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EXISTENCE OF CALCIUM CHANNELS AND INTERCELLULAR COUPLINGS IN THE TESTOSTERONE-SECRETING CELLS OF THE MOUSE

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SUMMARY

1. The electrophysiological properties of testosterone-secreting cells (i.e. Leydig cells) in the mouse were studied using patch electrodes. The cells appeared solitarily or in clusters after mechanical dissociation from testes. They were confirmed to be Leydig cells on the basis of 3β-hydroxysteroid dehydrogenase staining.

2. Under current-clamp conditions in the whole-cell configuration, Leydig cells immersed in standard saline were able to generate action potential-like responses. The active responses occurred after cessation of membrane hyperpolarization or when cells were held in a hyperpolarized condition and stimulated with depolarizing current pulses.

3. In Leydig cells under voltage clamp, depolarizations more positive than -50 mV evoked transient inward currents which decayed completely during the duration of depolarization (130 ms). No obvious outward currents were evoked by pulses less positive than 30 mV.

4. The inward currents were identified as Ca^{2+} current, since replacement of external Ca^{2+} with Mn^{2+} reversibly diminished the current whereas Ba^{2+} or Sr^{2+} substituted for Ca^{2+}.

5. With voltage pulses more positive than 40 mV, outward currents were evoked. The currents were dependent on K^+ concentration and were blocked by quinine or tetraethylammonium. The amplitudes of outward currents were increased with raised internal Ca^{2+} concentration.

6. Single-channel recordings of the outward currents revealed that the unitary conductance was 130 pS when internal K^+ was 131–143 mM and external K^+ was 5 mM. The open probability of the channel showed marked dependence on the membrane potential and the internal Ca^{2+} concentration. Thus, the current was identified as being Ca^{2+}- and membrane potential-dependent K^+ current.

7. Leydig cells within a cluster possessed distinct intercellular couplings. The mean coupling ratio obtained by applying two patch electrodes to a pair of cells was 0·84. Transfer of injected dye (Lucifer Yellow) to adjacent cells was also confirmed.

8. It was concluded that Leydig cells have at least two kinds of voltage-dependent channels in the membrane. The Ca^{2+} channel may be activated by physiological changes in membrane potential, leading to an influx of Ca^{2+}. The Ca^{2+}-dependent

K^+ channel hardly seems to be activated unless the internal Ca^{2+} concentration increases remarkably. It is presumed that intercellular coupling may play a role in synchronizing or intensifying the endocrine activities of Leydig cells located within a cluster.

INTRODUCTION

The membrane properties of endocrine cells have recently been extensively studied (Ozawa & Sand, 1986). It has been revealed that some cells are electrically coupled and that the membrane properties of several types of endocrine cells, especially those of aminergic or peptidergic cells, have features common to those of neuronal cells. Some kinds of endocrine cells elicit action potentials in response to electrical and/or chemical stimuli and the release of bioactive substances such as hormones or transmitters from the cells strongly depends on the intracellular concentration of Ca^{2+} (Petersen, 1980; Williams, 1981; Ozawa & Sand, 1986).

The membrane properties of steroid-secreting cells and their functional roles in steroidogenesis, however, are little known. Matthews & Saffran (1973) first reported that adrenocortical cells in neonatal rabbits were able to generate action potentials which were tetrodotoxin resistant and occasionally showed overshoot beyond the zero potential. The possibility of electrical couplings among adrenocortical cells has also been shown, and it has been revealed that aldosterone-secreting cells in the adrenal cortex of the cat displayed action potentials spontaneously or in response to applications of angiotensin II or increased concentrations of external K^+, depolarization in these cells being accompanied by increased aldosterone release (Natke & Kabela, 1979). In an adrenocortical cell line which secretes glucocorticoid in response to ACTH, calcium- and voltage-activated K^+ channels and calcium-dependent action potentials have been described (Tabares, López-Barneo & de Miguel, 1985; Tabares & López-Barneo, 1986). In progesterone-secreting cells of corpora lutea in the sheep, the dependence of resting membrane potential on external K^+ concentration and the dependence of progesterone secretion on external Ca^{2+} have been demonstrated, while neither the existence of electrical couplings among the cells nor generation of action potentials in them have been proved (Higuchi, Kaneko, Abel & Niswender, 1976). More recently, single testosterone-secreting cells (i.e. Leydig cells) were enzymatically isolated from rat testes and their membrane properties were studied using conventional microelectrodes (Joffre, Mollard, Régondaud, Alix, Poindessault, Malassiné & Gargouil, 1984). The cell membranes showed an almost linear current–voltage relationship over a range of -50 to 40 mV from the resting potential. Neither action potentials nor electrical couplings in the cells were observed.

