

Modulation of K^+ conductances by Ca^{2+} and human chorionic gonadotrophin in Leydig cells from mature rat testis

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1. Although the control of steroidogenic activity of the Leydig cell by the peptides luteinizing hormone (LH) and human chorionic gonadotrophin (hCG) is clearly mediated by cAMP, the extent to which Ca^{2+} controls the Leydig cell function is less well defined. In the present study, the whole-cell configuration of the patch-clamp technique was used to investigate the modulation of potassium conductances by calcium and hCG, in the Leydig cells from mature rat testis.
2. In symmetrical glutamate solutions, depolarizations elicited outwardly rectifying currents, which were mainly carried by potassium and were blocked by tetraethylammonium and 4-aminopyridine. For values of $[Ca^{2+}]_i$ below 10^{-8} M, transient currents of low amplitudes, insensitive to charybdotoxin (CTX) and iberiotoxin (IBTX), were activated above -40 mV. For $[Ca^{2+}]_i$ values of 10^{-7} M and above, noisy currents with slow activation kinetics were activated above 0 mV. These currents were sustained and were sensitive to CTX and IBTX.
3. Both current types were modulated by intracellular calcium. Ionomycin and a $[Ca^{2+}]_i$ elevation in the range from 10^{-9} to 10^{-7} M, both inhibited the CTX-insensitive currents, whereas a rise in the calcium concentration above 10^{-7} M increased the amplitude and shifted the threshold of activation of the CTX-sensitive currents to less positive levels.
4. hCG ($1-50$ i.u. ml^{-1}), in conditions where the chloride currents were strongly inhibited by 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulphonic acid (SITS), induced a partial inhibition of the CTX-insensitive currents but was unable to increase the CTX-sensitive currents.
5. No voltage-sensitive calcium current was recorded in control or hCG-stimulated cells.
6. The results indicate that hCG inhibits one kind of Ca^{2+} -modulated channel, perhaps as a result of a moderate $[Ca^{2+}]_i$ rise, but is unable to increase the intracellular Ca^{2+} concentration to the range in which large conductance Ca^{2+} -dependent channels are activated.

Leydig cells are located in the intertubular tissue of the mammalian testis. When stimulated by luteinizing hormone (LH) or human chorionic gonadotrophin (hCG), they secrete large amounts of testosterone. It is now well established that these hormones exert their predominant control through the adenylate cyclase pathway connected to the LH/hCG receptors by G_s proteins (Dufau, Baukal & Catt, 1982). However, like several adenylate cyclase-coupled receptors in other cell types, the LH/hCG receptors are also capable of modulating the cytosolic calcium (Segaloff & Ascoli, 1993).

The involvement of calcium in Leydig cell physiology is variable and sometimes controversial. The findings of Van Der Vusse, Kalkman, Van Winsen & Van Der Molen (1976)

indicate that calcium *in vitro* mimics the trophic effect of LH on mitochondrial pregnenolone production. The calcium ion association with the calcium-binding protein calmodulin facilitates the transport of cholesterol into mitochondria (Hall, Osawa & Mrotek, 1981). The calcium ionophore A23187 increases steroidogenesis in rat Leydig cells (Lin, 1985). Omission of calcium from the incubation medium results in a small (Mendelson, Dufau & Catt, 1975) or a strong decrease in the stimulation by LH/hCG of testosterone production in rat Leydig cells (Janszen, Cooke, Van Driel & Van Der Molen, 1976). However, there is no agreement on the effects of hormonal stimulation on $[Ca^{2+}]_i$. Two groups report that the stimulation by LH/hCG of mature rat Leydig cells progressively increases the intracellular free calcium concentration (Sullivan & Cooke, 1986;

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Kumar, Blumberg, Canas & Maddaiah, 1994), while another group found no effect (Tomic, Dufau, Stojilkovic & Catt, 1995). In the first two studies, the action of LH/hCG on the calcium signalling was mimicked by 8-bromo-cyclic AMP and protein kinase A, and it appeared either as an extracellular calcium-dependent (Kumar *et al.* 1994) or as an extracellular calcium-independent process (Sullivan & Cooke, 1986). Finally, the mechanisms of $[Ca^{2+}]_i$ elevation remain unclear. The calcium channel blockers, verapamil, diltiazem, nicardipine and cobalt, all inhibit steroidogenesis in Leydig cells from rat and mouse testis (Kumar *et al.* 1994). Bay K 8644, an activator of L-type calcium channels, was found to increase the cytosolic calcium concentration in one study on rat Leydig cells (Kumar *et al.* 1994) and to have no effect in another study (Tomic *et al.* 1995).

In all mammalian cell types studied, the cytosolic calcium controls ionic membrane conductances. In Leydig cells, the pattern of calcium-dependent conductances is not well known. Depolarizations in the presence of 10^{-7} M Ca_i^{2+} were reported to activate a calcium-activated chloride conductance in rat Leydig cells (Duchatelle & Joffre, 1987, 1990; Noulon & Joffre, 1993a) and a calcium-activated potassium conductance in mouse Leydig cells (Kawa, 1987). Depolarizations in the presence of low intracellular calcium were found to activate either a calcium-activated potassium conductance in mouse Leydig cells (Kawa, 1987) or an outwardly rectifying potassium conductance in rat Leydig cells (Duchatelle & Joffre, 1987, 1990). In addition, voltage-dependent calcium channels have been observed in mouse Leydig cells (Kawa, 1987).

In the present work, we used the whole-cell patch-clamp technique in rat Leydig cells to investigate the following points: (1) the concentration-dependent effects of calcium on potassium conductances; (2) the effects of an exposure to hCG on these conductances; and (3) the presence of voltage-dependent calcium channels.

METHODS

Leydig cell preparation

Leydig cells were dispersed from the testes of 60- to 90-day-old rats (Wistar AF) killed by decapitation as previously described (Duchatelle & Joffre, 1987; Noulon & Joffre, 1993b). These animals were raised under a controlled light-dark cycle (12 h:12 h) and constant temperature conditions (20 °C).

Briefly, testes were mechanically and enzymatically dissociated in a solution containing 0.5 mg ml^{-1} collagenase (0.6 – 0.8 U mg^{-1} ; Serva, Heidelberg, Germany) and 0.04 mg ml^{-1} trypsin inhibitor (soybean-type 1S; Sigma). The Leydig cells were purified from germ cells and red blood cells by density centrifugation on a discontinuous Percoll (Sigma) gradient. They were placed on 35 mm plastic Petri dishes, and cultured for a period ranging from 4 to 36 h in modified RPMI 1640 medium (Gibco). The Leydig cells were easily discernible from contaminating cells by their spherical and green-brown appearance, with a bright ring under a phase-contrast inverted microscope (Joffre *et al.* 1984a).

Electrophysiology

Membrane currents were obtained by the whole-cell configuration of the patch-clamp technique (Marty & Neher, 1983) with a patch-clamp amplifier (RK300; Biologic, France). Recording pipettes with resistances of 2–5 M Ω were pulled from borosilicate glass capillary tubing (GC150-TF10; Clark Electromedical Inc., Reading, UK) using a two-step vertical electrode puller (type PP-82; Narishige). They were coated with Sylgard 184[®] (Dow Corning), fire polished, and were connected to the head-stage of an amplifier through an Ag–AgCl pellet. Seal resistances ranging from 3 to 30 G Ω were obtained.

