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The Resting Potential of Mouse Leydig Cells: Role of an Electrogenic Na^+/K^+ Pump

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Abstract. Resting potentials (V_m) were measured in mouse Leydig cells, using the whole-cell patch-clamp technique. In contrast to conventional microelectrode measurements, where a biphasic potential was observed, we recorded a stable V_m around -32.2 ± 1.2 mV (mean ± SEM, $n = 159$), at 25°C, and an input resistance larger than 2.7×10^9 Ω. Although V_m is sensitive to changes in the extracellular concentrations of potassium and chloride, the relationship between V_m and these ions' concentrations cannot be described by either the Goldman-Hodgkin-Katz or the Nernst equation. Perifusing cells with potassium-free solution or 10^{-3} M ouabain induced a marked depolarization averaging 20.1 ± 3.2 mV ($n = 9$) and 23.1 ± 2.8 mV, ($n = 7$), respectively. Removal of potassium or addition of ouabain with the cell voltage-clamped at its V_m, resulted in an inwardly directed current, due to inhibition of the Na^+K^+ATPase. The pump current increased with temperature with a Q_{10} coefficient of 2.3 and had an average value of -6.5 ± 0.4 pA ($n = 21$) at 25°C. V_m also varied strongly with temperature, reaching values as low as -9.2 ± 1.2 mV ($n = 22$) at 15°C. Taking the pump current at 25°C and a minimum estimate for the membrane input resistance, we can see that the Na^+K^+ATPase could directly contribute with 17.7 mV to the V_m of Leydig cells, which is a major fraction of the -32.2 ± 1.2 mV ($n = 159$) observed.

Key words: Leydig cell — Resting potential — Patch clamp — Na^+K^+ pump — Q_{10}

Introduction

Leydig cells are distributed in the interstitium between the seminiferous tubules and are responsible for most of the testosterone production in mammals. The secretion process is under control of the luteinizing hormone (LH) and involves the activation of several second messenger molecules, like cAMP, GDP and calcium (Mendelson, Dufau & Catt, 1975; Baranão & Dufau, 1983; Dufau et al., 1984). Like in other endocrine cells, stimulation is accompanied by changes in the functioning of ion channels and membrane potential (Joffre et al., 1984b; Duchatelle & Joffre, 1987; Carnio & Varanda, 1995). In most cells, the membrane resting potential is generated mainly by an electrodiffusion of potassium ions through channels open at rest. The Na-K ATPase, although electrogenic, is considered to participate essentially in the maintenance of the ion gradients and to have only a minor direct contribution to the total membrane potential difference in excitable cells. The first determinations of the resting potential of rat Leydig cells were made by Joffre, Mollard, Régondaud, Alix, Poindessau, Malassiné and Gargouil (Joffre et al., 1984a, b). They reported a biphasic potential with a hyperpolarized phase ($MP_1 = -37.6$ mV) following impalement of the cell and a late stable potential at around -25.1 mV (MP_2). From their results they concluded that MP_2 is the actual resting potential, dependent on the extracellular K^+ concentration and chiefly determined by a Ca^{2+}-activated K^+ conductance and slightly by the activity of the Na/K pump. Kawa (1987), using the whole-cell configuration of the patch-clamp technique, reported a resting potential of value around -27 mV for mouse Leydig cells. Duchatelle and Joffre (1987, 1990), using the same technique in rat cells, found an average of -30.7 and -35 mV respectively, but made no experiments to investigate the mechanisms responsible for the genesis of this resting potential. The effects of Ca^{2+} and human chorionic gonadotropin (hCG) on K^+ currents where also analyzed by Desaphy, Rogier & Joffre (1996), leading to the conclusion that a Ca^{2+}-activated K^+ conductance may

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be largely inhibited at rest, which would explain the weak dependence of the resting membrane potential on the external K^+ concentration, observed by Joffre et al. (1984b). They also associated the low resting membrane potential of rat Leydig cells with the fact that a hyperpolarization-activated Cl^- current is mostly inhibited at rest.

In this paper, we describe experiments showing that the sodium pump may have a major contribution to the resting potential of Leydig cells freshly isolated from mouse testis. Using the whole-cell patch-clamp technique, we recorded a stable membrane potential of -32.2 ± 1.2 mV ($n = 159$; 25°C; $[Na^+]_{pipette} = 20$ mM), which is strongly dependent on temperature, with a Q_{10} of 1.87. In fact, the resting potential can reach values as low as -9 mV at 15°C ($[Na^+]_{pipette} = 20$ mM). Varying the sodium concentration inside the pipette strongly affects the membrane potential. Besides this, the cells undergo a marked depolarization when exposed to a K^+-free solution or when treated with 10^{-3} M ouabain. These same treatments were used to measure the current carried by the sodium pump and to disclose its relationship to the generation of the resting potential. The pump current is highly influenced by the temperature, with a Q_{10} of 2.3 ($[Na^+]_{pipette} = 20$ mM). From our results we conclude that the electrogenic pump has a major role in determining the resting potential of mouse Leydig cells.

