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POTASSIUM AND CHLORIDE CONDUCTANCES IN RAT LEYDIG CELLS: EFFECTS OF GONADOTROPHINS AND CYCLIC ADENOSINE MONOPHOSPHATE

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SUMMARY

1. The effects of gonadotrophins (luteinizing hormone and human chorionic gonadotrophin) and cyclic AMP on ionic conductances were investigated using the tight-seal whole-cell recording technique in Leydig cells freshly isolated from nature rat testis by enzymatic treatment.

2. In resting cells, the predominant ionic conductance is a voltage-dependent K^+ conductance resembling the delayed rectifier K^+ conductance of T-lymphocytes. This conductance is characterized by: (1) a time-dependent inactivation for potentials more positive than +20 mV, (2) a reversal potential near −65 mV, (3) a sensitivity to intracellular Cs^+, and (4) a sensitivity to extracellular TEA and 4-aminopyridine.

3. A Cl^- conductance is also present resembling the Cl^- background conductance in squid axons and heart cells. In resting cells, this conductance contributes only a small component of the total outward current obtained with depolarizing pulses.

4. Gonadotrophins (human chorionic gonadotrophin, porcine luteinizing hormone and ovine luteinizing hormone) have little effect on the K^+ conductance. They transiently increase a Cl^- conductance after a delay of up to 30 s. This response does not occur if the hormones are applied late in the whole-cell recording. Gonadoliberine (GnRH) does not affect the Cl^- or K^+ conductance.

5. Internal cyclic AMP (100 μM) mimics all these effects while internal application of a GTP–ATP mixture induces a similar response, which is, however, sustained rather than transient.

6. The Cl^- conductance was studied quantitatively with a GTP–ATP internal solution. This conductance is activated by depolarizing voltage steps to test potentials of −40 mV or more. Under these conditions, the instantaneous current observed as soon as the depolarizing pulse is applied displays outward rectification and reverses near E_{Cl}. During the pulses, a strong inactivation is observed for potentials greater than +40 mV. This conductance is independent of external and internal calcium.

7. It is concluded that the gonadotrophins act through a cyclic AMP-dependent

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process to activate a Cl^- conductance. This conductance is different to the hyperpolarization-activated Cl^- conductance and the calcium-activated Cl^- conductance also present in the membrane of resting cells.

INTRODUCTION

The Leydig cells are the testosterone-secreting cells of the mammalian testis. A large number of biochemical studies have contributed to elucidate the intracellular mechanisms involved in the stimulation–secretion coupling by gonadotrophins (for review see Dufau, 1988). Luteinizing hormone (LH) and human chorionic hormone (hCG) directly act on Leydig cells. There is a general agreement that these hormones bind to the same membrane receptors and increase the adenylate cyclase activity which, through the subsequent stimulation of mainly cyclic AMP-dependent events, stimulates steroidogenesis. In addition, it is now established that calcium is required for testosterone secretion (Janszen, Cooke, van Driel & van der Molen, 1976) and that LH–hCG stimulation induces a rise of free cytosolic calcium (Sullivan & Cooke, 1986).

Many electrophysiological studies have yielded results on ionic transport across the membrane of secreting cells. They have demonstrated that changes in membrane properties are involved in the stimulation by numerous hormones (see reviews: Petersen, 1980; Poisner & Trifaro, 1985). More recent investigations with voltage-clamp techniques (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) support the conclusion that the modulation of ionic conductances by hormones and neurotransmittors is based on a control of channels either directly or via second messengers, such as cyclic AMP, calcium or inositol trisphosphate.

Few studies define the membrane conductances of steroid-secreting cells. Only three of them communicate electrophysiological properties of the testicular Leydig cells. The earliest, performed on the rat testis with intracellular microelectrodes, have revealed a small negative membrane potential (near -25 mV) and a slow depolarizing effect of hCG (Joffre, Mollard, Régondaud, Alix, Poindessault, Malassiné & Gargouïl, 1984*a*; Joffre, Mollard, Régondaud & Gargouïl, 1984*b*). Using the whole-cell variation of the patch-clamp method, Kawa (1987) has determined the electrical properties of mouse Leydig cells and concludes that these cells have a voltage-dependent calcium channel and a calcium-dependent potassium channel. Recently we have demonstrated the presence of a voltage-dependent potassium conductance, a calcium-activated chloride conductance (Duchatelle & Joffre, 1987) and a hyperpolarization-activated chloride conductance (P. Duchatelle & M. Joffre, unpublished data) in the membrane of Leydig cells freshly isolated from the rat testis.

The purpose of the present study was to investigate the ionic conductances of the membrane of single rat Leydig cells by tight-seal whole-cell recording. We report experiments designed to re-examine the membrane properties of unstimulated and of LH–hCG-stimulated cells. We partly characterize two types of conductances in the membrane of resting cells respectively selective for potassium and chloride. We then examine their modulation by voltage, gonadotrophins, cyclic AMP and calcium. We find that gonadotrophins activate a chloride conductance, an effect which is

mimicked by cyclic AMP and a GTP–ATP mixture. This chloride conductance appears to be different to the calcium-activated conductance and to the hyperpolarization-activated chloride conductance which had also been detected in the membrane of resting cells (Duchatelle & Joffre, 1987). Preliminary accounts of this work have been published in abstract form (Duchatelle & Joffre, 1989).

METHODS

Leydig cell preparation

The Leydig cells were prepared as previously described (Joffre *et al.* 1984*a*) with slight modifications. They were isolated from the mature testes of 60- to 90-day-old rats (Wistar). These animals were raised under a controlled light–dark cycle (12 h : 12 h) and constant-temperature conditions (20 °C). They were killed by decapitation. Two testes were aseptically removed; they were decapsulated and placed in a 50 ml sterile Erlenmeyer flask containing 20 ml of sterile solution A (without bovine serum albumin and antibiotics) and collagenase (0·5–1 mg/ml) (Serva, Heidelberg, FRG, cl.histolyticum EC 3.4.24.3, 0·6–0·8 U/mg). The incubations were carried out in a shaking incubator (100–120 cycles/min, 37 °C, 30–60 min) until the seminiferous tubules were completely dispersed. Twenty millilitres of solution A (20 °C) were added to block the enzymatic treatment and the tubule fragments then removed by filtration through a nylon gauge (60 μm). The filtrates were centrifuged at 100 *g* for 8 min (20 °C) and the supernatants discarded. The sedimented interstitial cells were finally resuspended in 7 ml of solution A.

The dispersed cells were fractionated through a discontinuous Percoll (Pharmacia, Sweden) gradient, prepared in solution A (*d* (density) = 1·025, 1·050; 1·085) (Guillou, Martinat & Combarnous, 1985). The cell suspension containing up to 10^8 cells in 3·5 ml was layered on the top of the gradient and centrifuged for 20 min at 1500 *g* (4 °C). Under such conditions cell populations containing more than 60% Leydig cells could be isolated from 1·050–1·085 interfaces. The cells were pipetted, diluted with three volumes of solution A and centrifuged at 400 *g* (12 min, 20 °C), then washed twice using low-speed centrifugation (70 *g*, 4 min, 20 °C). They were suspended in RPMI-enriched medium (RPMI 1640, Flow Laboratories, UK) then counted in a haematocytometer.

The patch-clamp experiments were performed on cells stored for 1–4 h at room temperature then cultured on 35 mm plastic Petri dishes (Nunclon, Nunc, Denmark) in RPMI-enriched medium to allow attachment (90 min, 34 °C, humidified 95% air–5% CO_2).

Media

Solution A was a modified Earle's solution containing (mM): Na^+, 145; K^+, 5; Ca^{2+}, 2; Mg^{2+}, 1; Cl^-, 151; glucose, 11; HEPES-Na, 5 (Sigma, St Louis, MO, USA); bovine serum albumin, 0·1% (BSA; Sigma) and supplemented with streptomycin sulphate (100 μg/ml; Serva) and penicillin G (100 i.u./ml; Serva). The pH was adjusted to 7·4, then the medium was sterilized through a 0·22 μm pore filter system (Millipore).

RPMI-enriched medium (as Sharpe & Cooper, 1987) included HEPES (20 mM) and was supplemented with glutamine (2 mM; Flow Laboratories), sodium bicarbonate (20 mM; Serva), BSA (0·1%; Sigma), insulin (10 μg/ml; Sigma), transferrin (5 μg/ml; Sigma), streptomycin sulphate (100 μg/ml) and penicillin G (100 i.u./ml; Serva).

