1–24 (Sigma), was prepared from aliquots at 100 μM frozen in distilled water containing 50 mM acetic acid and 1% bovine serum albumin. ACTH was used at the final concentration of 10 nM by successive dilutions in the external solution.

Electrophysiology

Whole-cell membrane potential and membrane currents were recorded using usually the perforated-patch recording method with amphotericin B. However, as indicated in the text, some current-clamp recordings were performed using the standard whole-cell method. Experiments were carried out from day 2 to day 5 after plating on non-confluent isolated cells of 15–20 μM diameter that adhered to the plastic bottom of the culture dish. For experiments, a Petri dish was transferred from the incubator onto the stage of an inverted microscope and the culture medium was replaced by the control physiological solution. Further changes of external solution were then performed at a rate of 0.5 ml/min using a gravity perfusion system placed close to the cell (about 100 μM). Pipettes were pulled from thin-walled borosilicate glass (CG 150T-1.5 mM od., Harvard Apparatus, Edenbridge, UK) using a vertical puller (Kopf, Tujunga, CA) and were connected to the headstage of a patch-clamp amplifier RK 400 (Bio-Logic, Claix, France). For perforated-patch recordings, the tip of the pipette was dipped into the pipette solution for a few seconds, then the pipette was backfilled with the solution containing amphotericin B. Patch pipettes had a tip resistance of 2–4 MΩ in the control solution. Partition of the cell membrane by amphotericin B was continuously monitored by applying 20 mV hyperpolarizing pulses every 30 s from a holding potential of −60 mV. Experiments were started about 10 to 15 minutes after the seal was established when the increase of the transient capacitive current reached a steady-state value indicating a final series resistance of about 5 to 8 MΩ. The series resistance was not compensated because the voltage error introduced by the maximal activation of membrane currents was estimated to be

lower than 2%. Neither capacitive current nor leak current were subtracted from membrane-current recordings. In experiments where external Cl^- was lowered, a 3 M KCl-agar salt bridge was interposed between the Ag-AgCl reference electrode and the bath solution. In voltage-clamp experiments, current-voltage relationships were established from whole-cell membrane currents obtained by applying pulse protocols generated using the P-Clamp software (Axon Instruments, Burlingame, CA). In current-clamp experiments, membrane conductance was continuously monitored by injecting every 10 s constant hyperpolarizing 10 pA current pulses 2 s long. Current pulses were generated by an external programmable stimulator (SMP 311, Bio-Logic). Membrane conductance was calculated as $I/\Delta V$, where I was the injected current and ΔV, the evoked hyperpolarizing potential. All experiments were performed at room temperature (20–25°C).

Membrane-potential or membrane-current changes induced by hypotonic challenge or ACTH stimulation were monitored on both pen (Kipp & Zonen, Delft, the Netherlands) and tape (DTK 1204, Bio-Logic) recorders. Current signals were filtered at 1 kHz, digitized at 4 kHz with an analogue-to-digital converter (Labmaster TM 40, Scientific Solutions Inc., Solon, OH) and stored on the hard disc of a computer. For data analysis, Bio-Logic softwares were used. Results are expressed as mean ± SEM. When appropriate, data were tested for significance, using Student's test, where P values lower than 0.05 were considered to indicate significant differences.

Volume Measurement

Cell volume was determined on detached cells using a microscopic video imaging device including a video camera coupled to an inverted contrast microscope, a video monitor, a tape recorder and a computer equipped with Axon Imaging software (Axon Instrument). Since detached cells display a spherical shape, the measured parameter was the cell perimeter from which the cell volume was calculated. Variations of the cell volume were expressed in percent, 100% being the volume determined in control conditions. Every 10 or 30 s, one image of the cell was digitized, then stored on the hard disc of the computer. Subsequently, digitized images were displayed on the computer monitor and analyzed one by one using Analysis software (Soft Imaging System, Germany). For quantification, cell perimeter was traced with the mouse and a computer-generated cursor that determined the value of the measured parameter with an accuracy of about ± 4–5%. Each measurement was repeated three times and the cell volume calculated from these averaged data. In some experiments, cell volume change was monitored together with the membrane potential that was recorded under current-clamp conditions, as described above. Time-dependence relationships between both cell-volume and membrane-potential changes induced by hypotonic solution were achieved by synchronizing the digitization of each of the pictures with the hyperpolarizing membrane signal evoked every 10 s by current injections.

All these experiments were performed on detached cells because preliminary measurements carried out on adhering cells indicated that no change in the cell perimeter could be detected even by exposing these cells to hypotonic solutions where osmolarity was lowered by 50%. Likely, in these cells, hypotonic challenge should affect more the cell thickness [41, 48] than the cell perimeter. Two different methods were used to obtain detached cells. The one consisted of treating the cell culture for 2–3 min with trypsin (Gibco Laboratories, Paisley, UK). Once the cells detached, the Petri dish was transferred without delay onto the stage of the microscope where a recording pipette was positioned on a cell and the culture medium replaced by the control solution. Using such a method, hypotonic solutions induced large membrane depolarizations associated with detectable changes in cell volume. Nevertheless, when the effects of ACTH were tested on these detached cells (22 cells), no depolarizing membrane response was observed, whereas controls performed on the adhering cells indicated that ACTH-induced membrane depolarizations could be routinely recorded. The reasons why ACTH failed to activate membrane responses in freshly detached cells probably originated in the inability of receptors to bind this peptide after trypsin treatment. This problem was overcome when isolated cells obtained just after tissue dissociation were seeded into Petri dishes in which the bottom was coated with a thin layer of Sylgard. In Sylgard dishes, isolated cells did not adhere to elastomeric substratum, thus retaining their spherical shape and their capacity to generate membrane depolarizations in response to ACTH or hypotonic solutions up to 4 days after seeding.

Secretion Measurement

Secretion measurements were performed in 12-well testplates, each containing 1 ml of culture medium. On the third day, the culture medium was removed, the cell culture washed three times with Dulbecco solution before exposure for 2 h to 1 ml of different physiological solutions, namely, standard solution, standard solution + ACTH (10 nM), hypotonic solution (−60 mOsm), hypotonic solution (−100 mOsm), and high-K^+ solution (40 mM). After 2 h at 37°C, the cell medium was removed and cortisol content was determined by radio-immuno-assay, using specific antibody [9, 32]. At the end of each experiment, cells were counted (Coulter, Fullerton, CA). The data are presented as mean (± SEM) from measurements performed in 4 wells for each condition.