Materials and Methods

Cells

Cells were isolated from the testes of mice (70–80 days old) weighing 35–40 g, according to previously described methods (Kawa, 1987; Carnio and Varanda, 1995). Briefly, a mouse was killed by cervical dislocation, the testes were rapidly removed, decapsulated and placed in Hank's Balanced Salt Solution (HBSS). Leydig cells were then mechanically isolated by gently infusing solution into the testicular mass and the cells that entered into suspension were plated onto small pieces of glass slides (4 × 4 mm). After adherence to the glass, the cells were taken to a small lucite chamber (volume = 300 μl), mounted on the stage of an inverted phase contrast microscope (Nikon, TMD), for the electrophysiological experiments. The chamber was continuously perfused at a rate of 1 ml/minute and non-adherent cells and debris were washed away. Leydig cells are easily recognized by their size (16–18 μm in diameter), round shape and the presence of a bright halo under phase contrast microscopy, and were used for up to 3 hours after isolation. Experiments were carried out at room temperature (25 ± 1°C), or at the indicated temperature where noted. Temperature control was done with an automatic controller (TC 324B, Warner Instruments Corp.).

Electrophysiology

Conventional Microelectrodes

Ling-Gerhard type microelectrodes were pulled from borosilicate glass capillaries (Clark Electromedical, GC150F-15) in a David Kopf vertical puller and had resistances between 50–80 MΩ when filled with 1 M KCl. Potential differences were measured with a WPI (Duo 773) electrometer connected to the preparation via a 3 M KCl /agar bridge and Ag/AgCl electrodes. The microelectrode and cell membrane resistance were measured by applying hyperpolarizing current pulses (0.125 nA, 250 msec) every 2 seconds. The electrical potential differences were fed to an oscilloscope (Tektronix 5113) and recorded on paper (Narko MK-IV).

Patch Clamp

The whole-cell configuration of the patch-clamp technique (Hamill et al., 1981) was used both to measure membrane potential and pump current. A patch-clamp amplifier (Axopatch 200B, Axon Instruments) was connected to the preparation via salt bridges (2.5% agar in the pipette solution) and Ag/AgCl electrodes and set in current-clamp or voltage-clamp mode, depending on the experiment. Access resistance was in the range of 10–12 MΩ and electronically compensated up to 75%. Both membrane voltage and current were recorded in all experiments. When measuring membrane voltage, the total current passing through the membrane was kept at zero pA. Deviations were corrected by applying a holding current when necessary. When measuring current, the cell was clamped at its resting potential. The pump current (I_p) was measured as that part of the total current through the cell membrane that could be blocked by ouabain or by the absence of K^+ in the bathing solution. Micropipettes were pulled from the glass capillaries described above in a Narishige puller (model PP-83) and had resistances in the range of 4–8 MΩ. The signals coming from the amplifier were displayed on an oscilloscope screen (Tektronix 5113), low-pass-filtered at 50 Hz (8-pole Bessel filter; Frequency Devices) and sent to a computer through a TL-1 Labmaster (Axon Instruments) acquisition board. Data were sampled at 200 Hz and stored on a hard disc for later analysis, using the software Axotape 2.0 (Axon Instruments). Since the agar bridge and the pipette solution had the same electrolytes, errors due to junction potentials were minimized and offset at the start of the experiment with the pipette in the bath solution. In free-flow experiments, with the pipette in the solution, changing the bath from normal Hank's solution (145 mM NaCl) to one with 145 mM KCl (K^+ for Na^+ substitution) the offset potential deviates by no more than 3 mV. Therefore, no further corrections were applied to the data.

Solutions

Cells were maintained most of the time in standard Hank's Balanced Salt Solution (HBSS) with the following composition (mM): NaCl, 145; KCl, 4.6; $CaCl_2$, 1.6; $MgCl_2$, 1.1; Glucose, 10.0; HEPES 10.0; $NaHCO_3$, 5.0; pH adjusted to 7.4 with 1 N NaOH. Osmolality was around 300 mOsm/kg.H_2O. Where pertinent, the concentration of K^+ was changed by equimolar substitution with Na^+ and Cl^- by Aspartate. Nominally potassium-free solution was made without KCl.

The pipette solution was (mM): potassium aspartate, 130.0; NaCl, 10.0; Na_2ATP, 5.0; $MgCl_2$, 1.0; HEPES, 5.0; EGTA, 0.77; $CaCl_2$, 0.07 (free calcium concentration 10^{-8} M), pH 7.2 adjusted with KOH. In experiments where the intracellular Na^+ was set to 10 mM, NaCl was omitted from this solution; and when set to 0 mM, Na_2ATP was also substituted by MgATP. Osmolality was around 310 mOsm/kg.H_2O.