Characterization and control of the Leydig cell integrity

The integrity of the interstitial cells was controlled by morphological and histochemical tests after preparing the cells and their attachment onto the Petri dish. They had a viability greater than 95%, electromicroscopic characteristics and 3β-hydroxysteroid dehydrogenase activity of the Leydig cells, determined by the method of Sharpe & Cooper (1982). They produced testosterone in basal and in hCG-stimulated conditions (Joffre *et al.* 1984*a*). Their average diameter was $16{\cdot}6 \pm 0{\cdot}5$ μm ($n = 25$).

After rinsing four times with the external solution (solution A, Table 1) to remove the RPMI medium and most of the remaining germinal cells, the Petri dishes were fixed on the stage of an inverted microscope (Diavert, Leitz, Wetzlar, FRG) supporting the perfusion system and the

reference electrode. The cell observations were made at ×512 magnification. The Leydig cells were mainly spherical and largely single or in small clusters. They were slightly flattened. They had a characteristic bright ring under a phase-contrast microscope (Joffre *et al.* 1984*a*) and could thus easily be distinguished from germinal cells and fibroblasts.

Electrophysiology

The ionic current recordings were determined using the whole-cell configuration of the patch-clamp method (Marty & Neher, 1983) with a patch amplifier (EPC 7, List Electronic, Darmstadt,

TABLE 1. Composition of solutions (mM)

External solutions

	Na^+	K^+	Cs^+	Ca^{2+}	Mg^{2+}	Cl^-	Glutamate	EGTA	TEA
A	145	5	—	2	1	151	—	—	—
B	135	5	—	2	1	151	—	—	10
C	145	5	—	2	1	6	145	—	—
D	145	—	5	2	1	151	—	—	—
E	145	5	—	—	3·3	151·6	—	1	—

Internal solutions

	K^+	Na^+	Cs^+	Ca^{2+}	Mg^{2+}	Cl^-	Glutamate	EGTA	HEDTA	pCa
F	136	10	—	—	1	30·5	111·5	0·5	—	≈ 8
G	135	10	—	—	1	30·5	101·5	5	—	9
H	—	10	130*	—	1	30·5	111·5	0·5	—	≈ 8
I	138	10	—	1	—	30·5	96·5	—	9	7
J	132	10	—	—	2·96	30·4	111·5	0·5	—	≈ 8

All external solutions were buffered with 5 mM-HEPES–NaOH and were adjusted to pH = 7·4.
All internal solutions were buffered with 5 mM-HEPES–KOH and were adjusted to pH 7·7.
*Caesium glutamate was made from glutamic acid and caesium hydroxide at neutral pH. The Ca–EGTA and Ca–HEDTA equilibria were calculated using the dissociation constants given by Martell & Smith (1974) taking into consideration the Mg^{2+} and H^+ ions concentrations. K^+ concentration was always adjusted to 146 mM. Mg^{2+} was omitted in HEDTA-containing solution.

FRG). The patch electrodes were made from borosilicate tubing glass (Vitrex, Modulohm I/S, Denmark or GC 150TF-10, Clark Electromedical Instrument, UK) by a double pulling procedure using a vertical puller (PP 83, Narishige, Japan). They were coated with Sylgard 184 (Dow Corning, Seneffe, Belgium) and then fire-polished. Their tip resistances ranged between 1 and 3 MΩ when they were filled with 150 mM-KCl and were somewhat larger when using potassium glutamate. They were then connected to the holder through a Ag–AgCl pellet bathing in the internal solution. The reference electrode also consisted of a Ag–AgCl pellet bathing in the external solution. After obtaining a seal (seal resistances were always greater than 3 GΩ) the whole-cell configuration was achieved by applying an additional pulse of suction. Typical series resistance values (R_s) after rupturing the membrane ranged between 5 and 10 MΩ. In most recordings the R_s value increased during an experiment. A suction was then applied again to the pipette interior, in order to re-establish a good current pathway. If the error introduced by this resistance could not be effectively reduced by an additional suction, the cell was discarded. In the presence of hormones, the higher voltages steps elicited outward currents greater than 1 nA such that the currents reported are underestimated for these high voltages.

The control of voltage protocols and data collection was performed with an IBM personal computer (PC XT 286) equipped with a TECMAR (TM40, USA) analog-to-digital and digital-to-analog convertor, and with the aid of specific software (P Clamp 5, Axon Instruments, USA).

Voltage procedure. In most experiments the membrane potential was held at −40 mV. If the pipette and the bath solutions were different, appropriate corrections of the liquid-junction potential (Fenwick, Marty & Neher, 1982) were applied to the command potential so that the

effective membrane holding potential was −40 mV. Then 375 ms voltage pulses were applied at a frequency of one every 15 s. In the presence of the hormone, the pipette potential was stepped from −40 to +66 mV, the Na^+ equilibrium potential. Variations of this procedure are indicated in the figure legends. In some experiments, a ramp was applied. The voltage was changed from −100 to +100 mV, with a slope of 100 mV/s.

Data analysis. The cell current responses to voltage pulses were low-passed filtered at 3·3 kHz using a 3-pole Bessel filter. The currents were digitized on-line at 4 kHz. A total of 2000 points were recorded and stored on disc. Then the analysis of the data traces was performed with the aid of P Clamp software. Results are illustrated by representative recordings by the use of specific software (MatrixPlotter 2·3, Insight Development Corporation, USA). Current–voltage plots of currents were constructed either from P Clamp or from computer programs (GRXY and DESSIN; J. P. Poindessault, unpublished).

Solutions. In our previous study, the ionic currents were measured in Leydig cells dialysed with a chloride-rich solution ($E_{Cl} = 0$) (Duchatelle & Joffre, 1987). Under these conditions hyperpolarization-activated currents often developed within several minutes of recording (P. Duchatelle & M. Joffre, unpublished data). To suppress these spontaneous currents we decided to decrease the intrapipette chloride concentration and to hold the potential at E_{Cl}. Since the 'zero current' potential was near −35 mV, the holding potential was held at −40 mV. In such an experimental procedure, no spontaneous inward holding potential current was measured (see Fig. 5) and hyperpolarizing voltage steps did not induce time-dependent inward currents over 15 min periods (see Fig. 1).

The ionic compositions of the external and internal solutions used in this study are listed in Table 1. Their osmolarity ranged from 280 to 290 mosM. The Leydig cells which were plated onto plastic Petri dishes were always initially bathed in solution A. Then an isolated cell was chosen and superfused with solution A before rupturing the membrane, in order to prevent the deleterious effect of the solution flux. Then the superfusion of this cell was performed with the test solution which was ejected by gravity (20 cmH_2O pressure) from a plastic catheter with a tip opening of 250 μm. This perfusion system was mounted on a micromanipulator (Leitz) and placed 200 μm away from the cell. All experiments were carried out at room temperature (about 20 °C).

Statistics. Some data are expressed as means ± S.D. for n values.

Drugs and hormones

HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethane-sulphonic acid), EGTA (ethyleneglycol-bis-(β-aminoethylether)*N,N,N′,N′*-tetraacetic), HEDTA (*N*-(2-hydroxyethyl)ethylenediamino*N,N′,N′*-triacetic acid, ATP (adenosine 5′-triphosphate sodium salt), cyclic AMP (adenosine 3′5′-monophosphate sodium salt), glutamic acid, TEA (tetraethylammonium chloride) and 4-AP (4-aminopyridine) were purchased from Sigma: GTP (guanosine 5′-triphosphate sodium salt) was from Boehringer (Mannheim, FRG); sodium and potassium glutamate were from Fluka (Suisse); caesium hydroxide was from Aldrich (France) and caesium chloride from Serva (Heidelberg, FRG).

Human chorionic gonadotrophin (hCG) mainly originated from Organon (France) (Gonadotrophine Chorionique "Endo" (Pregnyl). Purified gonadotrophins were also used. They were generously given by Dr Combarnous from the Laboratoire de Physiologie de la Reproduction of l'Institut National de la Recherche Argonomique (Nouzilly, France): hCG (hCG-ConA IG 100-1); ovine LH (oLH-CY 1055) and porcine LH (pLH-CY 1349). hCG concentrations (μg/ml) were calculated from Mendelson, Dufau & Catt (1975) with hCG-Organon = 15000 i.u./mg. All these hormones, as well as gonadotrophin-releasing hormone (Sigma), were dissolved in the bathing solution and superfused around the cell with a plastic catheter.