The compensation circuitry of the RK 300 amplifier was used to cancel the pipette capacitance in the cell-attached mode. Membrane capacitance and series resistance were not compensated. Instead, they were determined in the whole-cell mode by fitting a capacitance current, recorded in response to a square pulse of 50 ms and +6 mV applied from –30 mV, with a first-order exponential, and by integrating the surface area of the capacitance peaks using Clampfit software (pCLAMP 5.5.1; Axon Instruments). The averaged series resistance and membrane capacitance (\pm s.e.m.) for fifty-nine cells were 12.0 ± 0.4 M Ω and 22.9 ± 1.0 pF, respectively. Since the amplitude of outward currents was low, no compensation of series resistance was employed.

Voltage-clamp signals were applied and recorded with the resulting currents by means of an IBM microcomputer (PC AT 286) equipped with an A/D–D/A conversion board (TM-40; Teckmar) and a specific software package (pCLAMP 5.5.1). The cell membrane currents were low-pass filtered at 3.3 kHz, digitized on-line at 4 kHz and stored. They were analysed off-line with the pCLAMP software after being filtered (cut-off frequency, 0.3 kHz).

The liquid junction potentials were measured as previously described (Fenwick, Marty & Neher, 1982), and they were of about –6 mV. All membrane voltages were corrected for this value. The current values were expressed as maximum current amplitudes measured within the first 100 ms of voltage step, except where mentioned. Except when anionic currents were recorded (for example in the presence of hCG), linear leak currents were observed between –86 and –66 mV, allowing us to calculate the leak conductance, and to correct the current–voltage relationships, except when an inward current was activated.

The current–voltage relationships were determined from step voltage protocols. The current traces illustrating the representative records and the current–voltage curves were drawn with a specific software package (Fig. P. 6; Biosoft, Cambridge, UK). Data are expressed as the means \pm s.e.m. for n experiments. The statistical significance of results was assessed by means of Student's t test (Multistat; Biosoft) performed on paired or unpaired data.

Solutions

Solutions for potassium current recordings. The compositions of external and internal solutions are given in Table 1. In order to inhibit chloride currents, glutamate and sulphate were substituted for chloride ions, so that the equilibrium potentials for glutamate and potassium ions were –3 and –83 mV, respectively. In these solutions, the pH was buffered with Hepes–NaOH (10 mM Hepes brings 4 mM Na^+), and the osmolarities were controlled to 300 ± 5 mosmol l^{-1} (external solution) and 280 ± 5 mosmol l^{-1} (internal solutions), as assessed by the freezing-point depression determined with an automatic semi-micro-osmometer (Knauer, Germany). The internal solutions were supplemented with 8–10 mM EGTA, and the free calcium concentration (pCa) in the

Table 1. Ionic concentrations (mM) of external and internal solutions

Solution	Potassium glutamate	Sodium glutamate	CaSO ₄	MgSO ₄	EGTA-(KOH) ₂	ATP-Mg	Hepes	pH	pCa
A	5	152	2	2	—	—	10	7.4	—
1	114	6	—	4	10	3	10	7.2	> 9
2	114	6	9.1	2	10	3	10	7.2	6
3	114	6	8.3	2	10	3	10	7.2	6.3
4	114	6	4.9	2.5	10	3	10	7.2	7
5	114	6	0.9	3	10	3	10	7.2	8
6	114	6	0.1	3	10	3	10	7.2	9
7	117	—	—	4	8	3	25	7.2	> 9
8	135	6	—	4	0.5	3	10	7.2	> 9

A, external solution; 1–8, internal solutions.

pipette solution was adjusted according to the calculation of Fabiato & Fabiato (1979) with the aid of a computer program.

Solutions for calcium current recordings. The electrodes were filled with solutions containing (mM): 74 CsCl and 56 caesium

glutamate or 130 caesium glutamate, 1 NaCl, 3 MgCl₂, 3 Mg-ATP, 10 EGTA and 10 Hepes (pH 7.2). The standard external saline contained (mM): 160 NaCl or 160 caesium glutamate, 10 CaCl₂, 1 MgCl₂, and 10 Hepes (pH 7.4). These solutions were chosen to maximize the inward currents through Ca²⁺ channels and to

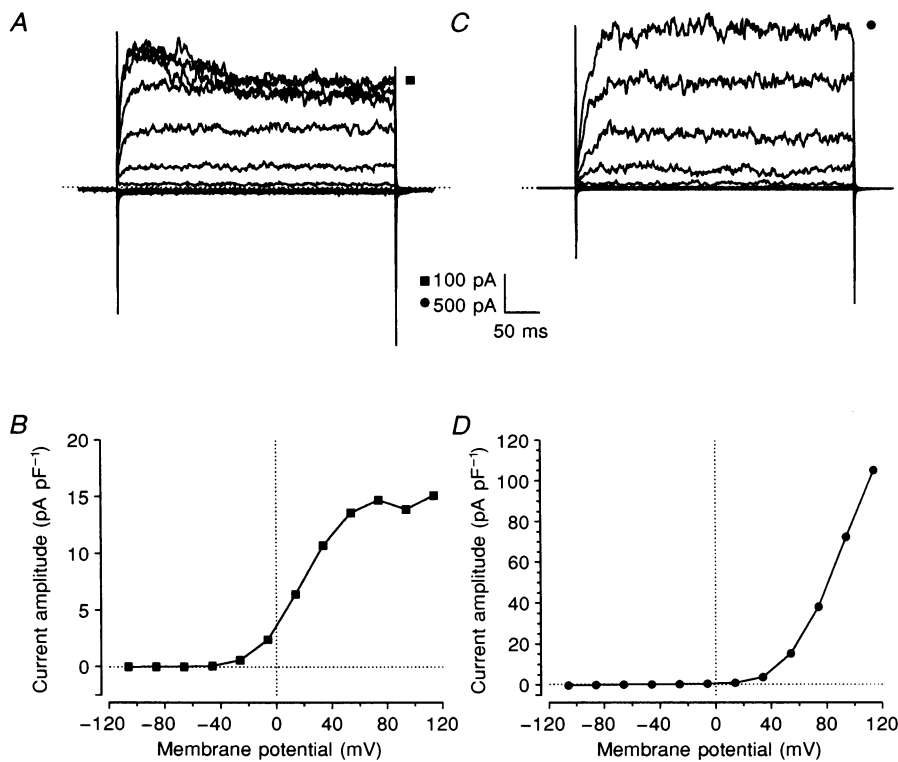


Figure 1. Membrane currents recorded in single rat Leydig cells dialysed with solutions at high (A and B) and low (C and D) pCa

A and C, current traces obtained in response to 500 ms voltage steps from -106 to +114 mV amplitude in 20 mV increments, applied from a holding potential of -46 mV, every 20 s. A, representative currents obtained in a cell (n = 7) dialysed with a pCa > 9 solution (solutions A/1). C, representative currents obtained in a cell (n = 7) dialysed with a pCa 6 solution (solutions A/2). The horizontal dotted lines indicate the zero current level. B and D, corresponding peak current-voltage (B) and steady-state current-voltage (D) relationships for the currents evoked in the cells in A and C, respectively. These currents are corrected for the leak currents.

minimize the outward currents through Cl^- and K^+ channels. In order to increase the currents flowing through the calcium channels, barium (100 mM) was substituted for the calcium ions. In this case, CaCl_2 was omitted and the NaCl concentration was decreased to maintain the ionic strength.

Before each experiment, the Petri dish was rinsed several times with the external solution. The test solutions were either added to the culture dishes for the times specified in the Results, or applied very rapidly by directing a streamline flow from the opening of a plastic capillary with an internal diameter of 250 μm positioned in the bath near the cell under investigation with a micromanipulator (Leitz, Germany). All experiments were performed at room temperature (20–23 °C).