The bathing solutions were exchanged by gravity superfusion of the whole chamber with the new solution and/or drug.

Solutions were prepared with double-distilled water and filtered through 0.22-μm filters (Millipore GSWP 02500) prior to use. Ouabain, Verapamil, Nifedipine and all salts were reagent grade from Sigma.

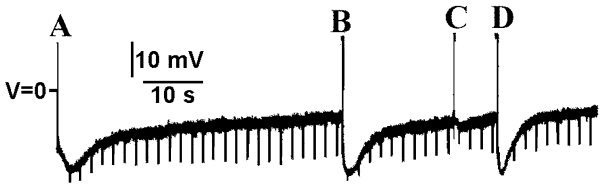

Fig. 1. Membrane potential of Leydig cell measured with a conventional microelectrode. (*A*) Time of impalement of the cell with the microelectrode. The time course of the potential is the same as seen by others: after an initial drop to around −16 mV (in this case), the cell transiently hyperpolarizes concomitantly with a decrease in the membrane resistance, and then depolarizes to a steady level. Hyperpolarizations can now be elicited by simply imposing new vibrations to the microelecrode. (*B* and *D*) Hyperpolarizations induced by long-duration pulses (around 250 msec). (*C*) Hyperpolarizarion induced by a short vibration (50 msec). Downward deflections are voltage responses to 0.125 nA current pulses (250 msec duration) applied every 2 seconds.

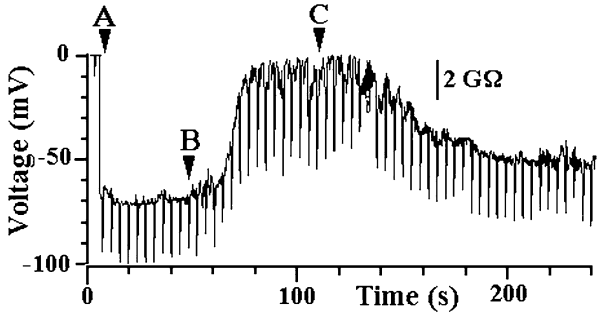

Fig. 2. Effect of ouabain on the resting potential of mouse Leydig cells. After the establishment of the whole-cell condition, the cell was clamped at 0 mV. At *A*, the amplifier was changed to the current-clamp mode and a membrane potential (−65 mV in this case), stable with time, was recorded. At *B*, the chamber was perfused with Hank's plus 10^{-3} M ouabain, and in *C*, the ouabain was washed out, leading to a recovery of the resting potential. Downward deflections are voltage responses to current pulses of 10 pA (250-msec duration) applied every 4 seconds.

Results

Conventional-Microelectrode Results

Penetration of Leydig cells with conventional microelectrodes always results in the recording of a biphasic membrane potential, with characteristics shown in Fig. 1. This result is very similar to what has been described by Joffre et al. (1984a). In our view, and in agreement with Joffre et al. (1984b), this biphasic time course is essentially an artifact due to the penetration of the cell by the microelectrode. To prove our point, a cell was impaled (*A* in Fig. 1) and then "tickle"[1] pulses were applied at different times (marked *B*, *C* and *D* in Fig. 1). *C* is a short-duration pulse (50 msec), while *B* and *D* are longer ones (250 msec). As can be seen, the cell hyperpolarizes after each pulse, and then the potential declines to a new steady level. This kind of response is dependent on the presence of external calcium and is not blocked either by verapamil or nifedipine (*data not shown*). Therefore, the hyperpolarization phenomenon can be explained by assuming an influx of calcium through a leakage pathway created by the electrode and activation of Ca^{2+}-dependent K^+ channels, known to be present in this type of cell (Kawa, 1987; Carnio & Varanda, 1995). Under these circumstances, it seems to us that the actual resting potential of Leydig cells could have been underestimated and that mechanisms other than diffusion potentials of ions could contribute to its final value.

[1]Tickle refers to a feature of the WPI Duo-773 electrometer, which permits the application of an audible frequency to the tip of the microelectrode, probably causing its vibration. It is used as an aid in pushing the microelectrode through the cell membrane.

Ouabain and K-free Solutions Strongly Depolarize the Cell

Figure 2 shows a similar type of measurement, but using a patch pipette sealed to the cell membrane. After the establishment of a GΩ seal (>10 GΩ), suction was applied to the interior of the pipette and the membrane ruptured, gaining access to the cytoplasm. Using this technique the input resistance of the cells was 2.7 ± 0.1 GΩ (n = 138), much larger than that measured with conventional microelectrodes (80 MΩ). The average capacitance of the cells was 19.3 ± 0.2 pF, which corresponds to a membrane area of approximately 1930 μm^2. Both parameters were measured by applying a 10-mV, 200 msec, hyperpolarizing voltage pulse, from a holding potential of −80 mV. The capacitance was calculated by integrating the current response.