RESULTS

Membrane currents of resting Leydig cells

The zero-current potential of the Leydig cells bathing in standard solutions (A/F; $E_{Cl} = -40$ mV) was immediately measured after rupturing the membrane within the pipette. It averaged −35 ± 4 mV ($n = 9$). It was more negative than those previously

measured with $E_{Cl} = 0$ (Duchatelle & Joffre, 1987). In the following experiments the holding potential was held at -40 mV.

Membrane currents recorded in standard conditions (solutions A/F, Table 1) in the presence of a low-calcium internal solution (10^{-9}–10^{-8} M) are illustrated in Fig. 1.

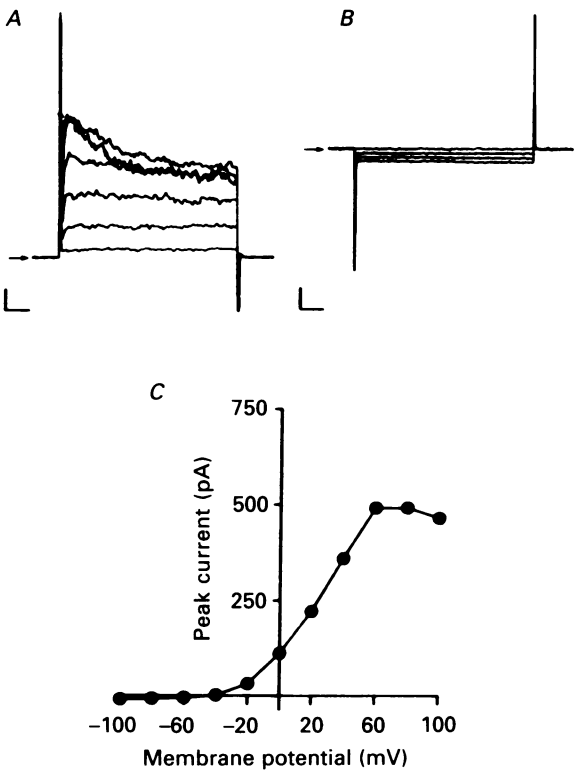

Fig. 1. Currents recorded from a freshly isolated rat Leydig cell in standard solutions (A/F, Table 1). *A*, current traces in response to depolarizing voltage steps of 20–120 mV amplitude in 20 mV increments, applied from a holding potential of -40 mV, every 15 s. *B*, responses to hyperpolarizing voltage steps of 0 to -60 mV amplitude in 20 mV increments, applied from a holding potential of -40 mV, every 15 s. *A* and *B* (and recordings in other figures): sampling interval, 250 μs; filter, 3·3 kHz. Calibration bars: 50 pA, 50 ms. Arrows: zero-current level. *C*, current–voltage relation for peak outward current. Values are not corrected for leakage current.

Depolarizing voltage steps activated voltage-dependent outward currents. Their amplitude progressively increased in response to larger depolarizing test pulses (Fig. 1*A*). For membrane potentials more positive than $+20$ mV, a time-dependent inactivation was seen. The currents were faster activated at stronger depolarizations and were of a more pronounced transient nature. Outward tail currents were measured upon subsequent repolarization to -40 mV. The magnitude of these tail currents was always small. They reached saturation as the pulse was increased near $+60$ mV.

Negative voltage pulses did not elicit any voltage-dependent current and only revealed a linear leak current (-100 to -40 mV), corresponding to an input

resistance of about 3 GΩ (Fig. 1*B*). A small outward current was progressively recorded at the holding potential (HP current). In the resting cells, this current was a few picoamperes.

When plotting the peak outward currents against membrane potential (Fig. 1*C*) the resulting current–voltage relationships revealed an outward rectification for

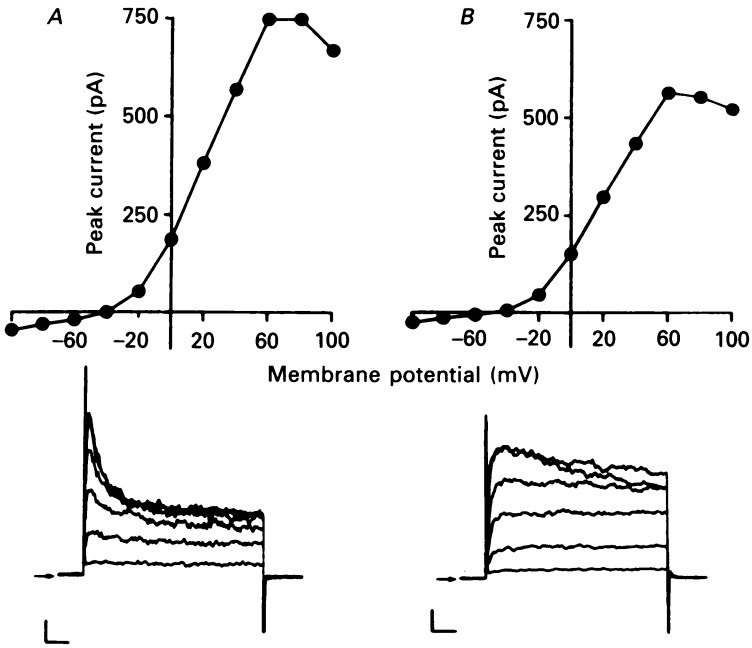

Fig. 2. Variations in the pattern of outward current in standard solutions (A/F). Bottom (*A* and *B*), current traces in response to depolarizing voltage steps of 20–100 mV amplitude in 20 mV increments, applied from a holding potential of −40 mV, every 15 s. The responses to hyperpolarizing voltage steps are as in Fig. 1. Calibration bars: *A*, 80 pA and 50 ms; *B*, 134 pA and 50 ms. Arrows: zero-current level. Top (*A* and *B*), current–voltage relation for peak outward current. Values are not corrected for leakage current. In spite of the differences between the patterns of outward current, the two curves are similar.

potentials ranging between −20 and +60 mV. The peak amplitude reached a maximum value near +60 mV, then remained constant. Such a plateau was never recorded when sodium was omitted from the internal solution (not illustrated).

Similar current–voltage curves were obtained from a wide range of identified Leydig cells (about 80%). It was occasionally noted that the patterns of outward currents elicited by depolarizing voltage pulses vary from one cell to another. Figure 2 illustrates two extreme behaviours with one example (about 10% of the cells; Fig. 2*A*) showing outward currents with fast activation and inactivation kinetics, without tail currents, and another example (about 10% of the cells; Figure 2*B*) where the outward currents rose more slowly during the pulses and inactivated less. The current–voltage relationships and the TEA sensitivity were similar in both classes. It was also notable that the maximum amplitude of the peak current, in

response to a +66 mV test pulse, varied from cell to cell between 130 and 1000 pA, and that this variability appeared to be independent of the pattern of outward currents, of the age of the rats and of the season.

The voltage dependence of the steady-state inactivation was determined by a conventional double-pulse protocol. The holding potential was set at −40 mV and

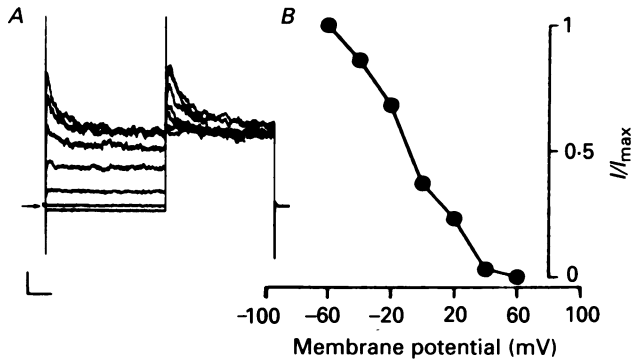

Fig. 3. Voltage dependence of steady-state inactivation of the outward current in standard solutions (A/F). *A*, current traces recorded in the following conditions: a 1 s voltage step of −20 to +100 mV amplitude in 20 mV increments was applied from a holding potential of −40 mV. It was followed with a 375 ms test pulse to +66 mV, a potential at which the outward current was fully activated. Calibration bars: 100 pA, 200 ms (pre-pulse); 100 ms (test pulse). Arrows: zero-current level. *B*, voltage dependence of steady-state inactivation. The data points are the peak of outward current values measured at +66 mV normalized to the maximal values (I/I_{max}). I and I_{max} are the net outward currents defined as the differences between the peak outward current and the current at the end of the test pulse, at the steady state.

pre-pulses of 1 s duration were applied. This duration was enough to reach a steady-state current at all potentials. The test pulse potential was always set at +66 mV.