Results

Membrane Depolarization Induced by Hypotonic Solution

The resting membrane potential of ZF cells measured in control solution was −61.1 ± 1.7 mV (n = 68). As reported by Dupré-Aucouturier et al. [13], this membrane potential often displayed disordered oscillations that were attributed to background activities of ionic channels related with the high input resistance of these cells (3.3 ± 0.3 GΩ, n = 53) [13]. When these oscillations were larger than ± 5 mV, cells were discarded.

To control a possible effect on the membrane potential of lowering $[Na]_o$, ZF cells were exposed to isotonic solutions in which 50 mM NaCl was replaced by 100 mM mannitol. In 15 out of 17 experimented cells, no drastic change in the membrane potential was observed, whereas in the remaining 2 cells, a membrane depolarization to about −37 mV, correlated with an increase in the membrane conductance, was detected. On the other hand, when mannitol was suppressed from isotonic solutions, hypotonic challenge induced large membrane depolarizations in these 17 cells. Since the present observations reveal that in the great majority of ZF cells (88%), isotonic Na^+-deficient solutions did not induce consequent changes in the membrane potential, subsequent

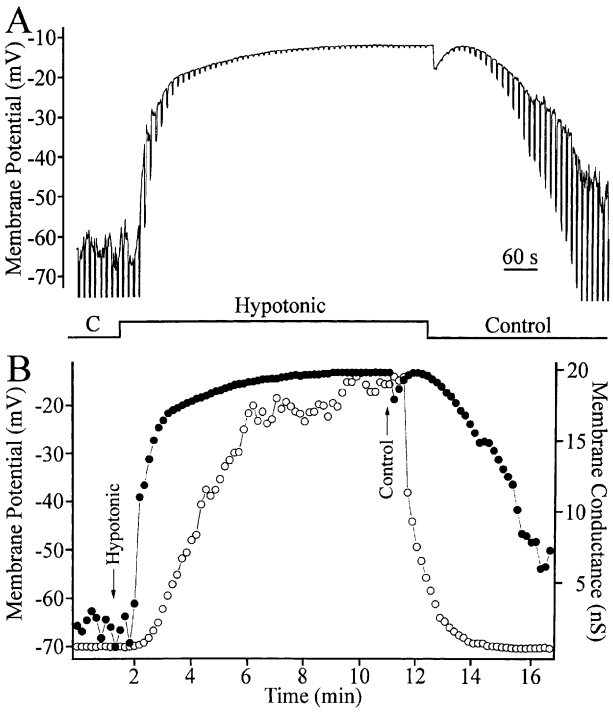

Fig. 1. Effect of hypotonic solution on the cell membrane potential. (***A***) Original recording of a membrane depolarization induced under hypotonic conditions. In this and the other membrane potential recordings, transient jumps of potential superimposed to the voltage trace were evoked by hyperpolarizing current pulses of 10 pA injected for 2 s every 10 s. For more clarity, membrane potential deflections were truncated at the beginning and at the end of this recording. (***B***) Quantification of both, membrane potential (*left ordinate*, ●) and input membrane conductance (*right ordinate*, ○) changes measured every 10 s. This graph drawn from the recording illustrated in ***A*** is representative of other recordings obtained in similar conditions. **C**: control solution.

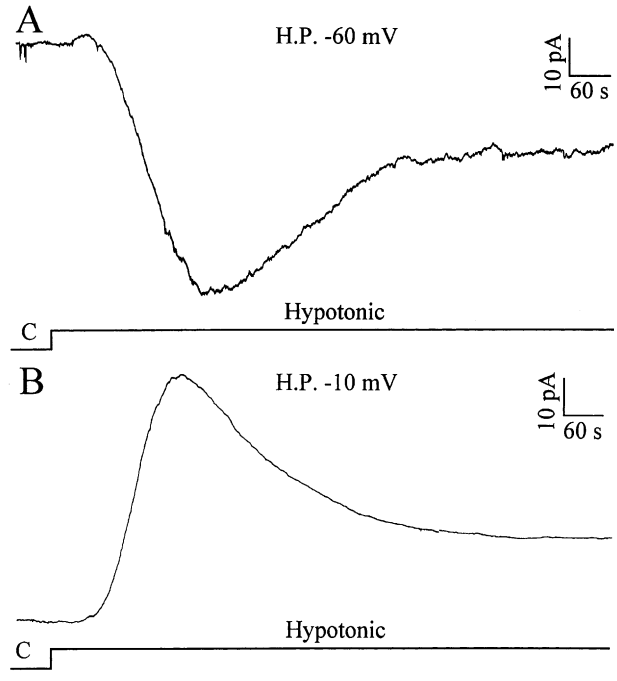

Fig. 2. Effect of hypotonic solution on the whole-cell membrane current activated from two different holding membrane potentials, −60 mV (***A***) and −10 mV (***B***). Holding control current was −3.5 pA in ***A*** and +10 pA in ***B***.

experiments were carried out by direct exposure of isolated cells to hypotonic solution in which the osmotic pressure was reduced by 100 mOsm.

Exposure of ZF cells to this hypotonic solution induced a maximum depolarization to −22 ± 1.3 mV (n = 48). Membrane depolarization began after a delay of 33 ± 5 s (n = 21) by a "fast" rising phase followed by a very slow one that ended with a steady plateau potential (Fig. 1*A*). In some cells, this plateau decayed slightly during the course of the experiments. The duration of this depolarizing phase, measured at 50 and 90% from its maximum value, was 28 ± 2 s and 94 ± 8 s (n = 21), respectively. Membrane depolarizations induced by hypotonic solution were accompanied by significant changes ($P < 0.0001$) in the membrane conductance, which increased up to 8.5 ± 1 nS (n = 48) from a resting value of 0.5 ± 0.03 nS (n = 48). In Fig. 1*B*, the membrane depolarization was superimposed with the membrane conductance change calculated from the record presented in Fig. 1*A*. This graph establishes the existence of a correlation between the two processes. However, in the beginning of the depolarizing phase (Fig. 1*B*), the large jump of the membrane potential was associated with only a weak increase in the membrane conductance. This was previously described by Dupré-Aucouturier et al. [13] and was attributed to the strong input resistance of these cells (3–4 GΩ), in which a weak membrane current produced by the activity of a few ionic channels could generate relatively large changes of the membrane potential. These membrane responses were fully reversible. As illustrated in Fig. 1*A*, recovery of the membrane potential always began by a quasi-instantaneous transient repolarization of a few mV, followed by a more or less pronounced depolarizing shoulder. The magnitude of this event, correlated with the change of the bath solutions, was very variable from cell to cell and its mechanism was not further explored.