A few seconds after breaking into the cell, the amplifier was turned to the current-clamp mode (*A* in Fig. 2) and a stable potential difference of −65 mV could be recorded for minutes. At *B*, 10^{-3} M ouabain was introduced into the solution perifusing the cell and a depolarization of 50 mV is seen, displacing the resting potential to near zero mV. Note that we are showing this particular experiment exactly because the cell resting potential is higher than the average and even so, the depolarization induced by ouabain has a magnitude larger than 2/3 of the recorded control level. Washing out the drug (*C*) reverses the effect, although in some cells, the recovery is not always complete. The average resting potential was equal to −32.2 ± 1.2 mV (n = 159), and the average depolarization induced by ouabain was 21.7 ± 4.0 mV (n = 7) and 14.3 ± 2.7 mV (n = 14) for the concentrations of 10^{-3} and 10^{-4} M, respectively. A similar depolarization is seen when the cell is peri-

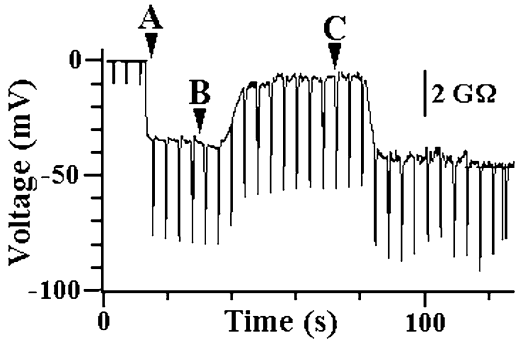

Fig. 3. Effect of K-free solutions on the resting potential of a Leydig cell. *A* marks the establishment of the whole-cell condition under current clamp. This cell had a resting potential difference equal to −35 mV, stable in time. In *B*, the solution superfusing the cell was made K^+-free and the cell depolarized to almost zero. *C* marks the return to control conditions, i.e., the potassium concentration was again 4.6 mM, and the potential returned to its initial value. Downward deflections are voltage responses to current pulses of 10 pA (250 msec duration) applied every 4 seconds.

fused with a solution nominally free of K^+ ions (*B* in Fig. 3), which is completely reversed upon reintroduction of normal Hank's solution to the perfusion chamber (*C* in Fig. 3).

The dependence of the resting potential on the extracellular K^+ and Cl^- concentrations is shown in Fig. 4*A* and *B*, respectively. The membrane potential varies around 15 mV per decade change in K^+ concentration, very far from what is expected for a membrane predominantly permeable to potassium. Chloride ions also affect the resting potential, but to an insignificant degree. In going from $[Cl]_o$ = 5 mM to $[Cl]_o$ = 150 mM, the resting potential changes by only about 2 mV.

The results shown in Figs. 2, 3 and 4 suggest that the resting membrane potential of Leydig cells cannot be explained simply in terms of an electrodiffusive movement of either potassium or chloride ions. Instead, the results in Figs. 2 and 3 are indicative that an electrogenic Na^+/K^+ pump must be directly contributing to this potential. To further establish this point, we measured the current carried by the sodium pump (I_p). The experimental design was the following: after the establishment of the whole-cell clamp condition, the amplifier was set to current-clamp mode for a brief period of time to measure the resting membrane potential. Then, the cell was clamped to this potential and the total current flowing through the membrane (I_m) was, as expected, null.

Figure 5 shows a typical record of I_m versus time. After the current reached stability, ouabain (10^{-3} M final concentration) was added to the chamber (*A*). Since we are blocking an outward current, I_m goes to a negative value around −7 pA in this particular case. Washing out the ouabain (*B*) reverses the effect with a time course of several seconds. Similarly, removal of K^+ from the bathing solution (*C* in Fig. 5) blocks the sodium pump and I_m reversibly increases to approximately −8 pA. Both ouabain (10^{-3} M) and potassium-free solutions produce an effect of the same magnitude, i.e., the maximum pump current in this particular cell should be around −8 pA.

Figure 6*A* is a dose-response curve for ouabain in the range of 5×10^{-6} M and 10^{-3} M. The drug has a maximum effect on the pump current at a concentration around 5×10^{-4} M. Assuming that the binding of ouabain to the sodium pump can be described by a first-order process, we can normalize the pump current measured at a given concentration of ouabain (I_D) in relation to the maximum pump current (I_p) through the Hill equation:

$$I_D = \frac{I_p \cdot D^n}{(K_D + D^n)} \quad (1)$$

where D is the concentration of ouabain; n is the Hill coefficient and K_D is the half-saturation concentration. Equation 1 can be linearized by plotting $[I_D/D]$ against I_D, as shown in Fig. 6*B*. The straight line represents the result of a linear regression made on the experimental points, when K_D is equal to 2.4×10^{-5} M, $n = 1$ and the total pump current I_p = 5.9 pA.