In the present study, we investigated and re-examined the following issues with regard to the electrical properties of testosterone-secreting cells in the mouse. (a) Are the Leydig cells electrically excitable? If so, what ionic channels underlie this excitability? (b) Are the Leydig cells which form clusters electrically coupled? If so, are the structures of the couplings also permeable to large molecules such as dyes? In order to explore these issues, we have devised a milder method for isolation of Leydig cells from mouse testes than that used previously and have employed patch-

electrode techniques (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). Some of the results obtained have already been presented in a preliminary form (Kawa, 1986).

METHODS

Preparation. Adult male mice of DDY (Deutschland–Densenbyokenkyasho–Yoken) strain weighing 32–46 g were killed by cervical dislocation. After ligation of the spermatic ducts and blood vessels, the bilateral testes were removed and the tunica albuginea of each testis was carefully cut away under observation with a dissecting microscope. Each testis was then placed in a recording chamber and about 1 ml of standard saline was injected into the intertubular spaces of the testis from the pointed tip of a polyethylene tube connected to a syringe. By this procedure, testosterone-secreting cells (i.e. interstitial cells or Leydig cells) were isolated in the recording chamber without significant contamination due to other kinds of cells. No enzyme was used for dissociation. After the Leydig cells had settled onto the base of the chamber (made of a cover-slip 0·15 mm in thickness), it was mounted on the movable stage of an inverted phase-contrast microscope (final magnification × 600, MTD, Nikon) and the chamber was perfused with experimental saline at a flow rate of 1–2 ml/min. The temperature of the chamber was monitored with a thermistor probe and maintained at 21–24 °C.

Recordings. The present experiments employed the whole-cell and the outside-out patch variants of the patch-clamp technique (Hamill *et al.* 1981). Details of our recording procedure have been described previously (Hagiwara & Kawa, 1984; Kawa, 1987). The resistance of the patch electrodes used ranged between 2 and 10 MΩ when filled with the internal solution. After obtaining a gigaohm seal with a cell, either a steady or a pulsatile negative pressure of up to 200 cmH_2O was applied so as to rupture the membrane at the tip of the patch electrode. If the seal resistance was observed to decrease to less than 1 GΩ during this procedure, the cell was discarded. For the study of electrical couplings among Leydig cells within a cluster, two patch electrodes connected to separate patch-clamp amplifiers were applied to each of two neighbouring cells and the membrane potentials of these cells were monitored in current-clamp mode while currents were passed into one of the cells through the patch electrode. In one series of experiments, the fluorescent dye Lucifer Yellow CH was injected into one of the cells in a cluster with a patch electrode filled with 5% Lucifer Yellow CH lithium salt (dissolved in distilled water). The ability of Lucifer Yellow to cross at least some gap junctions together with its very low rate of leakage through the cell membrane has already been documented (Stewart, 1981). The resistance of the electrodes filled with Lucifer Yellow ranged from 4 to 8 MΩ. The cells were sealed to the tip of the electrode well and the patched membrane was then ruptured easily by the same procedure as that described above. The chamber (depth 0·8 mm) containing the preparation was then covered with a coverglass (thickness 0·15 mm) and viewed and photographed using a Nikon Optiphot epifluorescence microscope (B excitation filter with an accessory filter, objective × 20). Errors caused by liquid junction potentials were corrected as previously described by Hagiwara & Ohmori (1982). Membrane potentials and current signals were displayed on a storage oscilloscope (5111, Tektronix) and photographed. Data averages were obtained with a signal processor (7T07A, Sanei, Japan).