Chemicals

Hepes, EGTA, Mg-ATP, Na-GTP, ionomycin (calcium salt) and tetraethylammonium hydroxide (TEA) were purchased from Sigma. Synthetic charybdotoxin (CTX) and iberiotoxin (IBTX) were purchased from Latoxan (France). These drugs were directly dissolved in the bath solutions, except for ionomycin, which was dissolved in DMSO (Merck, Germany) and was then diluted in the external solution to the chosen concentration. In control experiments, the maximal final amount of DMSO in the medium (1% v/v) did not affect the currents. Purified human chorionic gonadotrophin (hCG; 3000 i.u. mg^{-1} ; Pregnyl) was purchased from Organon (Séri-Fontaine, France).

RESULTS

Evidence for two different types of outwardly rectifying potassium conductances in rat Leydig cells

The outward currents were recorded in conditions where the chloride currents were strongly decreased by using glutamate as the major external and internal anion. The pipette solutions contained 10 mM EGTA, and the calcium concentrations were held at $\text{pCa} > 9$ (solutions A/1) and $\text{pCa} 6$ (solutions A/2).

Representative current traces are illustrated in Fig. 1. They were recorded in response to different voltage steps applied from a holding potential of -46 mV, close to the resting membrane potential of rat Leydig cells (Duchatelle & Joffre, 1990).

With the low-calcium solution, depolarizations induced voltage-dependent outward currents (Fig. 1A). The currents were slightly noisy and fully activated within 100 ms of the voltage pulse. They were sustained below $+20$ mV and generally transient above this potential. For the highest depolarizations, the currents activated faster, with a more pronounced transient pattern. The resulting peak current–voltage curve demonstrated a threshold of

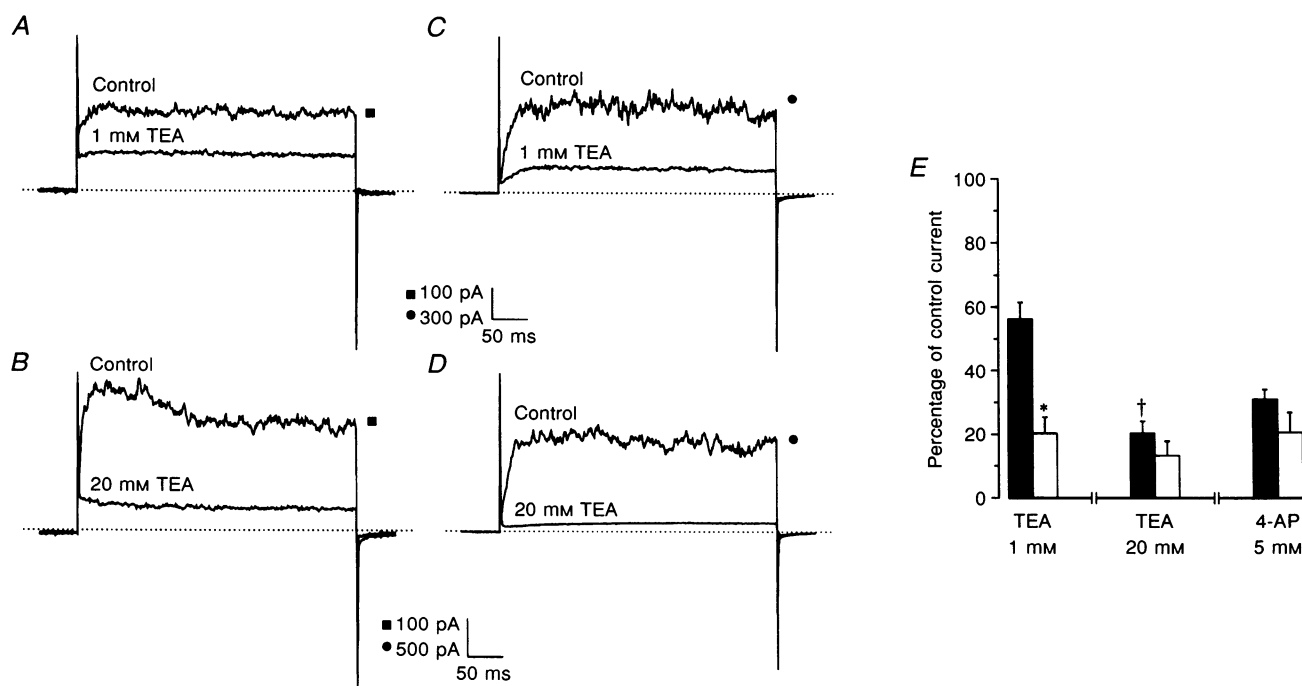


Figure 2. Effects of 1 and 20 mM tetraethylammonium on the outward currents recorded at high (A and B) and low (C and D) pCa

A–D, current traces evoked by depolarizing voltage pulses applied from a holding potential of -46 mV to $+74$ mV in 4 cells, before (Control) and in the presence of 1 mM (A and C) or 20 mM (B and D) TEA. A and B, the cells were dialysed with a $\text{pCa} > 9$ solution (solutions A/1). C and D, the cells were dialysed with a $\text{pCa} 6$ solution (solutions A/2). The horizontal dotted lines indicate the zero current level. E, comparison of the inhibiting effects of 1 mM TEA, 20 mM TEA and 5 mM 4-AP on the outward currents obtained at $+74$ mV at $\text{pCa} 6$ (□) and $\text{pCa} > 9$ (■). The inhibition is expressed as a percentage of the control current (means + s.e.m.) measured at $+74$ mV. $n = 3$ for all experiments except for the control of 1 mM TEA at $\text{pCa} 6$ ($n = 4$). * $P < 0.01$ vs. $\text{pCa} > 9$; † $P < 0.01$ vs. 1 mM in $\text{pCa} > 9$.

activation close to -50 mV, outwardly rectifying properties between -50 and $+50$ mV and a plateau of current for potentials above $+50$ mV (Fig. 1*B*). The characteristics of these currents were not modified by internal ATP, in the range from 0 to 3 mM, by internal pH between 6.8 and 7.7, and by 1 mM internal cAMP (not illustrated).

In about 10% of the cells dialysed with the low-calcium solution, the depolarizations above $+20$ mV induced sustained currents. Their amplitude, their noisy pattern, their current-voltage relationship, their sensitivity to TEA and 4-AP and their insensitivity to venom toxins (see below) suggests that these sustained currents resulted from the activation of the same conductance as the transient outward currents. This confirms our previous conclusions (Duchatelle & Joffre, 1990).

Figure 1*C* and *D* illustrates the second type of outward current recorded when the cells were dialysed with a pCa 6 pipette solution. In these cells, the holding currents were slightly outward and the hyperpolarizations elicited no

current. The depolarizations over $+14$ mV evoked outward currents of large amplitude. They were slowly activated, more noisy, and did not inactivate during the pulse. The associated steady-state current-voltage curve demonstrated strong outwardly rectifying properties extending above $+50$ mV, with a threshold of activation close to $+10$ mV.

Several experiments were performed to specify the ionic nature of these outward currents. TEA (1–20 mM), a widely effective potassium channel blocker, induced a strong voltage-independent inhibition of the outward currents recorded at pCa 6 (Fig. 2*C*, *D* and *E*). In contrast, the outward currents induced in the presence of pCa > 9 were less sensitive to 1 than to 20 mM TEA (Fig. 2*A*, *B* and *E*). Both currents were similarly inhibited by 5 mM 4-aminopyridine (4-AP) (Fig. 2*E*).