Membrane Potential Varies with the Sodium Concentration inside the Pipette

Changing the sodium concentration inside the pipette, and as a consequence inside the cell, greatly influences the resting membrane potential. Table 1 shows the average values of V_m for 4 sodium concentrations (nominally zero, 10, 20 and 50 mM), at 25°C.

Effect of Temperature on the Resting Potential and Pump Current

Both the pump current and resting membrane potential are strongly dependent on the temperature. Figure 7*A* shows an experiment where the membrane potential was measured while the temperature was changed from 15°C up to 35°C. In this case, the intracellular sodium concentration was 10 mM. As clearly seen, V_m is around −10 mV at 15°C and rises to −36 mV as the temperature goes to 35°C. In a few cells, the resting potential was almost zero mV at 15°C and also increased to around −30 mV when the temperature was set to 35°C. In those cases, it is tempting to suggest, that the resting membrane potential was set essentially by the pump activity. Figure 7*B* shows the Arrhenius plot for membrane potential, resulting in a Q_{10} of 1.87.

Figure 8*A* shows the Arrhenius plots for the pump current. The pump current has a Q_{10} of 1.92 when sodium in the pipette is 10 mM and 2.26 when sodium is 20 mM.

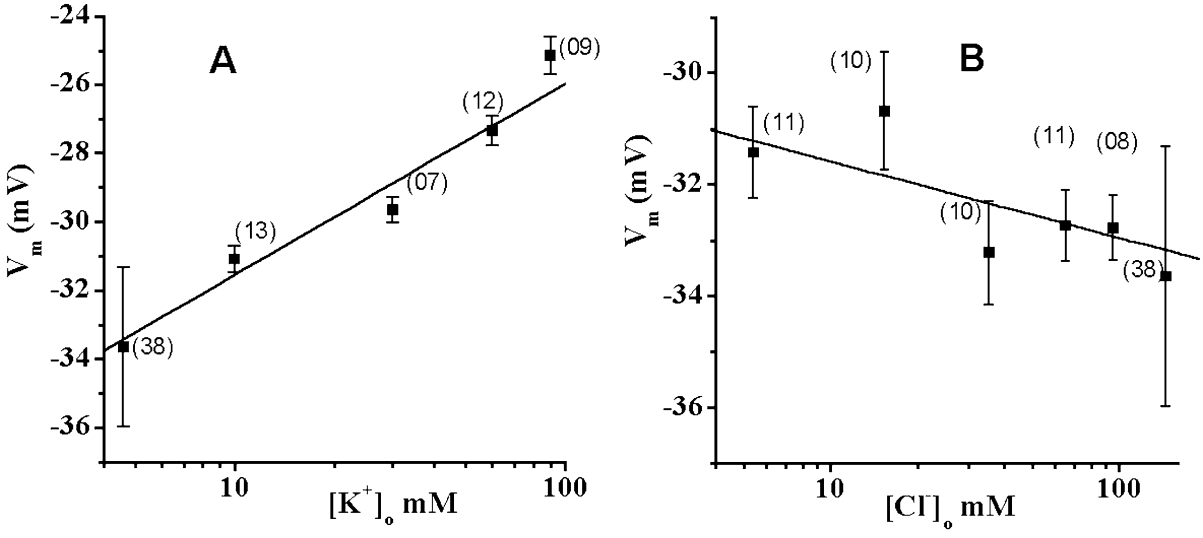

Fig. 4. Resting membrane potential dependence on the external concentrations of K^+ (*A*) and Cl^- (*B*) ions. Values are mean ± SEM of the number of cells indicated in parenthesis. All measurements were done at 25°C. Lines are best linear fits to the data points ($R = 0.96$ for (*A*) and 0.78 for (*B*)).

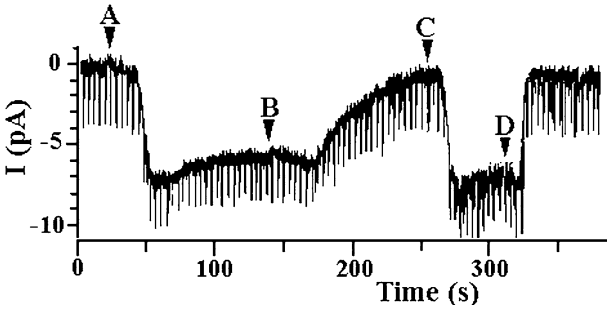

Fig. 5. Typical trace of pump current in Leydig cell. After establishment of the whole-cell condition, the membrane potential was measured and then the cell was clamped at its own resting potential (holding potential = −34 mV). At *A*, the cell bath was changed to Hank's plus 10^{-3} M ouabain. An inward current developed. After washing out the ouabain at *B*, the current returned to zero. At *C*, potassium was removed from the bathing solution and, again, an inward current developed. The effect was reversed upon reintroduction of potassium into the medium at *D*. Downward deflections are current responses to pulses of −10 mV, duration of 250 msec, applied every 4 seconds.