Cell identification. Histochemical identification of Leydig cells was accomplished by staining for 3β-hydroxysteroid dehydrogenase (3β-HSD) activity using a modification of the procedure described by Browning, D'Agata & Grotjan (1981). The Leydig cells were isolated in a chamber similar to that used for patch-clamp recordings. The extracellular saline in the chamber was removed gently and then replaced with 2% glutaraldehyde in order to fix the preparation for 3–5 min (at 23–25 °C). The chamber was then perfused several times with 0·07 M-phosphate buffer (pH 7·2) to wash away the fixative. The staining solution was composed of 0·07 M-phosphate buffer (pH 7·2) containing 1 mg nicotinamide/ml, 6 mg β-nicotinamide adenine dinucleotide/ml, 1·5 mg Nitro Blue Tetrazolium/ml, and 100 μg dehydroepiandrosterone/ml. The preparation in the

TABLE 1. Composition of solutions (mM)

A External solution*

	NaCl	KCl	$CaCl_2$	$MnCl_2$	$BaCl_2$	$SrCl_2$
Standard saline	126	5	10	—	—	—
10 mM-Mn^{2+} saline	126	5	—	10	—	—
10 mM-Ba^{2+} saline	126	5	—	—	10	—
10 mM-Sr^{2+} saline	126	5	—	—	—	10
30 mM-K^+ saline	101	30	10	—	—	—
120 mM-K^+ saline	11	120	10	—	—	—

B Internal solution

	KCl	CsCl	NaCl	$MgCl_2$	$CaCl_2$	K-EGTA	HEPES–KOH†	Free K^+‡	Free Ca^{2+}‡
KCl saline	120	—	—	0·8	2	4	8	130·6	$5{\cdot}8 \times 10^{-8}$
CsCl saline	—	120	—	0·8	—	5	10	13·4	$< 10^{-10}$
10^{-6} M-Ca^{2+} saline	100	—	—	1	18	20	10	143·2	$5{\cdot}4 \times 10^{-7}$
10^{-8} M-Ca^{2+} saline	100	—	—	1	2	20	10	143·1	$6{\cdot}2 \times 10^{-9}$
10 mM-K^+ saline	10	—	90	1	18	20	10	53·2	$5{\cdot}4 \times 10^{-7}$

* In external media, each saline also contained 1 mM-$MgCl_2$, 6 mM-sodium lactate, 10 mM-HEPES and 5 mM-NaOH to give pH $7{\cdot}3 \pm 0{\cdot}1$ at 21–24 °C.

† Final pH, $7{\cdot}2 \pm 0{\cdot}1$.

‡ Free K^+ and Ca^{2+} concentrations (M) were calculated using the dissociation constants in text.

chamber was then incubated with 0·3 ml of filtered staining solution for 50–60 min at 38 °C. The chamber was finally perfused with 0·07 M-phosphate buffer and then viewed using an inverted phase-contrast microscope.

Solutions. The compositions of the external and internal (inside the patch electrode) solutions are listed in Table 1. The osmolarities of internal salines seem lower than those of external salines by about 18% but these combinations of osmolarities appeared suitable for obtaining high seal resistance between the cells and the tips of the patch electrodes. The free-Ca^{2+} and free-K^+ concentrations were estimated by calculation with a personal computer (9801VM2, NEC, Japan) using the following dissociation constants (Durham, 1983). For EGTA (ethyleneglycol-bis-(β-amino-ethylether)*N*,*N*′-tetraacetic acid), $pK_{H1} = 9{\cdot}56$, $pK_{H2} = 8{\cdot}94$, $pK_{H3} = 2{\cdot}75$, $pK_{H4} = 2{\cdot}1$, $pK_{Ca} = 11{\cdot}38$, $pK_{CaH} = 5{\cdot}7$, $pK_{Mg} = 5{\cdot}62$ and $pK_{MgH} = 3{\cdot}8$ at 20 °C, where K_{H1}–K_{H4} are the four acid dissociation constants, and where K_{Ca} and K_{Mg} are the stability constants of $EGTA^{4-}$ and K_{CaH} and K_{MgH} are the stability constants of $EGTAH^{3-}$, for Ca^{2+} and Mg^{2+} respectively. For HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid), $pK_a = 7{\cdot}5$ for the acid dissociation constant. Chemicals for the staining solution, EGTA, HEPES and Lucifer Yellow CH, were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.). Quinine sulphate and tetraethylammonium chloride (obtained from Nakarai Chemical Co., Kyoto, Japan) were dissolved just before use.