In several cell types, different calcium-dependent potassium conductances can be separated by their sensitivity to venom toxins (Garcia, Galvez, Garcia-Calvo, King, Vasquez & Kaczorowski, 1991). IBTX (100 nM) and CTX (20 nM) were

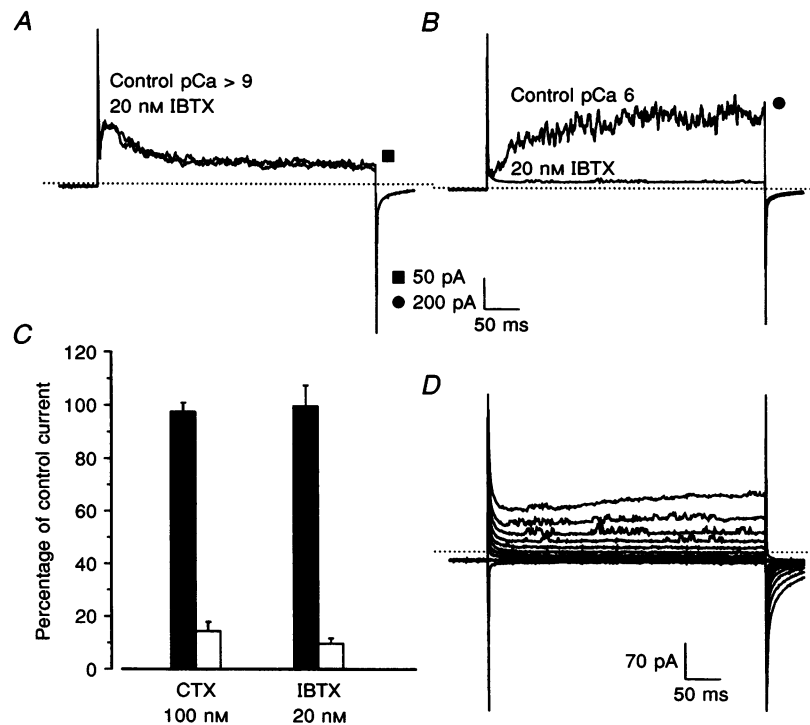


Figure 3. Effects of IBTX and CTX on the outward currents recorded at high and low pCa

A and *B*, current traces evoked by depolarizing voltage pulses applied from a holding potential of -46 mV to $+74$ mV in 2 cells, before (Control) and in the presence of 20 nM IBTX. *A*, the cell was dialysed with a pCa > 9 solution (solutions A/7); *B*, the cell was dialysed with a pCa 6 solution (solutions A/2). The horizontal dotted lines indicate the zero current level. *C*, comparison of the inhibiting effects of 100 nM CTX and 20 nM IBTX on the outward currents, in cells dialysed with a pCa > 9 solution (■; solutions A/7) and with a pCa 6 solution (□; solutions A/2). The inhibition is expressed as a percentage of the control current (means \pm s.e.m.) measured at $+74$ mV. $n = 3$ for all experiments except for IBTX at pCa 6 ($n = 4$). $P < 0.01$ for controls vs. CTX and IBTX at pCa 6. *D*, residual current traces elicited in the same cell as *B*, after superfusing the cell with 20 nM IBTX showing outward elementary events. The current traces were recorded in response to 500 ms voltage steps of -106 to $+114$ mV amplitude in 20 mV increments, applied from a holding potential of -46 mV, every 20 s.

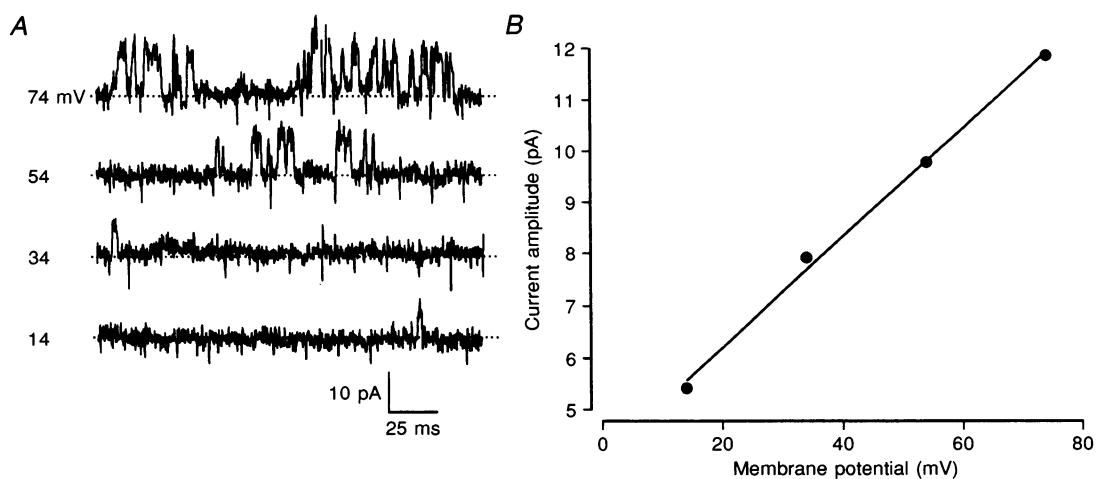


Figure 4. Single channel currents recorded in whole-cell configuration in a cell dialysed with a pCa 6 solution and superfused with 20 nM IBTX

A, single channel currents recorded at the membrane potentials shown to the left of each trace. The recordings were obtained from Fig. 3*D* after baseline subtraction. The currents were filtered at 1 kHz and digitized on-line at 4 kHz. *B*, current–voltage relationship derived from *A*. The current amplitudes have been estimated by eye. The linear regression line gives a unitary conductance of 106 pS.

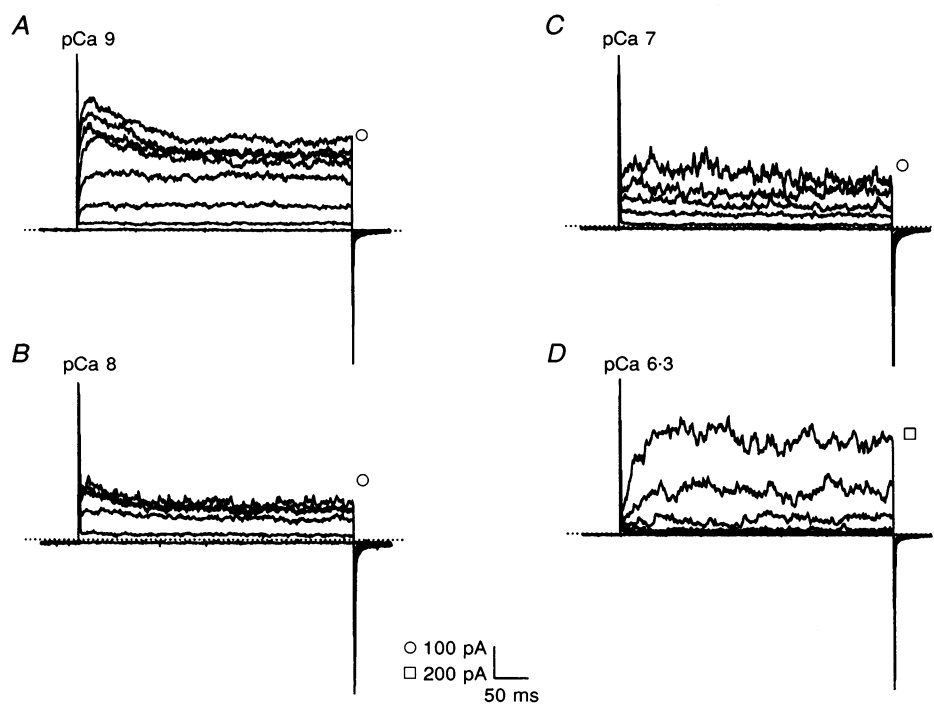


Figure 5. Membrane currents recorded in single rat Leydig cells dialysed with various pCa solutions