Figure 3*A* illustrates the currents with pre-pulse potentials varying between −60 and +60 mV. For the less-negative pre-pulses the peak currents became more and more inactivated. Full inactivation was reached at +60 mV. For all pre-pulse values, the steady currents were very similar and were always different to 0 mV. The corresponding steady-state inactivation is plotted in Fig. 3*B*.

Identification of the ionic nature of the outward current

The reversal potential of the outward currents was first estimated from the reversal of the tail currents, by a double-step protocol. The membrane potential was first changed from the holding potential to +66 mV, for 20 ms, to fully activate the outward currents and then shifted back to various second voltage steps. This procedure demonstrated that the reversal potential was near −65 mV (Fig. 4*A*).

The pharmacological separation of the outward currents into two components was achieved by adding 10 mM-TEA and by an exposure to a low-chloride solution. In these experiments, the currents were measured in response to +106 mV depolarizing pulses which led the membrane potential to E_{Na}. This is illustrated in Fig. 4*B* and *C*. The peak currents were markedly attenuated but not entirely suppressed by 10 mM-

TEA (solution B/F, Fig. 4*B*). The residual currents were still transient while the currents at the end of the pulse and the tail currents were diminished too. All effects were rapidly reversed. Such results were also obtained in the presence of 5 mM-4AP (not illustrated).

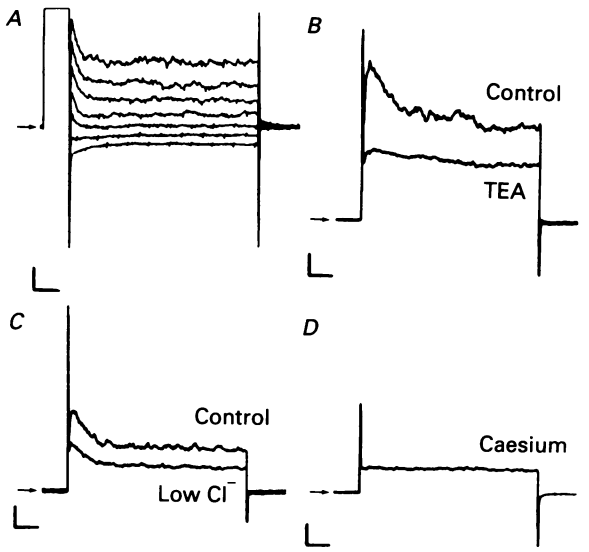

Fig. 4. Characterization of the outward current. *A*, reversal potential of outward current determined by the tail current. The cell was bathed in standard solutions (A/F). Traces of current were obtained first by applying a 20 ms depolarizing voltage step to +66 mV from a holding potential of −40 mV in order to activate fully the outward current. This was followed by test pulses of −40 to +20 mV amplitude in 10 mV increments and 150 ms duration. Tail current reversed quite near −65 mV. *B*, sensitivity of current to 10 mM-TEA. Current traces evoked in standard solutions (A/F) (Control) and in the presence of 10 mM-TEA (TEA) (B/F). Both traces were superimposed recordings evoked by depolarizing pulses to +66 mV (HP = −40 mV). The peaks of the outward current and the tail current were both affected by an exposure to TEA. Calibration bars: 100 pA, 50 ms. *C*, sensitivity of current to chloride ions. Superimposed current traces evoked in standard solutions (A/F) (Control) and during the perifusion of a low-chloride solution which induces a shift of E_{Cl} from −40 to +40 mV. Depolarizing pulses to +66 mV (HP = −40 mV). Calibration bars: 37 pA, 50 ms. *D*, current traces recorded in the absence of internal and external potassium (solutions H/D). Recording evoked by a depolarizing pulse to +66 mV (HP = −40 mV). Only a time-independent outward current was recorded during the voltage pulse. Calibration bars: 57 pA, 50 ms. Arrows: zero-current level.

When the Leydig cells were superfused with a low-chloride solution (solutions A/F then C/F, Fig. 4*C*), which induced a shift of E_{Cl} from −40 to +40 mV, the total outward currents were decreased, while the peak currents were not affected. At the same time, the current measured at the resting potential became inward.

In order to ascertain the presence of a chloride component in the outward currents, the evoked currents were also examined in cells simultaneously dialysed and exposed to a potassium-free solution (solutions D/H (Fig. 4*D*). In this way, K^+ ion movements were suppressed, as indicated by the disappearance of the peak current.

The current during the test pulse practically demonstrated no time-dependent characteristics, but inward tail currents were recorded upon subsequent repolarization to −40 mV.

Upon application of a Ca^{2+}-free solution ($0Ca^{2+}$ + 1 mM-EGTA), the outward currents were never affected, which indicated that the early transient outward

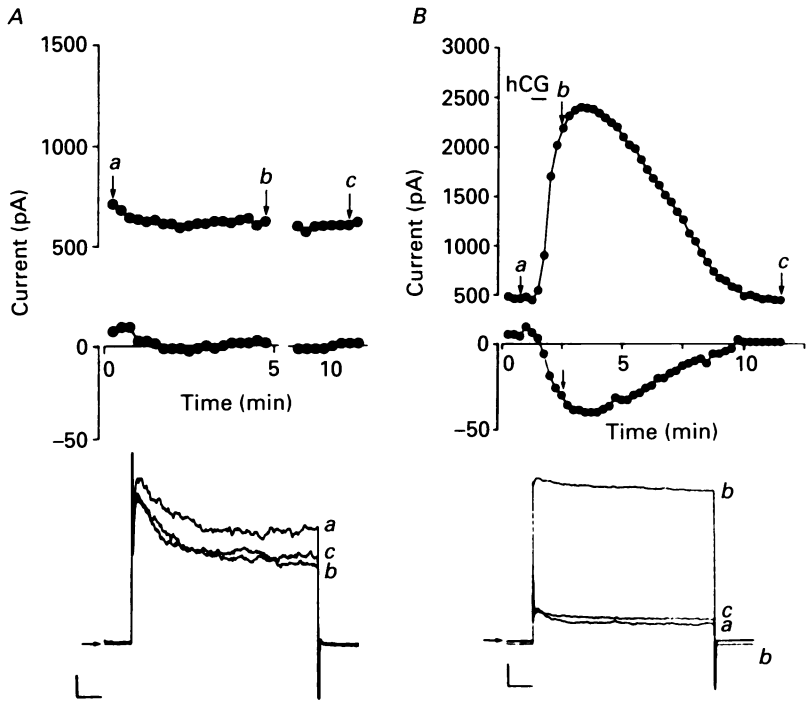

Fig. 5. Effects of hCG on currents. The cells were first bathed in standard solutions (A/F) and superfused by control solution alone (*A*) or by control solution in the presence of hCG (Organon, 50 i.u./ml, bar) (*B*). In this and in subsequent figures, the cells were stimulated with 375 ms depolarizing test pulses from −40 to +66 mV, every 15 s. Top, time course of peak outward (upper curves) and HP currents (lower curves). In *A* the data points are omitted during the current–voltage protocol. Bottom, current traces recorded as indicated on the figures (*a*, *b* and *c*). Calibration bars: *A*, 100 pA and 50 ms; *B*, 334 pA and 50 ms. Arrows: zero-current level.

currents as well as the chloride component were unlikely to depend on extracellular calcium.

Membrane currents in the Leydig cells following LH–hCG stimulation

In order to study eventual modifications of the above conductance, depolarizing voltage steps to +66 mV were applied from HP = −40 mV. The cells were initially bathed in standard solution and dialysed with a low-chloride and low-calcium solution (solutions A/F, Table 1; E_{Cl} = −40 mV). In spite of the fact that large-tip pipettes were used, the entire dialysis of the cells with the internal solution was not instantaneous. A typical time course of the evolution of cell currents using the control solution is illustrated in Fig. 5*A*).