In experiments where the membrane potential was recorded using the conventional whole-cell method (broken membrane), membrane responses to hypotonic challenge were similar to those obtained using perforated method. Nevertheless, the maximum values of both the membrane depolarization and conductance were higher, reaching −8.8 ± 2.9 mV (n = 10) and 13.1 ± 3.2 nS (n = 8) respectively (*not illustrated*). When these two parameters were compared with those obtained using the perforated-membrane recording method, t-tests indicated that only membrane depolarizations were significantly different, with $P \ll 0.05$. In broken-membrane

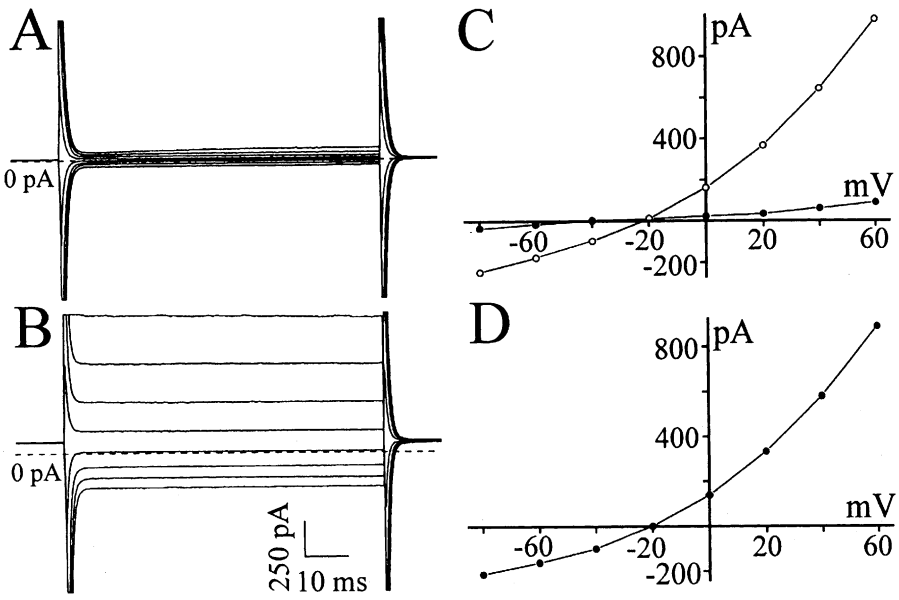

Fig. 3. Current-voltage relationships of the whole-cell membrane current induced by hypotonic solution. (***A***) Family of membrane currents obtained in control solution in response to voltage steps of variable amplitude applied for 75 ms between −80 and +60 mV by 20 mV increments from a holding membrane potential of −10 mV. (***B***) Family of membrane currents obtained at the peak of the transient outward current induced in the presence of hypotonic solution. (***C***) Current-voltage relationships of membrane currents illustrated in ***A*** and ***B*** in control condition (●) and under hypotonic conditions (○). (***D***) Current-voltage relationships of the volume-induced membrane current obtained by subtraction procedures.

conditions, the Nernst equation applied to the ratio of Cl^- concentrations between pipette and hypotonic solutions gave a calculated Cl^- equilibrium potential of −7.3 mV.

When osmolarity of the bath solution was reduced by 60 mOsm instead of 100 mOsm as above described, only 57% of cells exhibited a membrane depolarization to −30.5 ± 1.5 mV (n = 27) correlated with an increase in the membrane conductance, which reached 6.8 ± 1.2 nS (n = 19). As compared to results obtained with hypotonic solution where osmolarity was reduced by 100 mOsm, t-test revealed that membrane depolarizations were significantly different ($P \ll 0.05$), whereas membrane conductances were only marginally different ($P = 0.069$).

Membrane Current Induced under Hypotonic Conditions

Membrane responses to hypotonic challenge were further characterized by voltage-clamping ZF cells at different holding potentials. From a holding membrane potential of −50 or −60 mV, near the resting membrane potential (Fig. 2*A*), hypotonic solution induced a transient inward current, whereas from a holding potential of −10 mV (Fig. 2*B*), the membrane current activated by hypotonic change was a transient outward current. Maximum currents recorded from holding potentials of −60 and −10 mV were 83 ± 10 pA (n = 11) and 66 ± 5 pA (n = 57), respectively. These observations indicate that the reversal potential of the membrane current induced by hypotonic challenge ranges between −10 and −50 mV. The reversal potential of this ionic current was determined from current-voltage relationships obtained by applying short voltage steps (75 ms duration) between −80 and +60 mV in control (Fig. 3*A*) and under hypotonic conditions (Fig. 3*B*). These membrane responses were generated from a holding potential of −10 mV to inactivate the voltage-dependent currents described in these cells, namely a large transient K^+ current [4, 25], a transient and a maintained Ca^{2+} current [16, 23]. Figure 3*A* is representative of membrane currents recorded in control conditions in a ZF cell in response to these voltage steps. Figure 3*B* shows currents traces recorded using a similar stimulating protocol applied at the peak of the transient outward current induced by hypotonic solution. These membrane currents activated instantaneously and did not display time dependence during their time course, even for high depolarizing potentials. Current-voltage relationships established from this recording revealed that, in control condition, the membrane current was quasi-linear and crossed the voltage axis at −40 mV, while at the maximum of the membrane response induced by hypotonic solution, the large ionic current displayed an outward rectification and intercepted the voltage axis towards more positive potential (Fig. 3*C*). Using the subtraction procedure, the membrane current induced by hypotonic solution was zero at the

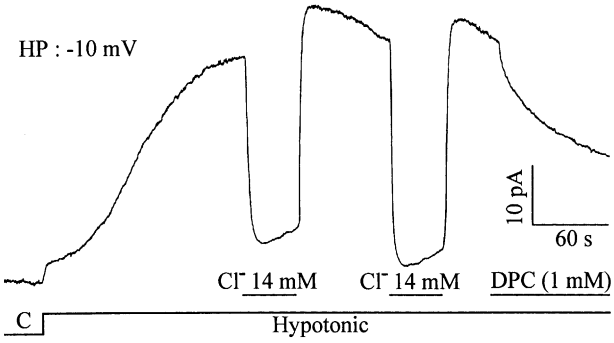

Fig. 4. Effect of lowering $[Cl]_o$ on the membrane current induced by hypotonic solution, from a holding membrane potential of −10 mV. At the end of this recording, the blocking effect of DPC (1 mM) on this outward current is shown. Holding control current: +3 pA.

potential of −20 mV (Fig. 3*D*). Averaged from 49 cells, the reversal potential measured at the peak of this transient volume-sensitive current was −21 ± 0.7 mV.