A–D, current traces obtained in response to 500 ms voltage steps from -106 to $+114$ mV amplitude in 20 mV increments, applied from a holding potential of -46 mV, every 20 s. They have been recorded in 4 different cells dialysed with solutions containing 10 mM EGTA and calcium sulphate yielding: pCa 9 (solutions A/6; *A*); pCa 8 (solutions A/5; *B*); pCa 7 (solutions A/4; *C*); and pCa 6.3 (solutions A/3; *D*). The horizontal dotted lines indicate the zero current level.

unable to inhibit the potassium currents elicited at $pCa > 9$ (Fig. 3A and C). In contrast, these toxins strongly inhibited the currents recorded at $pCa 6$ (Fig. 3B and C).

IBTX and CTX act on calcium-activated potassium channels by decreasing their open probability without modifying the elementary conductance (Candia, Garcia & Latorre, 1992). Sometimes, the residual whole-cell currents showed single channel events (Fig. 3D), from which we could determine the amplitude *vs.* membrane potential relationship (Fig. 4). These currents had the elementary conductance of the well-characterized depolarization-induced, large conductance, calcium-dependent potassium channel.

Calcium dependence of both types of the outwardly rectifying potassium conductances in rat Leydig cells

The calcium dependence of the currents was investigated either by clamping the calcium concentration to various

levels with different calcium-EGTA-buffered solutions (solutions 1-6, Table 1) or by dialysing the cells with a solution containing 0.5 mM EGTA and applying 10 mM external ionomycin.

In the range $< 10^{-9}$ to 10^{-8} M calcium, the outward currents induced by depolarizations showed little sensitivity to $[Ca^{2+}]_i$ and had similar properties (Fig. 5A and B). When the calcium concentration was increased from $< 10^{-9}$ to 10^{-8} M, the threshold of activation was shifted to negative potentials by about 5 mV. Simultaneously, the amplitude of the outward currents and the whole conductance decreased (Fig. 6A and C).

In the presence of 10^{-7} M calcium, the amplitude of outward currents was the same as that recorded with 10^{-8} M calcium for potentials below +80 mV. In contrast, for potentials above +80 mV, the inactivating phase disappeared, and the

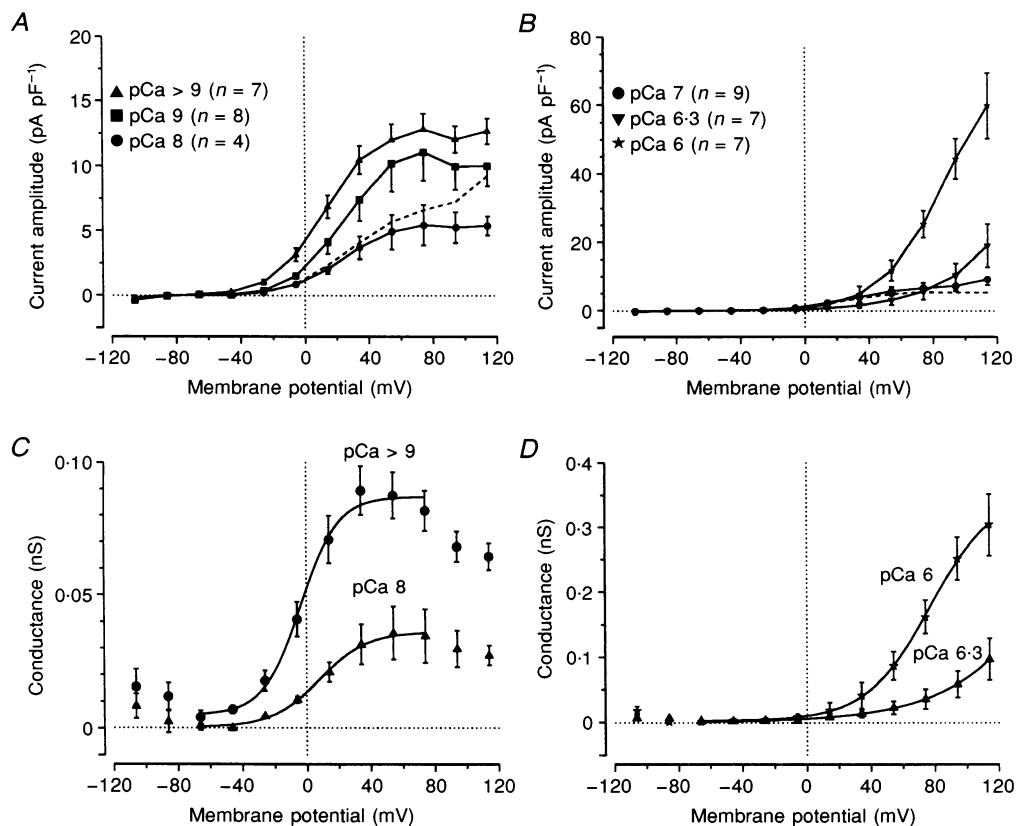


Figure 6. Voltage dependence of the potassium conductances recorded at different values of pCa . A and B, amplitude of the normalized ($pA pF^{-1}$) peak currents (A) and steady-state currents (B) plotted against membrane potentials. The values are expressed as means \pm s.e.m. for n cells. The dashed lines indicate the current-voltage curves for $pCa 7$ (A) and $pCa 8$ (B). All currents are corrected for leakage. C and D, conductance-voltage relationships in Leydig cells dialysed with high (C) and low pCa (D). The conductances are expressed as the current amplitude divided by the driving force according to $E_K = -83$ mV. The curves were obtained with a computer by fitting the values between -66 and $+74$ mV (C) or -66 and $+114$ mV (D) with the Boltzmann equation: $G = G_{max}/(1 + \exp((V - V_{50})/K))$, where G_{max} is the limiting maximum conductance, V_{50} the potential of half-maximal activation and K the slope factor. At $pCa > 9$, $G_{max} = 87$ pS, $V_{50} = -3.6$ mV and $K = 11.8$ mV; at $pCa 8$, $G_{max} = 36$ pS, $V_{50} = 7.9$ mV and $K = 15.2$ mV. Values at $pCa 6.3$ ($G_{max} = 1621$ pS, $V_{50} = 227.5$ mV and $K = 38.5$ mV) and at $pCa 6$ ($G_{max} = 355$ pS, $V_{50} = 77.2$ mV and $K = 20.0$ mV) were obtained by extrapolation.

amplitude of the outward currents and their noisy appearance increased (Figs 5C and 6B).

These changes were strengthened in the presence of 5×10^{-7} and 10^{-6} M calcium. The depolarizations evoked noisier currents of large amplitude, which did not inactivate (Fig. 5D). A rise in the calcium concentration speeded up the activation of the outward currents, increased their amplitude and shifted the threshold of activation to less positive potentials. The changes in the steady-state current–voltage curves and in the conductances confirmed the calcium dependence of the outwardly rectifying currents (Fig. 6B and D).