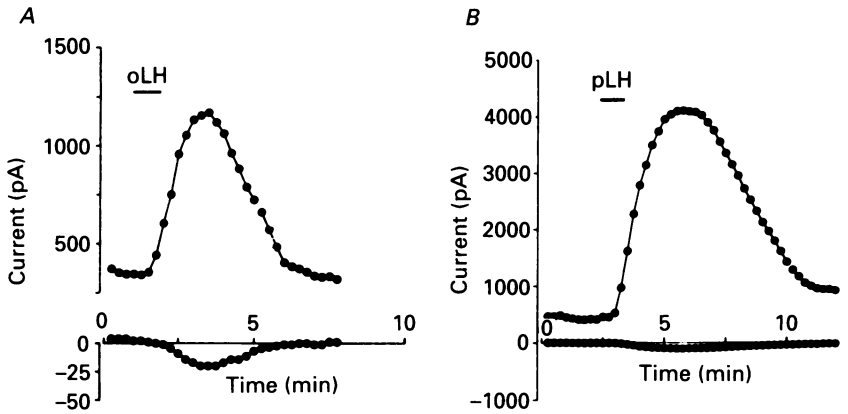

Fig. 6. Effects of ovine and porcine LH (oLH and pLH) on currents. The cells were first bathed in standard solutions (A/F) except for a period (bar) during which the hormone dissolved in the control solution was perifused. Time course of peak outward (upper curves) and HP currents (lower curves). *A*, response to ovine LH (CY 1055, 3·3 μg/ml). *B*, response to porcine LH (CY 1349, 3·3 μg/ml).

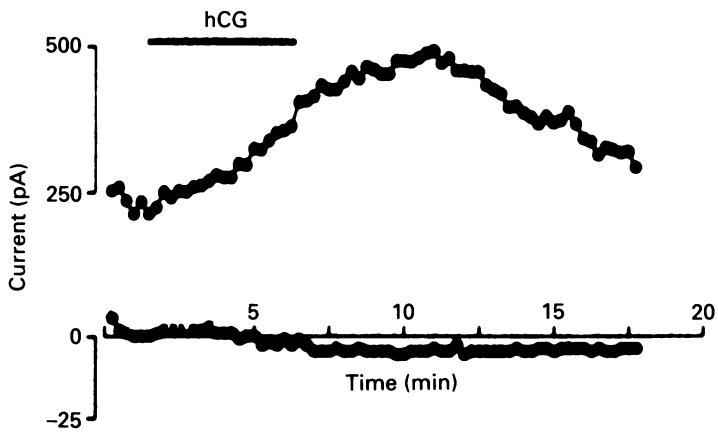

Fig. 7. Effects of hCG at low concentration on currents. The cell was first bathed in standard solutions (A/F) except for a period (bar) during which the hormone dissolved in control solution was perifused. Time course of peak outward (upper curve) and HP currents (lower curve). hCG: Organon, 0·0033 μg/ml.

Within 2 min of achieving the whole-cell recording configuration, the magnitude of the holding potential currents and of the peak outward currents slightly decreased, then both currents remained stable over 10 min, indicating that the filling solution had equilibrated with the cytoplasmic space.

A response to a pulse of hCG (Organon; 50 i.u./ml; 1 min application) is illustrated in Fig. 5*B*. Within 30 s following the exposure to the hormone, the outward currents at +66 mV increased. This corresponded to a development of an inward current at the holding potential. This hCG effect was transient, the currents returning to their

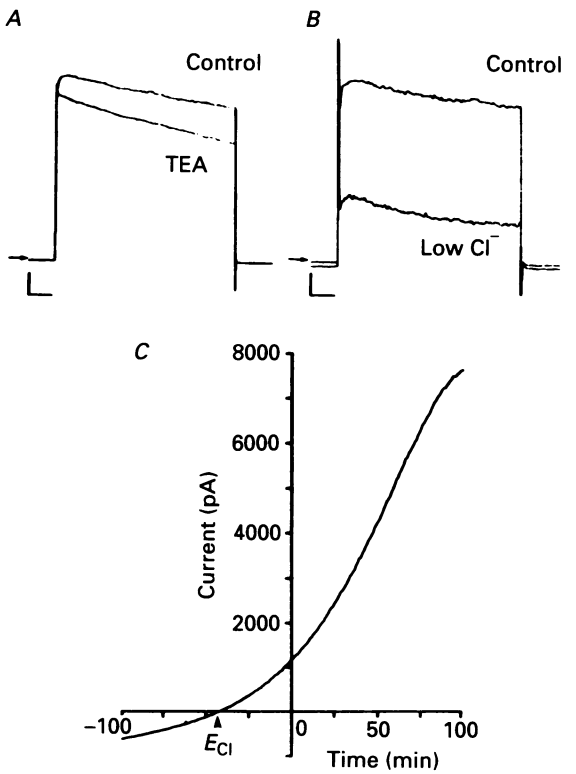

Fig. 8. Characterization of the gonadotrophin-induced current. *A*, sensitivity of hCG-induced currents to 10 mM-TEA using standard solutions (B/F). The cell was stimulated by hCG (ConA-IG 100-1; 3·3 μg/ml). Superimposed current traces evoked at the maximum of the hormone effect (Control) and in the presence of 10 mM-TEA (TEA) in response to +66 mV (HP = −40 mV). TEA slightly decreased the outward current without affecting the HP current. The tail current was suppressed. Calibration bar: 555 pA, 50 ms. *B*, sensitivity of hCG-induced currents to a low-chloride solution. The cell was stimulated by hCG (Organon, 50 i.u./ml) (solutions B/F). Current traces evoked at the maximum of the hormone effect (Control) and in the presence of a low-chloride solution (low Cl^-). The shift of E_{Cl} from −40 to +40 mV largely decreased the outward current and increased the HP current. Calibration bar: 167 pA, 50 ms. Arrows: zero-current level. *C*, current relation for the hCG-stimulated outward current recorded with standard solutions (A/F). The cell was stimulated by hCG (Organon, 50 i.u./ml). The membrane potential was ramped from −100 to +100 mV at a rate of 100 mV/s before the exposure to hCG then at the maximum of the effect. The current–voltage curve was then obtained by subtracting the current in the absence of hCG from that in its presence. The hCG-stimulated component reversed near E_{Cl} (E_{rev} = −41 mV).

initial levels within a 10–15 min period. Similar responses were recorded when a longer pulse of hCG was used. No response was obtained following a second exposure to hCG or when the pulse of hormone was delayed by 10 min.

Such responses were specific to LH–hCG. They were also recorded in the presence of ovine and porcine LH (Fig. 6*A* and *B*) while exposures to GnRH (gonadoliberine) (10^{-7}–10^{-4} M) were ineffective.

The patterns of the response to high concentrations of hCG greatly varied from one

cell to another. All cells responded to the higher concentrations of hormone (5–50 i.u./ml) while about 10% of the cells were activated by low concentrations (0·05 i.u./ml). With 50 i.u./ml hCG, the maximal amplitude of the outward currents varied from 0·5 to 4 nA and the corresponding HP currents from 10 to 100 pA. When

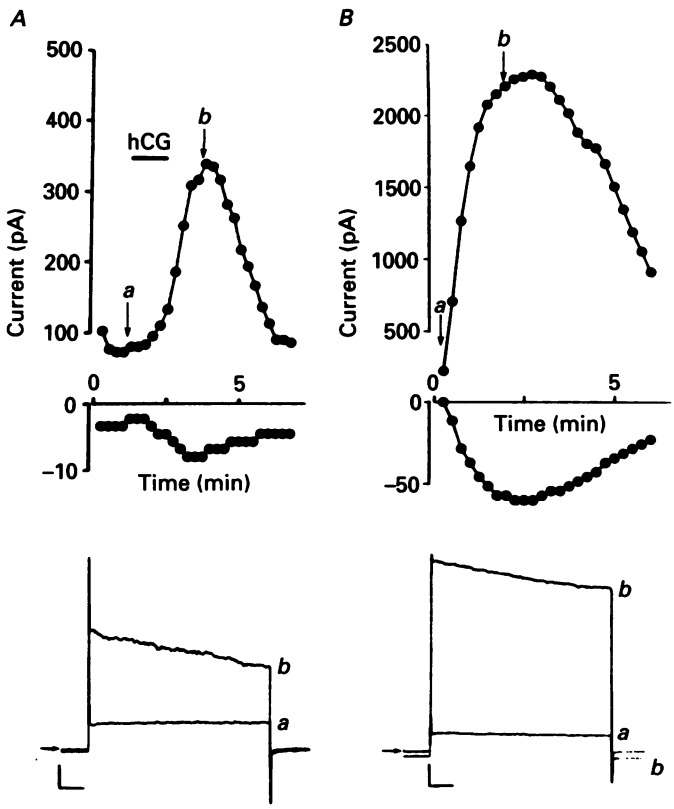

Fig. 9. Effects of hCG (Organon, 50 i.u./ml) and cyclic AMP (100 μM) (*B*) in cells dialysed and perifused with K^+-free solutions (D/H). Top, time course of peak outward (upper traces) and HP currents (lower traces). Bottom, current traces recorded as indicated on the figures (*a* and *b*). Calibration bars: *A*, 67 pA and 50 ms; *B*, 278 pA and 50 ms. Arrows: zero-current level.

using the lower concentrations, the efficiency of hCG at increasing the currents was enhanced by a longer exposure (Fig. 7). In such conditions, the time to peak of the response was delayed and the whole response duration was lengthened. On average this time to peak varied as follows with hCG concentration: 50 i.u./ml, 153 ± 43 s, $n = 10$; 0·5 i.u./ml, 210 ± 27 s, $n = 4$; 0·05 i.u./ml, 655 ± 59 s, $n = 5$.