Effect of Low-Cl^- Solution on the Membrane Current Induced under Hypotonic Conditions

The above results suggest that membrane responses induced by hypotonic solutions result from the activation of a Cl^- current. Indeed, under hypotonic conditions, the maximum depolarization recorded in current clamp and the reversal potential of ionic current obtained in voltage clamp were similar around −20 mV, a value that would correspond to the supposed E_{Cl^-} in these cells. This hypothesis was tested using an experimental approach that consisted in changing $[Cl]_o$ during the membrane response induced by hypotonic solution. As illustrated in Fig. 4, the outward membrane current induced under hypotonic conditions from a holding potential of −10 mV was strongly depressed when the external Cl^- concentration was lowered to 14 mM. Brief applications of this solution produced, in a reversible manner, an instantaneous decrease near 0 pA of the membrane current, in agreement with the expected shift of E_{Cl^-} towards more positive values. This suggested that in these experimental conditions, the reversal potential of this ionic current could be close to −10 mV.

Pharmacology

Membrane responses observed under hypotonic conditions were sensitive to different Cl^--channel inhibitors. Thus, in current-clamp experiments, DPC (1 mM), applied during the depolarizing plateau (Fig. 5*A*), repolarized the cell membrane by 82 ± 5% (n = 13). This effect was accompanied by a decrease in the membrane conductance (Fig. 5*A*) and, in this

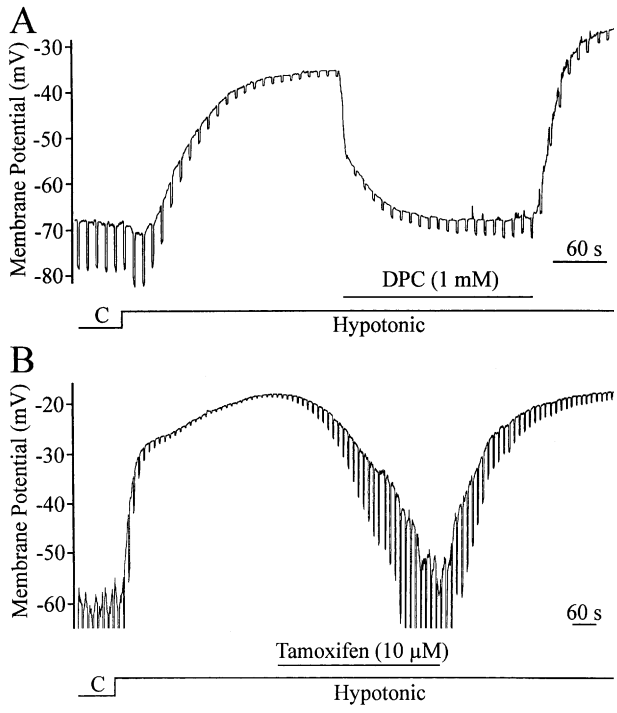

Fig. 5. Effect of two different Cl^- -channel inhibitors, DPC (1 mM) in ***A*** and tamoxifen (10 mM) in ***B***, on membrane depolarizations induced by hypotonic solution.

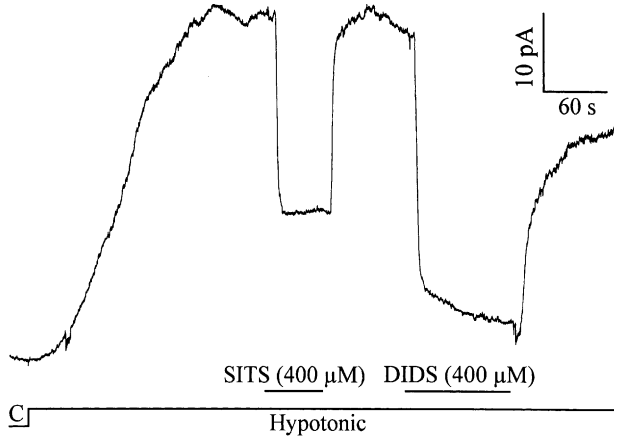

Fig. 6. Effect of two different Cl^- channel inhibitors (SITS and DIDS) on the membrane current induced by hypotonic solution from a holding membrane potential of −10 mV. Used at similar concentrations (400 μM), DIDS was a more potent inhibitor of the membrane current than SITS. Holding control current: +5 pA.

recording, was fully reversible. In the presence of other inhibitors, such as 9-AC (1 mM) or DIDS (250-400 μM), membrane depolarizations were depressed by 55 ± 23% (n = 4) and 73 ± 10% (n = 8) respectively. Another compound, tamoxifen, known to inhibit volume-activated Cl^- channels in many cell types, was also tested. Used at concentrations of 8–10 μM, this anti-estrogen repolarized the cell mem-