The dual effect of calcium on the two types of outwardly rectifying potassium conductances was confirmed in experiments in which ionomycin was added to the external medium. As shown in Fig. 7A, outward currents recorded in response to test pulses below +50 mV were decreased after exposing the cell to ionomycin. Above +50 mV, ionomycin elicited a current increase which was due to non-inactivating channels and which was associated with large current fluctuations. These results show that, when the calcium

concentration increases, inhibition of the calcium-inhibited potassium conductance and stimulation of the calcium-activated potassium conductance occur simultaneously. The resulting current–voltage curves confirm these observations (Fig. 7B).

Effects of hCG on the outwardly rectifying potassium conductances in rat Leydig cells

The effects of hCG on the ionic conductances have been previously investigated in Leydig cells which were bathed in a chloride-rich external solution and which were dialysed with a low-calcium solution lacking ATP and containing either 0.5 or 10 mM EGTA (Duchatelle & Joffre, 1990). In these experiments, hCG appeared mainly to increase a cyclic AMP-dependent chloride conductance. In the present experiment, the cells were bathed in chloride-free solutions and were dialysed with a solution containing 0.5 mM EGTA, allowing the cytosolic calcium to vary freely. The internal medium also contained 3 mM Mg-ATP and, in some experiments, 200 μ M Na-GTP. The external solution, in some cases, also contained a chloride channel blocker (4-acetamido-4'-isothiocyanatostilbene-2,2'-disulphonic acid SITS, 1 mM). The cells were then superfused by the

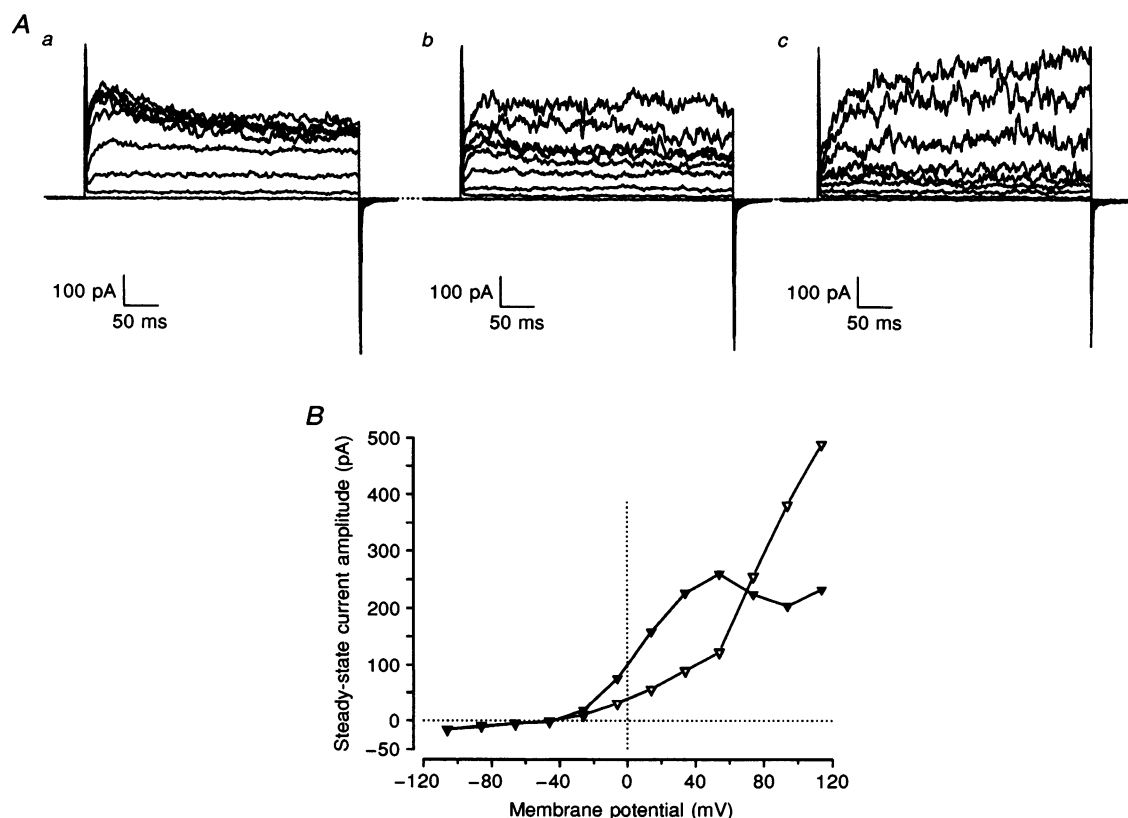


Figure 7. Effects of the application of $10 \mu\text{M}$ ionomycin on the outward currents obtained in a cell dialysed with a $p\text{Ca} > 9$ solution in the presence of 0.5 mM EGTA

A, current traces recorded in response to 500 ms voltage steps from -106 to $+114$ mV amplitude in 20 mV increments, applied from a holding potential of -46 mV, every 20 s (solutions A/8). They were obtained before (a) and after a first (b) and then a second (c) application of a 90 s pulse of ionomycin performed at 25 min intervals. B, current–voltage curves for the steady-state currents measured in the same cell as in A before the first application (a, ▼) and after the second application (c, ▽) of ionomycin. These currents are not corrected for leakage.

standard saline in the presence of 1, 10 or 50 i.u. ml^{-1} hCG (0.33, 3.3 or 16.5 $\mu\text{g ml}^{-1}$).

Despite the fact that glutamate was substituted for chloride ions in the pipette and the external solutions, hCG induced currents resembling those recorded in the presence of chloride ions, as previously described (Noulin & Joffre, 1993b) (Fig. 8A and B, $n = 15$). The currents were rapidly blocked by SITS ($n = 5$; not illustrated). This observation, the kinetics of the outward currents and the reversal potential of the currents close to the equilibrium potential for glutamate corroborate the finding that hCG activated the cyclic AMP-dependent chloride channels. This suggests that glutamate ions flow through this channel. In these experiments the residual currents were noisy and of low amplitudes. These findings were confirmed in additional experiments, in which the anionic conductance was blocked with 1 mM SITS before stimulating the cells with hCG ($n = 6$) (Fig. 9). In these cells, a clear blockade of outward currents was observed after 5–6 min exposure to hCG (Fig. 9). This inhibition was also obtained in the absence of SITS (Fig. 8C and D).

In total, an inhibition of the potassium conductance was observed in ten cells bathed in a medium in which the chloride conductance was strongly inhibited by SITS, which was applied either before or after exposing the cell to hCG ($n = 11$). In such conditions, the peak of outward current decreased from 10.0 ± 1.2 to 7.9 ± 0.9 pA pF^{-1} ($-18.9 \pm 2.6\%$, $P < 0.01$, paired data). For comparison, the peak of outward current decreased from 11.1 ± 2.2 pA pF^{-1} ($n = 8$) in the presence of 10^{-9} M calcium to 5.4 ± 1.6 pA pF^{-1} ($n = 4$) in 10^{-8} M calcium ($P < 0.01$, unpaired data). The inhibition induced by hCG was independent of the presence of GTP in the pipette solution and of the hCG concentration (range, 1–50 i.u. ml^{-1}), but was correlated with the amplitude of the peak current in the control conditions ($r = 0.7$; $P < 0.05$; $n = 10$). Comparing current–voltage curves obtained in the presence of SITS, in the control conditions and at the maximal effect of hCG, demonstrated that hCG has an inhibitory influence on the potassium conductance without shifting the threshold of activation. As a control for the specificity of the effects, we tested a heat-inactivated hCG. The inhibition was never observed under these conditions ($n = 7$).