Discussion

In several types of secreting cells, the action of secretagogues or second messengers has been studied in relation to changes in cell membrane potential (Petersen, 1992). Nevertheless, the mechanism that generates and maintains the resting potential are peculiar to each cell type. As a general rule, excitable cells have a resting potential around −90 mV, primarily determined by an electrodiffusion of K^+ ions (Hodgkin & Katz, 1949; Hodgkin & Horowicz, 1959). The vomeronasal chemoreceptor neuron of the frog is clearly an exception, where an electrogenic pump contributes with a large fraction to the resting membrane potential (Trotier & Døving, 1996), as is also the case for T-lymphocytes of mice (Ischida & Chused, 1993); human neutrophils (Bashford & Pasternak, 1985) and rat mast cells (Bronner et al., 1989).

As first described by Joffre et al. (1984a, b), in rat Leydig cells, the measurement of the resting potential with conventional microelectrodes gives ambiguous results, most probably due to artifacts introduced by the impalement. This hypothesis is supported by the results shown in Figs. 1 and 4. Vibrating the microelectrode produces transients that have the same time course and magnitude as the transient produced by penetration of the microelectrode per se. In fact, the magnitude of the hyperpolarization can be increased by increasing the time of vibration imposed to the microelectrode. This phenomenon is dependent on the presence of calcium in the extracellular solution and is not abolished by blockers of calcium channels, such as verapamil and nifedipine. Besides this, the relationship between the resting potential and the extracellular K^+ or Cl^- concentrations (Fig. 4) cannot be described by either the Nernst or the Goldman-Hodgkin-Katz equation, as also shown in Joffre et al. (1984b). These data not only suggest that Leydig cells are permeable to K^+ and Cl^- ions, as demonstrated before, but strongly points to the involvement of other mechanisms, besides electrodiffusion, in the generation and maintenance of the resting potential.

Although these cells do have Ca^{2+}-activated potassium channels in their membrane, these channels have a very low opening frequency when the cell is not stimulated (Kawa, 1987; Carnio & Varanda,

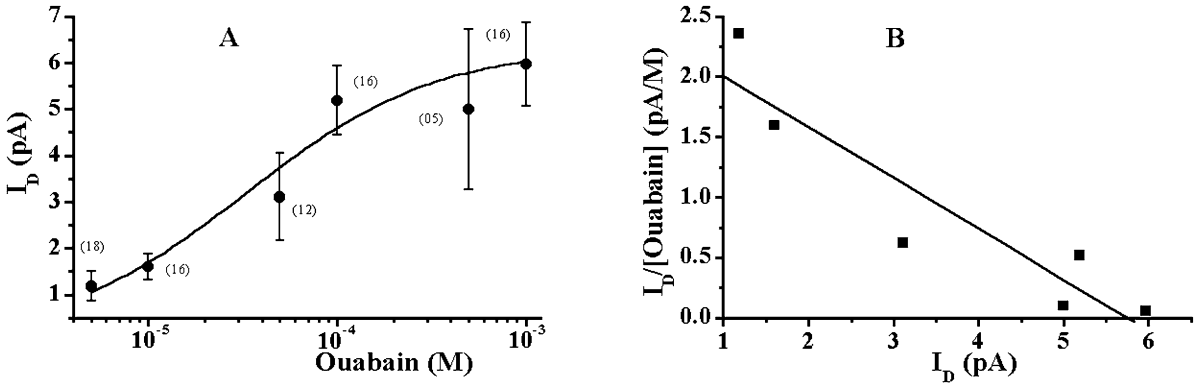

Fig. 6. Dose-response curve for ouabain. (*A*) The continuous line represents the best fit of equation 1 to the data points. (*B*) A Scatchard-type plot for the same data; the continuos line was calculated by linear regression. The correlation coefficient was −0.92 and the calculated values of K_D and I_p were 2.4×10^{-5} M and 5.9 pA, respectively. The values on the ordinate were multiplied by 10^{-5}.