RESULTS

Under phase-contrast microscopy, Leydig cells isolated from adult mice appeared almost spherical with diameters of 16–19 μm and exhibited bright margins with fine indentations. The cells which settled on the base of the chamber were either solitary or formed clusters consisting of two to ten cells. The major contaminants were erythrocytes which were readily distinguishable by their discoid shape and small diameter of 7–8 μm. Occasionally, spermatogenic cells residing in the seminiferous tubules of the testis also appeared in the chamber. However, as these spermatogenic cells had smooth margins and characteristic nuclei, it was possible to discriminate them without difficulty (Hagiwara & Kawa, 1984). To confirm the identification of Leydig cells, specific staining for 3β-hydroxysteroid dehydrogenase was performed. After incubation in the staining solution, almost all the cells showing the characteristic morphological features described above stained dark brown while erythrocytes and other contaminating cells remained unstained. Positively stained cells accounted for more than 80% of the cells in the chamber, discounting the erythrocytes.

Potential changes under current clamp

Leydig cells settling on the base of the recording chamber occurred solitarily or in clusters. For the following experiments in which intracellular isopotentiality was critical, solitary cells were used rather than clustered cells, since there was a possibility that the latter might be electrically coupled. The average zero-current potential of Leydig cells immersed in the standard saline was -27 ± 9 mV (S.D., $n = 19$). The input resistance of the cells measured with a hyperpolarizing current pulse (20 pA, 300 ms) was $3{\cdot}3 \pm 2{\cdot}0$ GΩ (S.D., $n = 10$). As the measured input resistance may consist of the real input resistance of the cell and the leakage resistance at the electrode tip in parallel, the measured value may be an underestimate unless the leakage is comparatively negligible. Thus, the real value may have been around 6–8 GΩ as judged from the measured near-maximum values. By the same consideration, the real resting potential in the absence of leakage may be more negative than the mean zero-current potential obtained above.

In Leydig cells under current-clamp conditions, an inflexion in the rate of membrane potential occurred after cessation of a hyperpolarizing current pulse which brought the membrane potential to a value more negative than −80 mV (Fig. 1*A*). The traces shown in Fig. 1*B* are another example of such 'off-responses' of the membrane potential observed on a faster time scale. After the end of a hyperpolarizing current (50 pA, 350 ms), the membrane potential initially changed