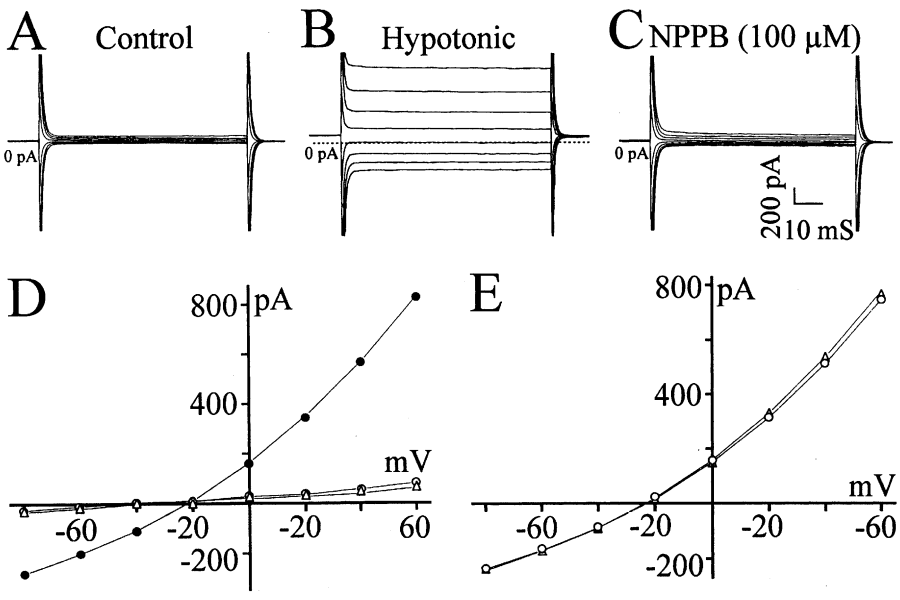

Fig. 7. Effect of NPPB (100 μM) on the membrane current induced by hypotonic solution from a membrane potential of −10 mV. Top recordings correspond to families of membrane currents obtained as indicated in Fig. 3 in control solution (***A***), at the peak of ionic current induced by hypotonic solution (***B***) and at the maximum inhibition produced by NPPB (***C***). (***D***) Current-voltage relationships of the whole-cell membrane currents drawn from recordings at top (Control, ○ Hypotonic, ● and NPPB, Δ). (***E***) Using subtraction procedures, these current-voltage curves reveal that the membrane current induced by hypotonic solution (○) perfectly superimposed the membrane current suppressed by NPPB (Δ).

brane by 66 ± 9% (n = 8). This is illustrated in Fig. 5*B* where the blocking effect of tamoxifen on the membrane potential was correlated with a large decrease in the input membrane conductance that returned to its basal value. In this cell, the effects of tamoxifen were fully reversible.

In voltage-clamp experiments performed from a holding potential of −10 mV, the effects of DPC (1 mM) on the membrane current induced by hypotonic solution are illustrated in Fig. 4. Figure 6 reveals that DIDS (400 μM) inhibited more potently the membrane current than did SITS (400 μM) and that the effects of these two compounds were reversible. From current-voltage curves, SITS and DIDS inhibited the membrane current in a voltage-dependent manner because the blocking effect of these compounds was more pronounced in the depolarizing direction (*not illustrated*). Furthermore, as shown in Fig. 7, NPPB (100 μM) was a potent inhibitor of the membrane current induced under hypotonic conditions. In this figure, current traces were obtained in control solution (Fig. 7*A*), during the maximum current induced under hypotonic conditions (Fig. 7*B*), and after the membrane current was blocked by NPPB (Fig. 7*C*). Current-voltage curves presented in Fig. 7*D* correspond to total membrane current recorded in these three different conditions, whereas current-voltage curves shown in Fig. 7*E* were obtained after the substraction procedure. These curves indicate that the membrane current induced by hypotonic solution and the membrane current suppressed by NPPB were similar. The blocking effect of this drug on the membrane current was voltage-independent.

The effects of different Cl^- channel blockers on the membrane current induced under hypotonic conditions were compared with those described by us on the ACTH-induced Cl^- membrane current [13]. This Cl^- current was strongly inhibited by DPC and 9-AC (1 mM each) and was sensitive to DIDS (400 μM) and NPPB (100 μM). In the present study, the effects of these various Cl^- channel inhibitors were tested, together with those of SITS (400 μM) and tamoxifen (8–10 μM), on the membrane current induced under hypotonic conditions and re-examined for quantification under ACTH stimulation. As shown in Fig. 8, all these inhibitors blocked more or less efficiently the membrane current activated in these two different experimental conditions. For each compound used, t-test indicated that the differences were not significant, except for 9-AC where this difference was marginally significant (P = 0.061).

Cell-Swelling Measurements

Cell volume was simultaneously measured with the membrane depolarizations induced by hypotonic solution. ZF cells, detached or seeded on Sylgard layer, responded to hypotonic challenge by a membrane depolarization very similar to that recorded on adhering cells. From a mean resting membrane potential of −63 ± 4.1 mV (n = 22), hypotonic solution induced a membrane depolarization to a maximum value of

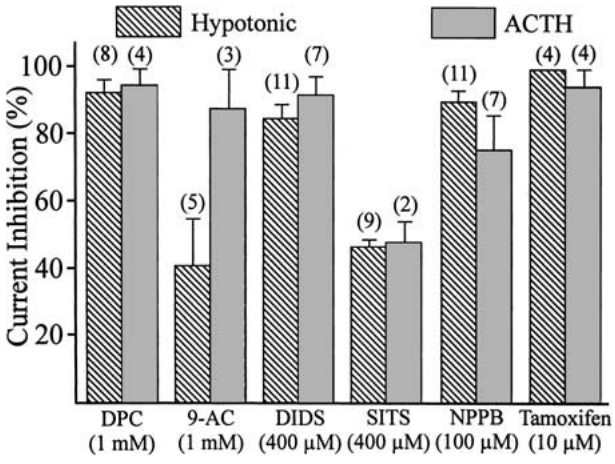

Fig. 8. Comparison of the effects of different Cl^- current inhibitors on both, the volume-sensitive (*hatched columns*) and the ACTH-induced membrane currents (*gray columns*). Results are expressed in percent of inhibition of the membrane current recorded under hypotonic conditions from a holding membrane potential of −10 mV. For each compound used, *t*-test detected no significant differences. Numbers of experiments are indicated above each column.