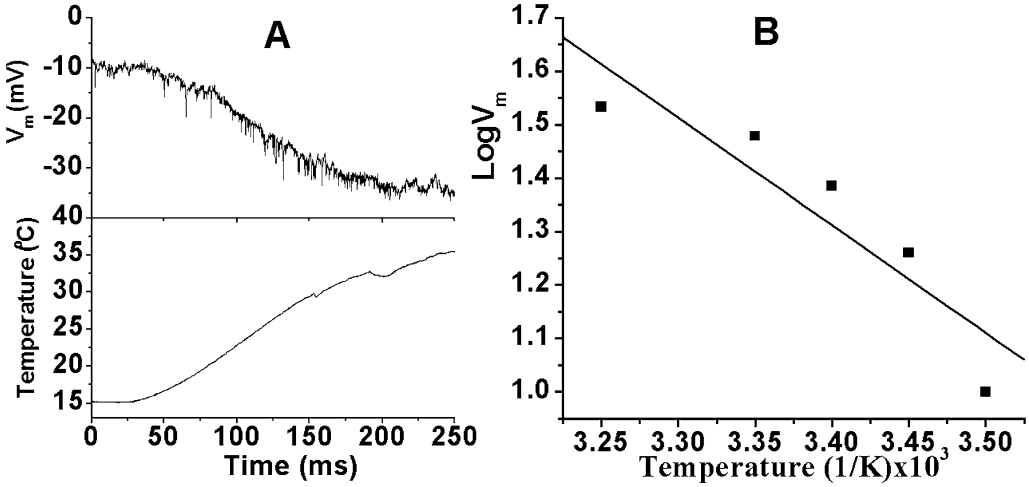

Fig. 7. Temperature dependence of the resting membrane potential. (*A*) Typical response of V_m (upper panel) to temperature rising continuously from 15 to 35°C. In this temperature range, the membrane potential changed from −10 to −36 mV. (*B*) Arrhenius plot of logV_m against temperature in °K. The Q_{10} of the process is equal to 1.87. Points represent the mean of at least 6 experiments and the line is the best linear fit to the experimental points ($R = 0.98$).

1995) and their activity should not contribute decisively for the maintenance of a stable resting potential. This is expected since the resting intracellular calcium concentration in Leydig cells is around 5×10^{-8} to 2×10^{-7} M (Sullivan & Cooke, 1986; Kumar et al., 1994; Tomic et al., 1995), somewhat bellow the K_D for activation of maxi-K^+ channels (Latorre et al., 1989) that are mainly activated by large depolarizations (+80 mV). Desaphy et al. (1996) showed that, in rat Leydig cells, an outwardly rectifying potassium conductance can be activated at potentials close to the resting potential, provided that there is a low intracellular calcium concentration. Based on their results, and on the reported calcium concentrations by others, they concluded that this conductance is largely inhibited at rest. Chloride currents are also present, but are mainly activated upon hyperpolarization of the cell, and are not calcium sensitive (Noulin & Joffre, 1993). These results argue against a major participation of these types of conductances in generating a stable resting potential and would explain the weak dependence of the resting potential on the external potassium concentration (Desaphy et al., 1996).

The observations shown in Figs. 2 and 3 support the hypothesis that an outward pump current is responsible for most of the polarization seen in Leydig cells. Ouabain, a specific inhibitor of the Na-K ATPase, blocked I_p and consequently induced a depolarization of the membrane, in a reversible way. A very similar response was seen when the extracellular solution was made free of K^+ ions, in accordance with the pump requirement for K^+. In fact, the depolarizations induced by ouabain or by the absence of potassium correspond to almost 70% of the cell's resting potential. Mullins and Noda (1963) and

Table 1. V_m and intracellular sodium concentration

Sodium concentration	V_m (mV) ± SEM	n
Nominally free	−13.4 ± 0.67	63
10 mM	−24.3 ± 2.1	12
20 mM	−32.2 ± 1.2	159
50 mM	−40.9 ± 2.4	24

Thomas (1969) postulate that a 3:2 Na^+/K^+ pump cannot contribute more than 10 mV to the cell's resting potential. We would like to point out that this might be true only if the Na^+ and K^+ fluxes, necessary to balance the pump fluxes, are exclusively diffusional. In fact, the fraction of the resting potential contributed by the pump can be much higher if only part of the Na^+ and K^+ balancing fluxes are made through an electroneutral exchanger (Bashford & Pasternak, 1985). Despite intense efforts, so far no one has described sodium currents in mouse or rat Leydig cells. Although purely speculative, the presence of a Na^+/H^+ or Na^+/Ca^{2+} exchanger would explain this issue. In order to further strengthen our point, we directly measured the current generated by the pump in the whole-cell clamp condition. Again, both ouabain and absence of K^+ in the extracellular bathing solution caused the appearance of an inward current (Fig. 5) whose magnitude is dependent on the concentration of ouabain (Fig. 6). The K_D for binding of ouabain in Leydig cells (2.4×10^{-5} M) is similar to values reported for other systems, like Purkinje cardiac cells (Cohen et al., 1987) and it seems that the pump needs to bind only one ouabain molecule in order to be blocked. The maximum current, at 25°C and intracellular sodium concentration of 20 mM, is around 6.5 pA, resulting in an average current density equal to 0.30 ± 0.05 pA/pF — again, close to values reported for other cells like Purkinje fibers (Sejersted, Wasserston & Fozzard, 1988), cardiac muscle cells (Hermans, Glitsch & Verdonck, 1994) or the vomeronasal chemoreceptor neurones of the frog (Trotier & Døving, 1996).