Identification of the ionic nature of LH–hCG-induced currents

During the hCG-induced response, the outward current recorded at +66 mV was somewhat reduced by 10 mM-TEA (Fig. 8*A*) such that the potassium components of the outward currents were similar before and after an exposure to hCG (compare Fig.

4*B* and 8*A*). Conversely, the hCG-induced currents were very sensitive to a change of E_{Cl}. A shift of E_{Cl} from −40 to +40 mV simultaneously induced a marked decrease of the outward currents and an increase in the inward HP currents (Fig. 8*B*). Under these conditions, the pattern of the remaining component of the outward currents was similar to that of the currents elicited at the resting state (Fig. 4*B*).

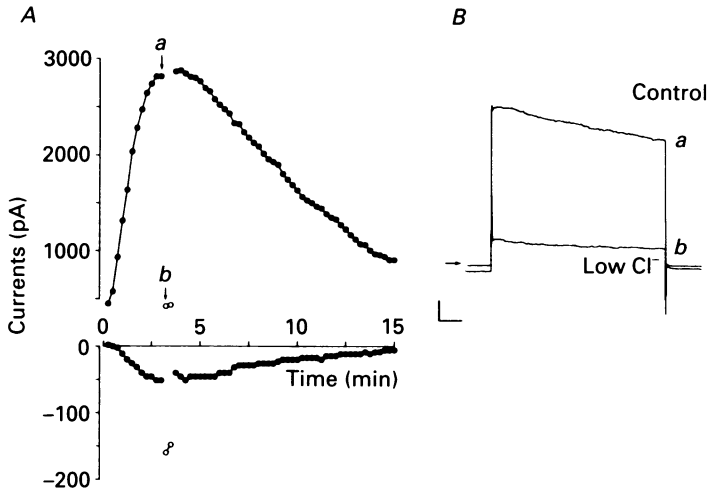

Fig. 10. Effects of internal cyclic AMP (100 μM) on currents. The cell was bathed in standard solutions (A/F) and dialysed with cyclic AMP. *A*, time course of peak outward (upper curve) and HP currents (lower curve). *B*, current traces evoked at the maximum of the cyclic AMP effect. Control ($E_{Cl} = -40$ mV) and in the presence of a low-chloride solution (Low Cl^-, $E_{Cl} = +40$ mV). Calibration bars: 400 pA, 50 ms. Arrow: zero-current level.

In addition, the effects of hCG were investigated in the absence of external and internal potassium (solutions D/A; Fig. 9). Under these ionic conditions increases in the outward and HP currents were still recorded. However, the response amplitude was lower than in K^+-containing solutions, and the kinetics of the response were slower. The outward component was instantaneously activated and was followed by a slow time-dependent inactivation.

To assess the chloride dependence of the LH–hCG-sensitive component of the outward currents, the reversal potential of this component was determined. Voltage ramps (100 mV/s) were applied to the pipette before and at the maximum of the hCG effects. The currents were then subtracted. The resulting currents appeared to reverse near E_{Cl} (−38 and −44 mV *versus* −40 mV) (Fig. 8*C*).

Intracellular mechanism of the LH–hCG-stimulated chloride conductance

It is now well established that LH–hCG stimulation of the Leydig cells mainly involves activation of adenylate cyclase and an increase in intracellular cyclic AMP. To test whether cyclic AMP affects the conductance of the resting cells, 100 μM-cyclic AMP was added to the internal solution (solutions A/F). Rupturing the membrane

within the pipette was followed by a rapid increase in outward currents during test pulses concomitant with the development of an inward current at the holding potential, followed by a gradual wane of both currents (Fig. 10). The response to cyclic AMP greatly varied from one cell to another. In a few cells the response was

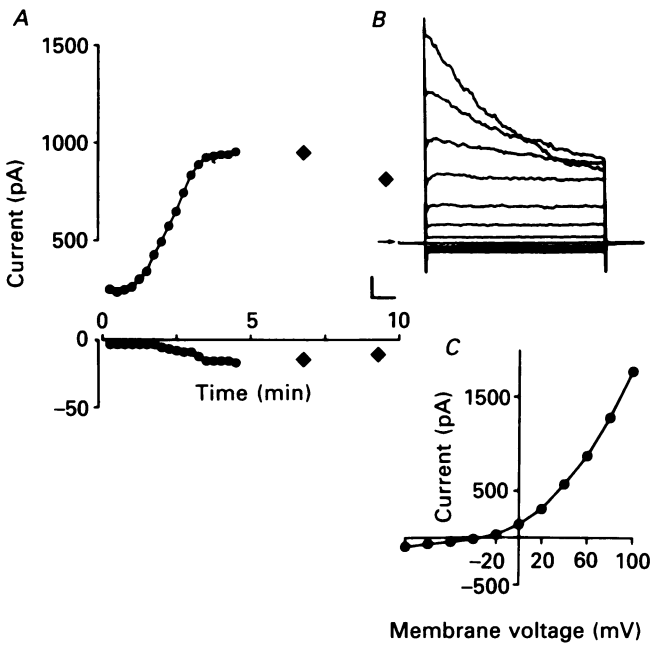

Fig. 11. Effects of internal GTP (200 μM) in the presence of ATP (2 mM) on currents. The cell was bathed in standard solutions and dialysed by a GTP–ATP mixture (A/J). *A*, time course of peak outward (upper curve) and HP currents (lower curve). Data points are omitted during current–voltage protocols. ◆, values corresponding to currents measured during the course of the *I–V* procedure at a potential of +66 mV. *B*, current traces in response to hyperpolarizing and depolarizing voltage steps of −60 to 100 mV amplitude in 20 mV increments, applied from a holding potential of −40 mV, every 15 s. Calibration bars: 200 pA, 50 ms. Arrow: zero-current level. *C*, current–voltage relation for peak outward current. Values are not corrected for leakage current. The reversal potential is near −40 mV, the chloride equilibrium potential.

sustained or slowly decreased with time. All the cells responded to higher concentrations of cyclic AMP (range 50–100 μM) while about 10% of the cells were activated by lower concentrations (5–20 μM).

In the presence of intracellular cyclic AMP, an exposure to 10 mM-TEA or to a low extracellular chloride solution (Fig. 10*B*) depressed the outward currents and increased the HP currents as in the presence of hCG. The response to cyclic AMP in the absence of potassium was similar to that recorded under standard ionic conditions (compare Fig. 9*B* and 10*A*).

When the internal solution was supplemented with 2 mM-ATP and 0·2 mM-GTP and the Mg^{2+} concentration was simultaneously increased to 3 mM (solutions A/J), a gradual increase in both holding and test currents was obtained (Fig. 11*A*).