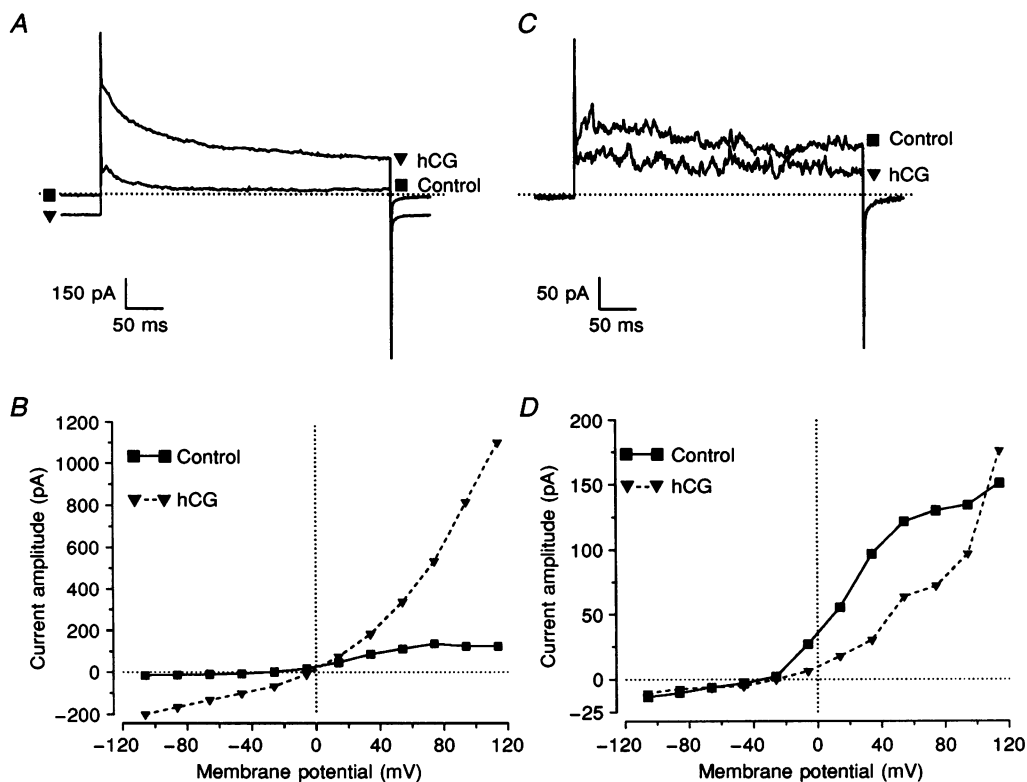


Figure 8. Effects of the application of 50 i.u. ml^{-1} hCG on the outward currents obtained in a cell dialysed with a pCa > 9 solution in the presence of 0.5 mM EGTA

A and C, current traces evoked by depolarizing voltage pulses applied from a holding potential of -46 mV to $+74$ mV in 2 cells, before (Control) and in the presence of 50 i.u. ml^{-1} hCG. The internal solution contained 3 mM ATP and no GTP (solutions A/8). A, representative example of activation of the cAMP-activated glutamate conductance observed in 5 of 7 cells; C, example of inhibition of the potassium conductance as observed in 2 of 7 cells. The horizontal dotted lines indicate the zero current level. B and D, current–voltage curves for the peak currents measured in the same cells as in A and C, respectively, before and after the application of 50 i.u. ml^{-1} hCG. These currents are not corrected for the leak currents because of the development of the hCG-dependent anionic conductance at the negative potentials.

These results also indicate that, in rat Leydig cells, the hormone hCG did not recruit enough calcium to activate the calcium-activated potassium conductance.

Lack of voltage-dependent calcium channels in the membrane of rat Leydig cells

More than seventy Leydig cells isolated from fifteen different rat testes were examined for voltage-dependent calcium channels. These experiments were performed on Leydig cells, either stimulated for 1–2 days with 1–10 i.u. ml⁻¹ of hCG dissolved in the culture medium or bathed in the control medium and then superfused with 1–50 i.u. ml⁻¹ of hCG. The patch-clamp recordings were obtained in situations where the external medium contained either 10 mM calcium salts or 100 mM barium salts. The pipette solutions also contained 3 mM Mg-ATP and, sometimes, 200 μM Na-GTP. The holding potential was -40 or -80 mV. No T-type or L-type calcium current was recorded.

DISCUSSION

Two classes of Ca²⁺-dependent K⁺ channels

This study shows that two types of Ca²⁺-dependent K⁺ channels with opposite sensitivities to [Ca²⁺]_i are present in the Leydig cells from rat testis. In cells dialysed with a low-calcium solution, we observed a delayed, inactivating outwardly rectifying potassium conductance with an activation threshold near -40 mV. TEA and 4-AP, two blockers of delayed-rectifying and transient potassium currents in excitable cells inhibited the whole-cell outward currents in Leydig cells. From this point of view, the Leydig

cell currents resemble those resulting from activation of conductances called K_n in T-lymphocytes and K_o in macrophages (Gallin, 1991; Schlichter, Papahill & Schumacher, 1993). However, in contrast to I_{Kn} and I_{Ko}, the currents in the Leydig cells are insensitive to charybdotoxin and do not entirely inactivate.

Like K_n and K_o (Bregestovski, Redkozubov & Alexeev, 1986; Grissmer & Cahalan, 1989; Schlichter *et al.* 1993), the outwardly rectifying potassium conductance of the Leydig cells is inhibited in a dose-dependent manner by internal calcium in the range of 10⁻¹⁰ to 10⁻⁷ M. The calcium decreases the peak amplitude of the voltage-dependent currents as well as the maximal conductance. For these reasons, the resting potassium conductance of Leydig cells also resembles the delayed outwardly rectifying potassium conductance of swine granulosa cells (Mattioli, Barboni & Seren, 1991).

For 5 × 10⁻⁷ and 10⁻⁶ M cytosolic calcium, noisy currents are elicited by depolarizations. In contrast to the resting potassium conductance, these currents are strongly inhibited by a low concentration of TEA, suggesting that they involve large conductance, calcium-activated potassium channels (Blatz & Magleby, 1984; Yellen, 1984). These currents are also specifically inhibited by charybdotoxin and iberiotoxin (Miller, Moczydlowski, Latorre & Phillips, 1985; Galvez *et al.* 1990). These calcium-activated K⁺ channels are widely represented in a variety of exocrine and endocrine cells, including the Leydig cells from mouse testis (Kawa, 1987; Carnio & Varanda, 1995).