The contribution of the electrogenic Na^+/K^+ pump to the resting potential of cells was nicely analyzed in the works of Mullins and Noda (1963) and of Thomas (1969). According to them, the maximum contribution of the pump to the total membrane potential would be around 10 mV. This number was calculated based on the following assumptions: 1) only Na^+ and K^+ are the determinants of the membrane potential and 2) the steady-state intracellular concentrations of those ions are maintained by the pump and are dependent on the pump rate. Not considered in the above treatment is the fact that the contribution of the pump to the cell's resting potential may, in fact, depend on the ratio between the pump conductance (G_p) and the diffusional conductance of the membrane (G_d), as pointed out by Läuger (1991).

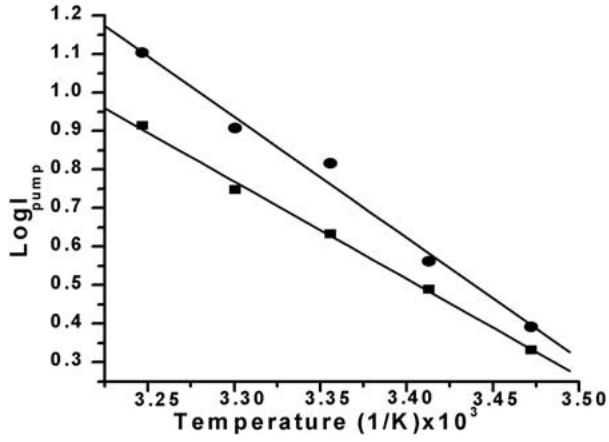

Fig. 8. Temperature dependence of the pump current. I_{pump} was measured as described in Material and Methods for temperatures ranging from 15 to 35°C in steps of 5°C. Measurements were made with 20 mM (●, Q_{10} = 2.26) or 10 mM Na^+ (■, Q_{10} = 1.92) in the pipette solution. Lines are best linear fits to the data points; in both cases, R = 0.99. Each point represents the mean of at least 6 measurements.

What, then, makes Leydig cells different from other cells? In our view, the input resistance of the cell is a key factor in determining the relative contribution of an electrogenic pump to the cell's resting potential. In cells where the input resistance is low, the contribution of I_p to the membrane potential difference may be of only a few millivolts (Thomas, 1969) but in other cells, where the specific membrane resistance is larger than 10^9 Ω, an electrogenic pump may significantly contribute to the cell's resting potential. Measurements made by us and others with conventional microelectrodes (Joffre et al., 1984a,b), report values for the input resistance of Leydig cells around 30 to 80 MΩ. Under this condition, the contribution of an electrogenic pump to the resting potential would be minimal (0.2–0.5 mV). However, using patch-clamp pipettes, Kawa (1987) reports input resistances in excess of 3.3 GΩ, in agreement with our mean value of 2.7 GΩ. In fact, taking into consideration that this input resistance is in parallel to a leakage resistance in the pipette tip, the real cell membrane resistance should be higher than 6–8 GΩ. Therefore, if we take a very conservative approach and consider 6.5 pA as the pump current (at 25°C) and 2.7 GΩ as the input resistance of these cells, the pump may contribute as much as 17.5 mV to the total resting potential of −32 mV. This value is very close to the average depolarization produced both by the potassium-free solution (18.7 mV) and 10^{-3} M ouabain (21 mV). In fact, at 35°C, and taking the average pump current equal to −12.7 pA, measured at this temperature, the contribution of the pump would be 34.3 mV to a resting potential of −34.5 mV. In other

words, the Na^+-K^+-ATPase is directly responsible, in the worst case, for at least half of the Leydig cell's membrane potential, in clear contrast to what was previously described by Joffre et al. (1984a,b). This situation is not particular to Leydig cells. T lymphocytes (Ischida & Chused, 1993), rat peritoneal mast cells (Bronner et al., 1989) and neutrophils (Bashford & Pasternak, 1985) are known examples of cells having the same mesenchymal origin as Leydig cells, and in which the membrane potential is set primarily by the sodium pump.

A major role for the Na/K pump in generating the resting potential can also be derived from the temperature dependence of both membrane potential and pump current. Cooling to 15°C strongly depolarized the cells to values around −9 mV and lower. The Q_{10} of this process was 1.87. The temperature also affected, in a similar way, the current generated by the pump, with a Q_{10} of 1.92 in the range of 15–35°C when sodium in the pipette was 10 mM and 2.26 for sodium equal to 20 mM. This value is in good agreement with those observed in rabbit sino-atrial node (Sakai et al., 1996), sheep Purkinje fibers (Glitsch & Pusch, 1984) and smooth muscle cells from mesenteric arteries (Nakamura et al., 1999). Taken together, our results strongly suggest that the electrogenic Na^+/K^+-ATPase plays a major role in determining the resting potential of mice Leydig cells, which cannot be explained based solely on electrodiffusive movement of ions. This situation arises mainly because of the high membrane resistance observed in Leydig cells when at rest.

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