This response appeared to be sustained. Only a small and slow decline followed the maximal response. The voltage dependence of the outward currents was analysed at the steady state (Fig. 11*B* and *C*). Current traces show no outward relaxations in response to lower depolarizing voltage pulses while both an activation and an

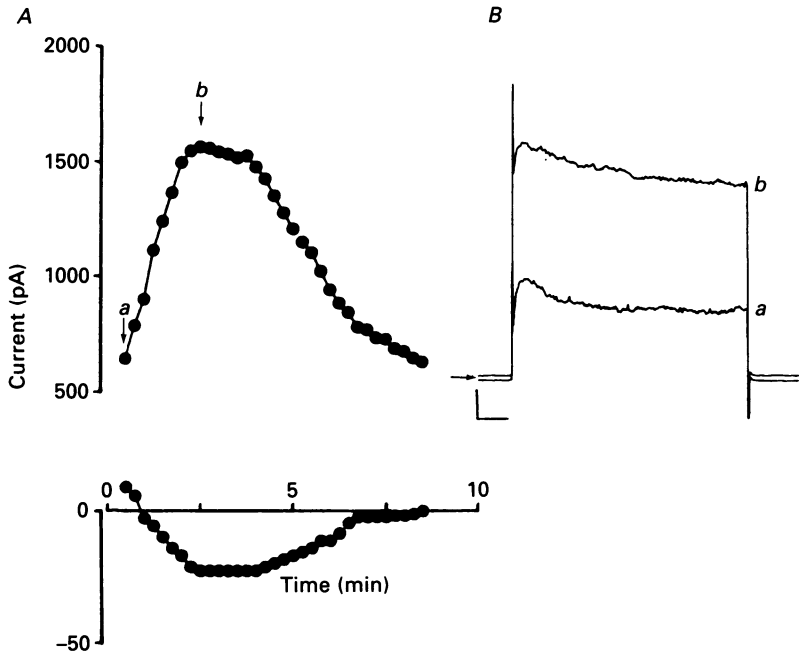

Fig. 12. Effects of internal cyclic AMP (100 μM) in the absence of internal and external calcium on currents. The cell was dialysed with cyclic AMP in the presence of 5 mM-EGTA solution and bathed in a solution containing 1 mM-EGTA (E/G). *A*, time course of peak outward (upper curve) and HP currents (lower curve). *B*, current traces evoked at the rupture of the membrane within the pipette (*a*) and at the maximum of the cyclic AMP effect (*b*). Both traces are superposed recordings evoked by pulses to +66 mV (HP = −40 mV). Calibration bars: 200 pA, 50 ms. Arrow: zero-current level.

inactivation were encountered for membrane potentials more positive than +40 mV. Peak currents demonstrated strong outward rectification properties (Fig. 11*C*). It is also notable that no time-dependent current was measured in response to hyperpolarizing voltage steps (Fig. 11*B* and *C*). These currents were not obtained in the presence of ATP alone.

To test whether intracellular or extracellular calcium ions were involved in the response to the messengers, LH–hCG stimulation and cyclic AMP effects were performed in the absence of calcium (solutions E/G). Neither response was in any way affected by this experimental procedure (Fig. 12).

Conversely, an increase in the intracellular calcium from about 10^{-8} to 10^{-7} M (solution A/I) induced long-lasting outward current relaxations in response to depolarizing voltage steps. This was followed by slow inward tail currents upon returning to the holding potential (Fig. 13*A*). Moreover, an inward HP current was always induced. The outward currents were decreased and the inward tail currents

simultaneously increased during an exposure to a low-chloride solution (Fig. 13*B*). The steady-state current presented a high voltage dependence with an outward rectification (Fig. 13*D*) and reversed at a potential near E_{Cl} ($-35{\cdot}7 \pm 0{\cdot}9$ mV, $n = 3$) (Fig. 13*C*).

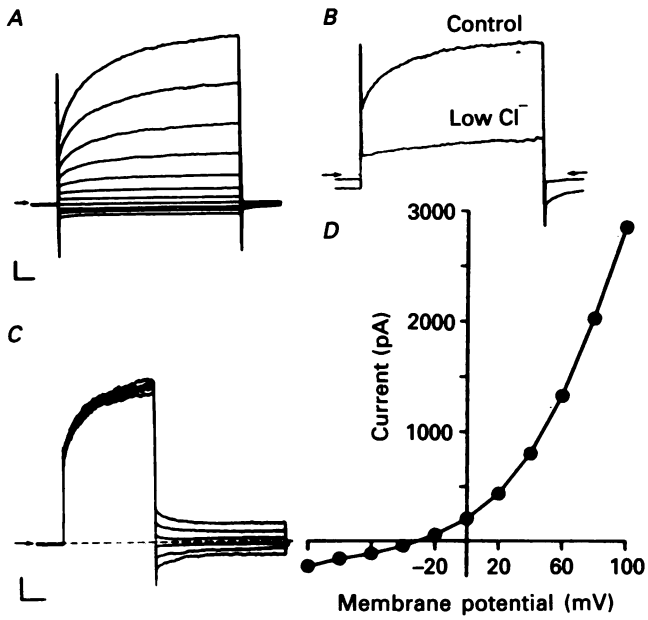

Fig. 13. Effects of internal calcium on currents. The cells were dialysed with a 10^{-7} M-calcium solution in the presence of standard solutions (A/I). *A*, current traces in response to hyperpolarizing and depolarizing voltage steps of -60 to 100 mV amplitude in 20 mV increments, applied from a holding potential of -40 mV, every 15 s. Depolarizing voltage steps induced long-lasting currents towards a steady-state level which were followed by inward tail currents upon returning to the holding potential. Hyperpolarizing voltage steps induced slowly decaying inward currents. Calibration bars: 400 pA, 50 ms. *B*, sensitivity of calcium-induced currents to a low-chloride solution. The cell was exposed to solution C. Current traces are superimposed recordings evoked by depolarizing pulses to $+66$ mV (HP $= -40$ mV). Control, in the presence of solutions A/I. Low Cl^- in the presence of solutions C/I. The shift of E_{Cl} from -40 to $+40$ mV largely decreased the outward current ('on' relaxation) and increased the HP current ('off' relaxation). Calibration bars: 250 pA, 50 ms. *C*, reversal potential of outward current determined by tail current analysis. The cell was bathed in solutions A/I. Traces of current were obtained by applying first a 20 ms depolarizing voltage step to $+66$ mV from a holding potential of -40 mV in order to activate fully the outward current. This was followed by test pulses of -60 to -10 mV amplitude in 10 mV increments and 570 ms duration. Tail currents reversed at -36 mV (interpolated potential value). Calibration bars: 200 pA, 100 ms. Arrows: zero-current level. *D*, current–voltage relation for steady-state currents.

DISCUSSION

Endocrine cells such as Leydig cells, luteal cells and adrenocortical cells secrete steroid hormones. While the biochemical mechanisms underlying these secretions are well known, by contrast the electrical properties of the plasma membrane and their

modifications by the stimulating hormones are far from being understood (Kawa, 1987; Matsunaga, Yamashita, Maruyama, Kojima & Kurokawa, 1987; Payet, Benabderrazik & Gallo-Payet, 1987; Durroux, Gallo-Payet & Payet, 1988; Cohen, McCarthy, Barrett & Rasmussen, 1988; Tabares, Urena & Lopez-Barneo, 1989). In the present study we investigated the electrophysiological properties of mature Leydig cells freshly isolated from rat testis, by the use of the whole-cell variation of the patch-clamp technique (Hamill *et al.* 1981). The results clearly show that the gonadotrophins (LH–hCG) increase a voltage-dependent chloride conductance. This effect is mimicked by internal cyclic AMP or by a combination of ATP and GTP.

Conductances in the resting Leydig cell

In the present investigation we corroborate results previously published (Duchatelle & Joffre, 1987) and we describe additional properties of the outward currents in the membrane of resting rat Leydig cells. (1) These currents are activated for depolarizing pulses more positive than −40 mV and (2) undergo inactivation at higher potentials (Fig. 1). (3) They reverse near E_K and (4) cell dialysis with a K^+-free solution containing Cs^+ completely abolishes the transient component of the currents (Fig. 4*D*). Moreover, (5) TEA and 4-aminopyridine diminish the currents as in several other cells (Rudy, 1988) and (6) omitting external calcium does not affect them (Fig. 12).

Unlike A-currents (Rudy, 1988) the transient currents elicited by depolarizing pulses are sensitive to TEA. We conclude that these transient currents are related to a delayed rectifier conductance as previously defined in human T-lymphocytes (Cahalan, Chandy, DeCoursey & Gupta, 1985; DeCoursey, Chandy, Gupta & Cahalan, 1987) and in macrophages (Ypey & Clapham, 1984; Gallin & Sheehy, 1985; Randriamampita & Trautmann, 1987). TEA also reduces the sustained current (Fig. 4*B*), a component which is still recorded after completion of inactivation at +60 mV. This indicates a fraction of K^+ channels which could fail to inactivate in the total outward current.

Tail currents appeared to revert between E_K and E_{Cl} (Fig. 4*A*) indicating some contribution of a chloride conductance elicited by depolarizing voltage steps. This conductance was revealed when a low-chloride solution superfused the cell and was clearly demonstrated in the presence of a blockade of K^+ conductance with Cs^+. This conductance may be the hormone-regulated conductance, as discussed below.