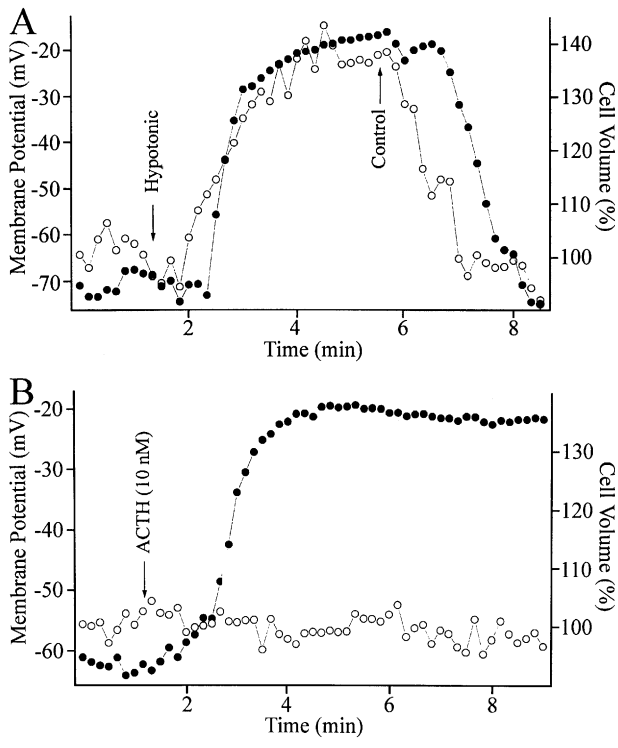

Fig. 9. Correlations between cell-volume and membrane-potential changes in isolated cells exposed to hypotonic solution and ACTH (10 nM). (***A***) Hypotonic solution produced a detectable increase in the cell volume *(right ordinate*, ○), which preceded the membrane depolarization *(left ordinate*, ●). (***B***) No change in the cell volume (*right ordinate*, ○) was detected during ACTH-induced membrane depolarization (*left ordinate*, ●). These figures (***A*** and ***B***) are representative of 4 experiments performed in similar conditions.

−21.5 ± 1.2 mV (n = 22). This membrane response was correlated with an increase in membrane conductance change that reached 9.3 ± 1.5 nS (n = 22) from 0.4 ± 0.04 nS (n = 22) in control conditions.

These membrane depolarizations were accompanied by a cell swelling that was detectable by eye on the video monitor. Taking the control volume as 100%, swelling caused by hypotonic challenge increased the cell volume up to 137 ± 3.8% (n = 13). Figure 9*A* illustrates the time course of both the membrane potential and the cell volume changes monitored every 10 s during the exposure of a detached ZF cell to hypotonic solution. This record, representative of 4 other experiments performed in similar experimental conditions, shows that the membrane depolarization is correlated with an increase in cell volume. However, it appeared that at the beginning of the response, the increase of the cell volume preceded the membrane depolarization. In this experiment, the membrane potential started to depolarize when the cell volume was higher than 110%. Full recovery of the cell volume was complete about 60 s after the cell was again exposed to the control solution (Fig. 9*A*).

We have previously shown that ACTH depolarized ZF cells by activating a Cl^- conductance [13]. For comparison, similar experiments were performed in the presence of this peptide on isolated cells seeded in Sylgard-coated Petri dishes. In these cells, ACTH (10 nM) triggered a membrane depolarization to −20 ± 1.5 mV (n = 4) from a resting potential of −62 ± 3.6 mV (n = 4), associated with membrane conductance changes that increased from 0.6 ± 0.15 nS (n = 4) in control conditions to 2.5 ± 0.3 nS (n = 4). As illustrated in Fig. 9*B*, which is representative of experiments performed in 4 different cells, no change in the cell volume was detected during these membrane responses. On the other hand, when a cell depolarized by 10 nM ACTH was exposed to hypotonic solution containing ACTH, a large increase in the cell volume was observed, associated with an additional membrane depolarization as shown in Fig. 10.

Measurement of Secretion

Previous studies established that ACTH-induced membrane depolarization was a crucial step in the steroidogenesis of isolated ZF cells. Dupré-Aucouturier et al. [13] reported that Cl^--current inhibitors diminished by >95% the cortisol secretion stimulated by ACTH, whereas these same inhibitors did not affect the basal secretion of this steroid. Furthermore, Enyeart et al. [14] showed that ACTH-stimulated cortisol secretion was prevented in the presence of Ca^{2+}-channel blockers, suggesting that Ca^{2+} was involved in the steroidogenesis and that these ions entered the cell by voltage-dependent Ca^{2+} channels likely activated by the membrane depolarization. The present study reveals that the membrane depolariza-

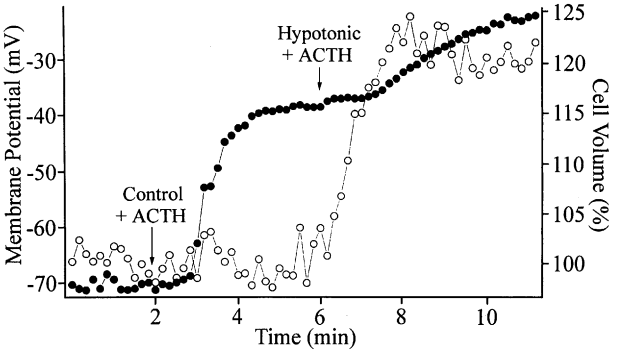

Fig. 10. Correlations between cell-volume and membrane-potential changes in isolated cells exposed first to ACTH (10 nM), then to hypotonic solution. In this recording, only hypotonic solution provoked a detectable increase in the cell volume (*right ordinate*, ○) accompanied by an additional membrane depolarization (*left ordinate*, ●).

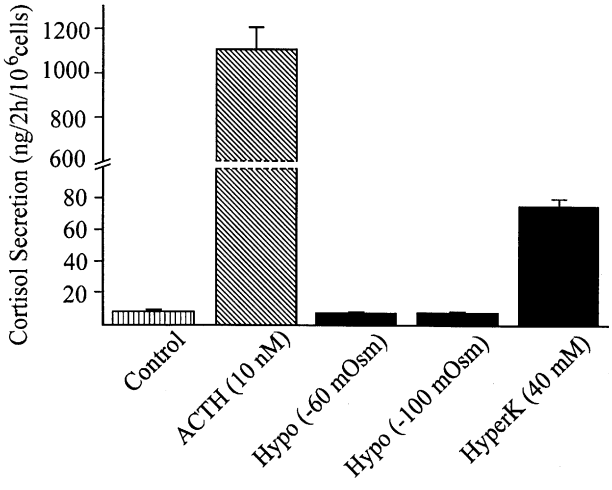

Fig. 11. Cortisol secretion measured in cell populations exposed to different media. Only ACTH strongly stimulated cortisol production. In hypotonic solutions deficient by 60 and 100 mOsm, cortisol production was similar to that measured in control solution. Compared to control or hypotonic conditions, cortisol production was increased by a factor of 9 in high-K^+ solution.

tions induced by hypotonic solution were very similar to those stimulated by ACTH [13]. To compare the secretory activities in these two situations, cortisol production was measured on cell populations incubated in the presence of ACTH and under hypotonic challenge. Figure 11 shows results from three different experiments performed using similar protocols. Basal secretion of cortisol detected in control condition was multiplied by a factor of 140 in the presence of ACTH. On the other hand, when the ZF cells were incubated in hypotonic solutions, cortisol secretion was not stimulated and was near the basal secretion. These results led us to consider the effects of high-K^+ solutions on the cortisol production, which is another way to depolarize ZF cells. Preliminary to this determination of cortisol secretion, isolated ZF cells were first exposed to high-K^+ solution (40 mM) and the membrane potential measured in current-clamp conditions. In 40 mM high-K^+ solution, the membrane potential depolarized quasi-instantaneously to -25 ± 1.5 mV ($n = 6$), a value nearly similar to that measured under hypotonic condition and more positive by 11 mV than that reported by Dupré-Aucouturier et al. [13] during ACTH stimulation. This depolarization was reversible and was accompanied by an increase in the membrane conductance. As shown in Fig. 11, cortisol secretion induced by this high-K^+ solution was only increased by a factor of 9.5 as compared to control, versus a factor of 140 when cells were incubated in the presence of ACTH.