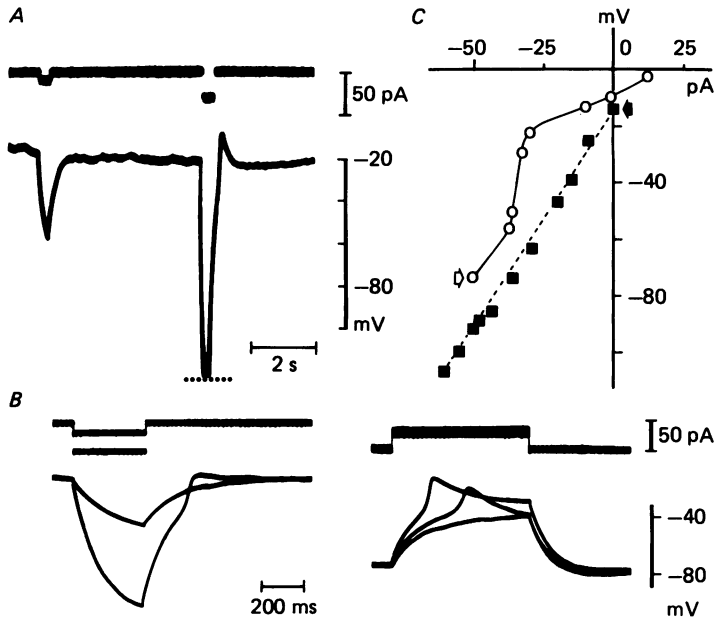

Fig. 1. Action potential-like responses in mouse Leydig cells. Two examples studied with a whole-cell patch electrode under current-clamp conditions. *A*, upper trace, current monitor. Lower trace, membrane potential. After cessation of stronger hyperpolarization (second pulse), an action potential-like response ('off-response') was observed. Part of the hyperpolarization was truncated (dotted line). *B*, another cell studied on a faster recording sweep. Two and three traces were superimposed in the left and right panels, respectively. When the cell was hyperpolarized with d.c. current (50 pA), it generated action potential-like responses during depolarizing pulses (right). *C*, *I–V* relations of the cell shown in *B*. Filled squares represent the relation (i.e. peaks of membrane hyperpolarization *vs.* membrane currents) in the absence of d.c. current. Filled arrow indicates the zero-current potential. Open circles represent the relation in the presence of hyperpolarizing d.c. current (50 pA, indicated by open arrow). The relationship (i.e. peaks of membrane depolarization *vs.* membrane currents) shows distinct non-linearity above −50 mV. External medium, standard saline. Patch electrode contained KCl saline.

with an approximately exponential time course from the previous level. At about −50 mV, the rate of change of the membrane potential increased abruptly and the membrane potential overran the level of zero-current potential. Then, in several hundreds of milliseconds, the membrane potential returned to the zero-current potential. When the membrane potential of the same cell was held hyperpolarized at −75 mV with constant d.c. current (Fig. 1*B*, right), the cell generated spike-like potentials in response to depolarizing overlapping current pulses. The threshold for the spike-like potentials was about −50 mV. Comparison of the *I–V* relationship of

this cell measured in the presence of hyperpolarizing d.c. current (50 pA; open circles in Fig. 1*C*) and in the absence of d.c. current (filled squares) show that the spike-like potentials were due to an increase in membrane conductance and that the mechanism of such conductance increase was hardly apparent or inactivated at depolarized potentials, as judged from the almost-linear plot shown by the filled squares in Fig. 1*C*. Off-responses (mean amplitude, 8·5 mV) and spike-like potentials were evoked in all of six cells with an input resistance of more than 1·5 GΩ. The amplitude of active responses in the membrane potential tended to be reduced as the input resistance of the cell decreased. In cases where the input resistance was below 1 GΩ, such active responses were rarely recognized, probably due to the prevalence of leakage shunting effects at the tip of the patch electrode.

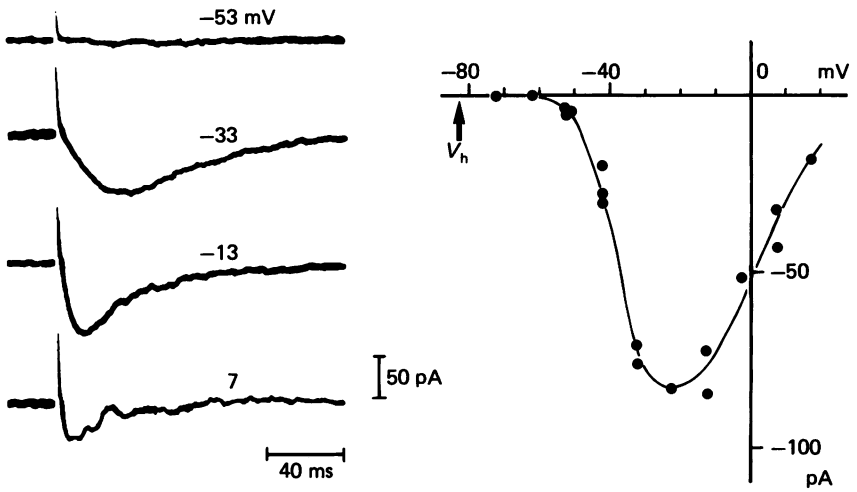

Fig. 2. Membrane currents of a Leydig cell in standard saline. Currents were recorded with a patch electrode under whole-cell voltage clamping. Left, specimen records. Figures indicate membrane potential (mV) during the pulse. Holding potential, −83 mV. Cell diameter, 18 μm. Patch electrode contained KCl saline. Right, *I–V* relations at the peak inward current. V_h represents the holding potential.