Some variability in the peak amplitude of the total outward currents and in the pattern of activation and inactivation is demonstrated. In all cases, neither the current–voltage curves, the threshold of activation or the TEA sensitivity are different. This variability is not correlated to the age of the rat or to a hypothetical seasonal cycle. The electrical properties of the rat Leydig cells appear to be very different to those of the mouse Leydig cells. Kawa (1987), using the same technique applied to mouse cells isolated by mechanical dissociation, demonstrated two currents: a calcium current evoked with depolarizing voltage steps more positive than −50 mV and an outward current carried by a calcium-activated potassium channel, elicited with voltage pulses more positive than +40 mV. In our experiments these currents have not been demonstrated. It may be argued that these conductances are masked by the large chloride conductances evoked by depolarizing

voltage pulses and/or by an increase in intracellular calcium. Kawa observed action potential-like responses in current-clamp conditions, a response which has never been demonstrated on rat Leydig cells by the intracellular method (Joffre *et al.* 1984*b*) and confirmed by the current-clamp technique (P. Duchatelle & M. Joffre, unpublished data). Altogether these results suggest that the electrical properties of Leydig cells from rat and mouse are different or that these properties are modified by enzymatic or mechanical isolation.

Changes induced by gonadotrophins and second messengers

To investigate the control of the conductances by gonadotrophins we first decided to dialyse the cells with purely ionic internal solutions in order to obtain a response to the stimulation relying only on the physiological content of the cytoplasm. Since the second messenger and metabolite contents are diluted with time, the time course of the LH–hCG effects must be expected to be transient, as experimentally observed. In these conditions a current response to LH–hCG is recorded within a 30 s latency. This response is specific to gonadotrophins and does not occur in the presence of gonadoliberine.

A main conclusion of the present work is that a transient increase in a chloride conductance underlies the effects of gonadotrophins, while the potassium delayed rectifying conductance is not affected. This is substantiated by the following results: (1) Outward currents induced by depolarizing voltage steps are slightly reduced by TEA whereas they are very markedly decreased by an application of a low-chloride perfusing solution (Figs 8*B* and 10). (2) The patterns of the residual currents observed in low internal and external chloride solution (Fig. 8*C*) are similar to those recorded in the resting cell. (3) The value of the reversal potential of the component increased by gonadotrophins is near E_{Cl}. (4) hCG-induced currents may be obtained with Cs^+-dialysed cells (Fig. 9).

A similar increase in the outward current is recorded when the cells are perfused with cyclic AMP (Fig. 10). The response to hCG as well as to cyclic AMP is transient. Since the LH–hCG effects are mimicked by the main second messenger of these hormones, the results strongly support the hypothesis that gonadotrophins increase the chloride conductance by stimulating a cyclic AMP-dependent pathway.

In contrast, the effects of a GTP–ATP mixture are more sustained (Fig. 12), effects which are not recorded upon application of internal ATP alone. GTP alone is known to stimulate the adenylate cyclase of Leydig cells in the absence of stimulating hormone (Khanum & Dufau, 1988). This suggests that the GTP–ATP-induced increase in the chloride conductance might occur by a cyclic AMP-dependent mechanism.

The response to hCG is decreased in magnitude in the absence of potassium and/or in the presence of caesium. In contrast this substitution does not affect the response to cyclic AMP (Fig. 9). In the absence of potassium, the binding of hCG to receptors is not affected while the testosterone secretion and the cyclic AMP production are decreased (Dufau & Catt, 1978). Our results clearly demonstrate that potassium and caesium do not affect the gating of chloride channels, since the current response to cyclic AMP is not affected. In contrast, the results in the presence of hCG suggest that the absence of potassium or the presence of caesium decreases the efficiency of

the receptor–G_s protein–adenylate cyclase system to produce cyclic AMP. The same hypothesis can explain the results of Dufau & Catt (1978).

Nature of the gonadotrophins-stimulated conductance

Previous studies demonstrated the presence of two chloride conductances in the membrane of resting Leydig cells. First, these cells possess a hyperpolarization-activated chloride conductance (P. Duchatelle & M. Joffre, unpublished data). This conductance is unmasked after some time of dialysis in the presence of a chloride-rich solution, suggesting control of this conductance by a metabolite. In the present work, low internal chloride solutions were used to minimize the hyperpolarization-induced current. Under these conditions, the hyperpolarization-induced current was never recorded in response to hCG, LH, cyclic AMP and GTP–ATP (Fig. 11*B* and *C*). It was sometimes observed after a delay following a response to these stimulating factors. This suggests that a cyclic AMP-dependent process inhibits the conductance as in *Aplysia* neurons (Lotshow & Levitan, 1987).

A second chloride conductance is activated in the presence of calcium-enriched internal solution (Duchatelle & Joffre, 1987; Fig. 12). This conductance shows the characteristics of the well-known calcium-activated conductance demonstrated in several cells (Barish, 1983; Owen, Segal & Barker, 1984; Marty, Tan & Trautmann, 1984; Evans & Marty, 1986; Bader, Bertrand & Schlichter, 1987; Randriamampita, Chanson & Trautmann, 1988; Taleb, Feltz, Bossu & Feltz, 1988). This conductance is not activated by GnRH, LH, hCG or cyclic AMP.

It is notable that gonadotrophins activate a chloride conductance which presents properties different to those of the previously described conductances. This gonadotrophin-induced conductance is activated by depolarizing voltage steps greater than −40 mV, has a high chloride selectivity, displays outward-rectifying instantaneous currents at the onset of voltage steps and is characterized by a time-dependent inactivation for potentials more positive than +40 mV. It is independent of external calcium. This conductance is similar to the background chloride conductance present in the membrane of squid axons (Inoue, 1985) and in heart cells (Harvey & Hume, 1989). In Leydig cells this conductance seems to be a small component of the resting conductance. This conductance is activated by a cyclic AMP-dependent process, as are the chloride conductances described in heart cells (Harvey & Hume, 1989), in T- and B-lymphocyte cell lines (Chen, Schulman & Gardner, 1987; Bosma, 1989), in mast cells (Penner, Matthews & Neher, 1988) and in several epithelial cells (Gögelin, 1988 (review); Giraldez, Sepúlveda & Sheppard, 1989; Gray, Greenwell & Argent, 1988; Schoppa, Shorofsky, Jow & Nelson, 1989).

The LH–hCG response is followed by a transient increase in the HP current (Fig. 5). The question arises as to the origin of this current. Hyperpolarizing voltage steps to −100 mV do not induce any time-dependent current (Fig. 10*B* and *C*). This rules out the participation of the hyperpolarization-activated currents in this response (P. Duchatelle & M. Joffre, unpublished data). These results support the idea that the chloride conductance which gives outward currents in response to depolarizing voltage steps is also the cause of the non-inactivating inward currents recorded upon subsequent repolarization to −40 mV. This inward current presumably results in an increase in the internal chloride concentration following the large cyclic AMP-

induced outward currents (Fig. 5*B*), such that E_{Cl} shifts to values less-negative than −40 mV. It is notable that the HP current is enhanced when a high internal chloride solution is used.

The stimulation of Leydig cells by gonadotrophins initiates testosterone secretion. Our results demonstrate that these hormones activate a cyclic AMP-dependent chloride conductance, independently of a calcium pathway. The experiments with microelectrodes suggest that the Leydig cell membrane is slightly permeable to chloride ions which are passively distributed in accordance with the membrane potential (Joffre *et al.* 1984*b*). They also demonstrated the slow depolarizing effect of hCG and cyclic AMP, an effect which was assumed to be linked to a blockade of a calcium-activated potassium permeability, but which can be re-interpreted as due to an increase in Cl^- permeability in the light of the present experiments. This interpretation remains tentative since the physiological value of E_{Cl} is unknown. Furthermore leakage currents and a possible activation of a Ca^{2+}-dependent K^+ channel by Ca^{2+} ions flowing through the leak may produce substantial errors in the determination of the resting potential.

Functional significance for secretion

Hormonally regulated chloride channels have been demonstrated in the apical membrane of epithelial cells. They are involved in epithelial secretion stimulated by cyclic AMP-dependent messengers (see review: Gögelin, 1988). In heart cells, the chloride conductance controlled by adrenaline is involved in the chronotrophic effect of this neurotransmitter (Harvey & Hume, 1989). The cyclic AMP-activated chloride conductance in mast cells may provide the driving force for calcium influx (Penner *et al.* 1988). It is presently difficult to attribute a function to the cyclic AMP-dependent chloride conductance in the stimulation–secretion process of the Leydig cell. One possible speculation is to consider that chloride movements are involved in the steroid release.

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