Discussion

Cl^- Dependence of Membrane Responses Induced under Hypotonic Conditions

The present study establishes that isolated ZF cells are sensitive to osmotic changes. They respond to a hypotonic challenge by a membrane depolarization correlated with a large increase in the membrane conductance caused by the activation of a volume-sensitive Cl^- current. First, our observations confirm that the exposure of ZF cells to hypotonic solutions induces a detectable cell swelling that is associated, after a delay of about 30–40 s, with a membrane depolarization. Similar observations were reported in rat brain endothelial cells, where the increase in cell diameter occurred always before the activation of volume-sensitive Cl^- current [46]. Second, this depolarization involves Cl^- for the following reasons. In current-clamp experiments performed using the perforated-membrane recording method, membrane depolarizations induced under hypotonic conditions reached -22 ± 1.3 mV ($n = 48$) and -30.5 ± 1.5 mV ($n = 27$) in bath solutions where osmotic deficit was 100 and 60 mOsm, respectively. The shift of membrane depolarizations measured in these two hypotonic solutions (8.5 mV) was higher by only 4 mV than the shift of E_{Cl^-} calculated from the ratio of external Cl^- concentrations (4.6 mV). When the experiments were carried out using the conventional recording method (broken membrane), the membrane depolarization induced in hypotonic solution deficient by 100 mOsm (-8.8 ± 2.9 mV, $n = 10$) was very close to calculated E_{Cl^-}, which was -7.3 mV. The results obtained in current clamp strongly suggest that under hypotonic conditions, the cell membrane depolarizes towards a potential that would correspond to E_{Cl^-}. However, this hypothesis is not supported by other results obtained in voltage-clamp experiments. Although the membrane current induced by hypotonic stimulation reversed at

-21 ± 0.7 mV ($n = 49$), a value similar to that reached by the membrane depolarizations (-22 ± 1.3 mV, $n = 48$), the effects of low-Cl^- hypotonic solutions on this membrane current were more ambiguous. In these low-Cl^- hypotonic solutions, E_{Cl^-} was likely positive because from the ratio of external Cl^- concentration changes, it was shifted by 49 mV towards positive potentials. In these conditions, the membrane current elicited from a holding potential of -10 mV should be an inward current. Our results indicated that the membrane current observed in low-Cl^- solution, although strongly depressed as compared to control hypotonic solution, was never clearly inward. Its reversal potential was estimated at about -10 mV, a potential more negative than the expected E_{Cl^-}. These discrepant observations between current- and voltage-clamp experiments lead us to suppose that under voltage-clamp conditions, where a membrane current continuously flows through the cell membrane, a redistribution of Cl^- would shift E_{Cl^-} towards more negative value. However, such a hypothesis would need further experiments to be verified.

These membrane responses were sensitive to various compounds known to inhibit Cl^- channels. Amongst these compounds, DPC, 9-AC, DIDS, SITS and NPPB, classically used to inhibit Cl^- membrane conductances, blocked more or less efficiently the membrane responses observed under current- or voltage-clamp conditions. Tamoxifen, described to block more selectively volume-activated Cl^- current in some cell types, was also a potent inhibitor of the membrane responses induced in ZF cells under hypotonic conditions. Consequently, all these observations demonstrate that a volume-sensitive Cl^- current is involved in the membrane depolarizations triggered by hypotonic challenge.

Comparison with ACTH-induced Membrane Responses

As before reported, ACTH induced in bovine ZF cells a membrane depolarization dominated by the increase of a Cl^- membrane conductance [13]. This previous observation is similar to that described in the present study, where hypotonic solution induced a Cl^--dependent membrane depolarization. Under hypotonic conditions, maximum depolarization (-22 ± 1.3 mV, $n = 49$) was more positive by 14 mV than that observed with ACTH (-36 ± 1 mV, $n = 56$). This can be attributed to the shift of E_{Cl^-}, which was more positive by 10 mV in hypotonic solution. The maximum membrane conductance measured in hypotonic solutions (8.5 ± 1 nS, $n = 48$) was significantly higher ($P \ll 0.05$) than that detected in the presence of ACTH (1.8 ± 0.2 nS, $n = 48$) [13]. Since the input membrane conductance of a cell is directly proportional to the number of open channels and to their elementary conductance, such a difference raises the question of whether the membrane responses observed under hypotonic conditions and ACTH stimulation result from the activation of one or two distinct types of Cl^- channels.

The membrane current activated under hypotonic conditions was very comparable to the membrane current activated by ACTH stimulation. These Cl^- currents displayed no time dependence and showed a weak outward rectification. It was not possible to discriminate them by pharmacological methods. Because basic biophysical and pharmacological properties of these two membrane currents were very similar, we hypothesized that volume-sensitive Cl^- currents described in the present study would be also activated by ACTH, suggesting that the same class of Cl^- channels would be involved in these membrane responses. However, membrane depolarizations were correlated to cell volume change under hypotonic stimulation, but not under ACTH stimulation. This observation does not prove that Cl^- channels involved in the membrane responses induced by ACTH stimulation are distinct from volume-sensitive Cl^- channels. Indeed, volume-sensitive Cl^- channels can be activated by different factors, such as cell swelling, reduction of intracellular ionic strength [29] and by application of GTPγS [11]. Recently, Estevez, Bond and Strange [15] have shown that the activation of a volume-sensitive Cl^- current in neuroblastoma cells by this GTP analog occurred in the absence of cell swelling. This signifies that this type of Cl^- channel can be activated by intracellular metabolic pathways that by-pass the increase in cell volume.