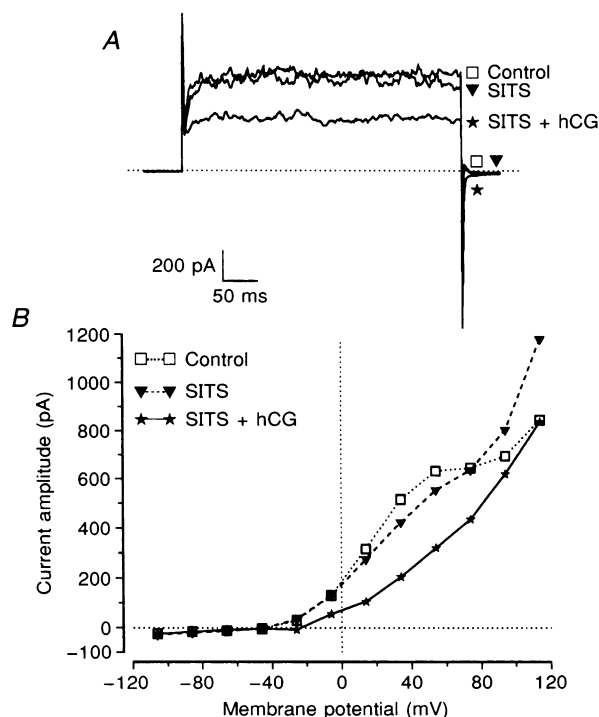


Figure 9. Effects of the application of 10 i.u. ml⁻¹ hCG in the presence of 1 mM SITS on the outward currents obtained in a cell dialysed with a pCa > 9 solution in the presence of 0.5 mM EGTA

A, current traces evoked by depolarizing voltage pulses applied from a holding potential of -46 mV to +74 mV, before (□) and in the presence of 1 mM SITS (▼), and in the presence of 1 mM SITS with 10 i.u. ml⁻¹ hCG (★). The internal solution contained 3 mM ATP and 0.2 mM GTP (solutions A/8). The horizontal dotted line indicates the zero current level. B, current-voltage curves for the currents measured in the same cell and same conditions as in A. These currents are not corrected for the leak currents because of the development of the hCG-dependent anionic conductance at the negative potentials.

Physiological relevance

The present findings show that an outwardly rectifying potassium conductance is activated at potentials close to the resting membrane potential of the Leydig cells, provided that there is a low cytosolic calcium concentration. The resting calcium concentration was estimated to be 5×10^{-8} to 2×10^{-7} M in rat Leydig cells (Sullivan & Cooke, 1986; Kumar *et al.* 1994; Tomic *et al.* 1995). Confrontation of these numbers with the Ca^{2+} sensitivity observed with internal Ca^{2+} buffers would indicate that this conductance may be largely inhibited at rest. This, and the fact that the hyperpolarization-activated chloride conductance is not activated at rest, would explain the low resting membrane potential of the cells, measured either with the intracellular microelectrode technique (Joffre *et al.* 1984a) or with the 'zero current' mode of the patch-clamp technique (Duchatelle & Joffre, 1990); this would also explain the weak dependence of the resting membrane potential on external potassium (Joffre, Mollard, Régondaud & Gargouil, 1984b). However, the possibility should be kept in mind that, as in human T-lymphocytes (Schlichter *et al.* 1993), the calcium-dependent inhibition of the resting potassium conductance could be modulated by the cytosol. In rat Leydig cells, we can exclude cAMP, ATP and H^+ from such a role. However, the fact that ionomycin clearly reduced the inactivating K^+ current in cells dialysed with a weak Ca^{2+} buffer indicates that the channels are not blocked under resting conditions.

This study also demonstrates a calcium-activated potassium conductance in the membrane of rat Leydig cells, as previously observed in mouse Leydig cells (Kawa, 1987; Carnio & Varanda, 1995). Since the calcium-activated potassium currents, in the presence of 10^{-7} M calcium, are elicited for potentials above +80 mV, we conclude that, in rat Leydig cells, this conductance is not involved in the control of resting membrane potential.

The addition of hCG, under conditions in which the cAMP-dependent hyperpolarization-activated chloride conductance is strongly inhibited, produces a progressive reduction in the amplitude of the outward potassium currents without shifting the membrane potential at which the outward currents are activated. With a dose of 1–50 i.u. ml^{-1} (3.3–15.6 $\mu\text{g ml}^{-1}$), a maximum inhibition of about 20% takes place in 5–6 min.

LH inhibits a delayed rectifier potassium current in swine granulosa cells (Mattioli *et al.* 1991). As in the rat Leydig cells, the level of maximum current inhibition in the granulosa cells is independent of the LH concentration between 1 and 50 $\mu\text{g ml}^{-1}$. This inhibitory effect of LH depends on the intracellular calcium. It is likely that, in rat Leydig cells, the inhibiting effect of hCG is also mediated by a moderate increase in the intracellular calcium concentration.

The present results indicate that the calcium-activated potassium conductance of rat Leydig cells is not activated upon stimulation by LH/hCG. It is possible to argue that

cell dialysis during whole-cell recording limits the response to hCG. On the other hand, the finding that hCG induces a membrane depolarization in undialysed whole cells recorded with the microelectrode technique (Joffre *et al.* 1984b) is in agreement with blockade of a potassium conductance.

The present results suggest that the regulation of K^+ conductances by Ca^{2+} is dramatically different in rat Leydig cells and in mouse Leydig cells. In the mouse, the calcium-activated potassium conductance in Leydig cells is activated upon stimulation by LH/hCG (Carnio & Varanda, 1995), whereas no such effect was found here. A clue to this difference may be given by the present finding that voltage-dependent Ca^{2+} channels are lacking in rat Leydig cells, whereas they are present in the mouse (Kawa, 1987). It is tempting to propose that in mouse, the activation of large conductance Ca^{2+} -dependent K^+ channels is secondary to the activation of voltage-dependent Ca^{2+} channels, and that this pathway is absent in the rat.

Like several adenylate cyclase-coupled receptors in many cell types, the LH/hCG receptor is also capable of activating the inositol phosphate–calcium signalling pathway, through a guanyl nucleotide regulatory protein (Segaloff & Ascoli, 1993), and these gonadotrophins stimulate the phospholipid turnover of rat Leydig cells (Lowit, Farese, Sabir & Root, 1982). However, this response has not been consistently observed (Ascoli, Pignataro & Segaloff, 1989). As noted in the Introduction, conflicting reports have appeared concerning the effects of LH/hCG on $[\text{Ca}^{2+}]_i$ in rat Leydig cells. Whereas no effect was found in single cells (Tomic *et al.* 1995), LH/hCG was reported to increase $[\text{Ca}^{2+}]_i$ in cell suspensions (Sullivan & Cooke, 1986) and in cells plated on coverslips (Kumar *et al.* 1994). It is now well established that the rat Leydig cell populations isolated by elutriation and Percoll gradient centrifugation are heterogeneous (Bhalla, Rajan, Burgett & Sohal, 1987; Tomic *et al.* 1995), and that there are numerous paracrine and autocrine relationships between the cells (Saez, 1994). Also, these findings are interpreted as corresponding to the secretion by LH/hCG of autocrine and paracrine factors, which, in turn, should act on the Leydig cells by specific receptors coupled to the inositol phosphate–calcium signalling pathway and inducing an increase in the intracellular calcium. For example, it is established that GnRH (gonadotrophin-releasing hormone) and endothelin-1 induce rapid and transient elevations of the cytosolic calcium in some rat Leydig cells (Tomic *et al.* 1995). Such considerations might also explain the fact that Bay K 8644 induces an increase in the cytosolic calcium of plated Leydig cells (Kumar *et al.* 1994), but does not do so in single Leydig cells (Tomic *et al.* 1995).

Thus, the present study supports the view that, in rat Leydig cells, the LH/hCG stimulation involves an early blockade of the resting potassium conductance. Since this conductance is insensitive to ATP, pH and cAMP, there is strong evidence that this blockade involves a $[\text{Ca}^{2+}]_i$ rise, which presumably results from some weak activation of the

inositol phosphate–calcium signalling pathway. This occurs with an increase in the gonadotrophin and cAMP-regulated chloride current that is voltage but not calcium dependent.

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Acknowledgements

This work was supported by grants from Centre National de Recherche Scientifique and Direction de Recherche et Etudes Doctorales and by a research fellowship to J.-F. Desaphy from the Ministère Français de la Recherche et de l'Enseignement Supérieur.

Received 5 December 1995; accepted 30 April 1996.