In adrenal ZF cells, membrane depolarizations induced by ACTH were mimicked by a membrane-permeable form of cAMP [13]. In this cell type, ACTH is known to increase the PKA activity [35], suggesting that these Cl^- channels would be dependent on phosphorylation steps by PKA stimulation. Several studies reviewed by Nilius and Droogmans [27] have shown that volume-sensitive Cl^- channels could be activated or modulated by different classes of protein-kinases, such as protein tyrosine kinases [45] or protein kinase A (PKA) [12]. For instance, in carotid body type-I cell, cAMP activates a volume-sensitive Cl^- current that is abolished in the absence of intracellular ATP [6]. Amongst the diversity of Cl^- channels described in the literature, the cystic fibrosis transmembrane regulator (CFTR) and multidrug-resistance P-glycoprotein (Pgp), which belong to the superfamily of ATP-binding cassette (ABC) transporter, would be possible candidates. CFTR, which functions as a protein kinase A-activated Cl^- channel [1, 8] is known to regulate different ion channels, amongst which is a volume-sensitive Cl^- channel [44]. Nevertheless, as discussed by Dupré-Aucouturier et al. [13], CFTR is probably not expressed in the membrane of

ZF cells because mRNAs coding for this channel protein were never detected in adrenal tissue. In addition, CFTR would downregulate the volume-sensitive Cl^- channel [44] by protein-protein interactions, this regulation being PKA-independent [2]. On the other hand, P-glycoprotein has been detected in the adrenal cortex [39] where it would participate in the secretion of steroid hormones [3]. Although disputed [27], this ABC transporter could also regulate the activity of a volume-sensitive Cl^- channel [42, 49]. But still, Vanoye et al. [43] reported that in the cells expressing P-gp, the steady-state Cl^- current activated by cell swelling was reduced by the stimulation of PKA. Taken together, these observations are not in favor of the activation or the modulation by these ABC transporters of the volume-sensitive Cl^- current described in ZF cells.

These different data reveal that the control of volume-sensitive Cl^- channels can be achieved by a great diversity of mechanisms that are still incompletely understood. Although the results exposed in the present study argue in favor of one class of Cl^- channel, further experiments are necessary to elucidate this question.

Secretion

In the present study we show that contrary to ACTH, cortisol production by bovine ZF cells was not stimulated under hypotonic conditions. In adrenal and other secretory tissues, the synthesis and the secretion of steroid hormones involve complex mechanisms that are not completely understood. Thus, in adrenal ZF cells, the most potent stimulator of steroidogenesis is ACTH. This hormone binds to membrane receptors coupled to G-proteins whose αs subunit activates the cAMP-dependent protein kinase (PKA) signaling pathway [35]. Although the targets of this protein kinase remain to be characterized, phosphorylation of cellular proteins would be requisite for the synthesis of steroid hormones [38]. Beside these effects on the cellular metabolism, ACTH induces membrane depolarization by inhibiting a background K^+ current [13, 24] and by activating a cAMP-dependent Cl^- current [13]. This depolarization is a necessary condition in the activation of the steroidogenesis, since in the presence of inhibitors of cAMP-dependent Cl^- channels, cortisol secretion is inhibited [13]. It is accepted that in addition to phosphorylation steps, the steroidogenesis requires an increase of cytosolic Ca^{2+} concentration [5], which in bovine ZF cells would result from activation of voltage-dependent Ca^{2+} channels in response to ACTH-induced membrane depolarization [14]. The absence of increased cortisol production under hypotonic challenge indicates that in our hands, the membrane depolarization and the expected change in cytosolic Ca^{2+} concentration were not a sufficient condition to stimulate the steroidogenesis in bovine ZF cells. However, when these cells were depolarized in high-K^+ solution, cortisol production, although weak compared to ACTH, was significantly higher than that measured under basal or hypotonic conditions. Contrary to glomerulosa cells, the effects of K^+-concentration changes on the membrane potential and steroid secretion have not been specifically studied in ZF cells. In adrenal glomerulosa cells, aldosterone production is controlled by angiotensin II, ACTH and K^+. The mechanism by which raised extracellular K^+ concentration operates would involve an increase in $[Ca^{2+}]_i$ that would exert a direct action on mitochondria [7, 34], where the first step of steroid biosynthesis occurs. In bovine ZF cells, we hypothesized that the differences in cortisol production measured in hyper-K^+ and hypotonic solutions would be caused by the nature of the stimulus inducing the membrane depolarizations. K^+ concentration changes evoke a membrane depolarization without cell swelling, whereas hypotonic solution provokes membrane depolarizations associated with cell swelling. A downregulation of $[Ca^{2+}]_i$ in response to hypotonic stimulation may be discarded. In several cell types, these solutions are known to increase $[Ca^{2+}]_i$ by activating mechanosensitive ion channels [26] or voltage-gated Ca^{2+} channels [22] or by modulating Ca^{2+} extrusion as reported by Startchik et al. [37] in adrenal bovine glomerulosa cells. Cell swelling can affect other cell functions. For instances, steroidogenesis in response to a stimulation begins in the mitochondria, where the cholesterol, the physiological precursor of steroid hormones, is delivered to the inner mitochondrial membrane, then is metabolized in pregnenolone. Mitochondria are known to be sensitive to changes in ionic composition of their environment, suggesting that hypotonic solutions, by producing cell swelling, could alter some mitochondrial functions that would affect the first steps of the steroidogeneis. However, this hypothesis contrasts with the effects of hypotonicity on secretory activities of adrenal glomerulosa cells. Thus, Wang et al. [47] demonstrated that aldosterone production and $[Ca^{2+}]_i$ were increased in bovine glomerulosa cells exposed to hypotonic environment. In rat adrenal glomerulosa cells, Makara et al. [21] detected no change in aldosterone production and $[Ca^{2+}]_i$ in response to a hypotonic challenge in control $[K^+]_o$ solution (3.6 mM). On the other hand, when $[K^+]_o$ was increased by 1.4 mM, hypotonic solutions triggered aldosterone production and positively modulated $[Ca^{2+}]_i$. The differences in the secretory activities of adrenal fasciculata and glomerulosa cells to hypotonic stimulations are at the present time unresolved and the present observations would provide additional clues to subsequent investigations concerning the mechanisms involved in the steroidogenesis of these cell types.

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