Membrane currents under voltage clamp

In order to investigate the ionic mechanisms of active responses in Leydig cell membranes, membrane currents were recorded using whole-cell patch clamp. Typical current recordings from a cell immersed in standard saline are shown in Fig. 2. Transient inward currents were evoked when the membrane potential was made more positive than about −50 mV and reached a maximum at around −25 mV. At more positive potentials, inward currents became small but no appreciable outward currents were observed at membrane potentials of up to +30 mV. Negative voltage pulses down to −113 mV did not produce appreciable voltage-dependent currents; there was no sign of inward rectification. The peak amplitudes of the inward currents were plotted against the membrane potentials (Fig. 2, right). Similar current–voltage curves were obtained from a total of five Leydig cells. The mean amplitude of inward currents at the peak was 71 ± 19 pA (S.D., $n = 5$).

Inward currents are carried by Ca^{2+}. The following results indicate that the inward currents are carried mainly by Ca^{2+}: (1) replacement of external NaCl with equimolar KCl did not alter the inward currents ($n = 3$); (2) inward currents were reversibly diminished when 10 mM-$MnCl_2$ replaced 10 mM-$CaCl_2$ in the external saline (Fig. 3*A*); (3) Ba^{2+} and Sr^{2+} were able to carry inward currents (Fig. 3*B* and *C*). The

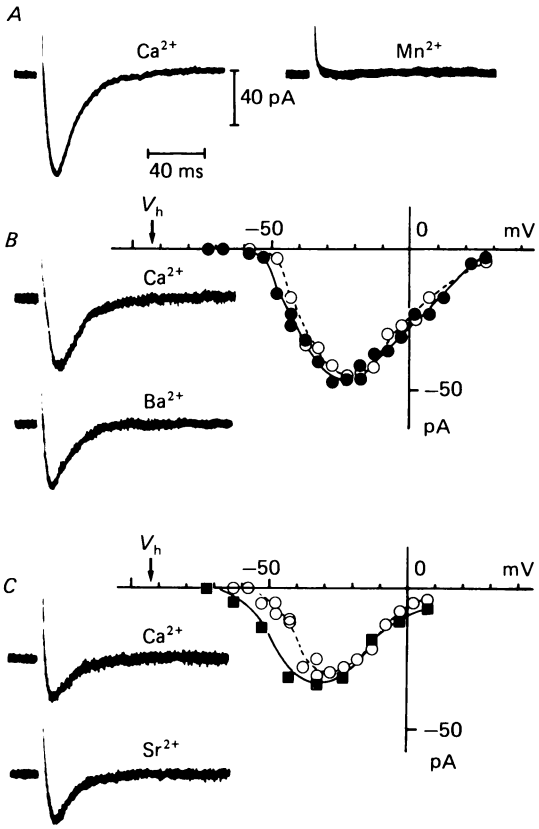

Fig. 3. Effects of replacement of external Ca^{2+} with Mn^{2+}, Ba^{2+} and Sr^{2+} on the inward currents. Patch electrode contained KCl saline. *A*, left, inward current near-maximum in standard saline. Current was evoked by a voltage pulse to −23 mV. Right, membrane current after replacement of external Ca^{2+} with equimolar Mn^{2+} (10 mM-Mn^{2+} saline). Same voltage pulse was applied. Holding potential, −83 mV. *B*, left, membrane currents at −23 mV in standard saline (upper trace) and after washing in 10 mM-Ba^{2+} saline (lower trace). Right, *I–V* relations in standard saline (○) and in 10 mM-Ba^{2+} saline (●). V_h represents holding potential of −93 mV. Cell diameter, 19 μm. *C*, same as *B* except that the test solution for this cell was 10 mM-Sr^{2+} saline (■). Cell diameter, 18 μm. Calibration in *A* applies to all traces shown.

I–V relationships of the Ba^{2+} currents and Sr^{2+} currents were shifted in a negative direction along the abscissa by about 4 and 10 mV, respectively. This was presumably due to the weaker effects of Ba^{2+} and Sr^{2+} on the external surface charge of the membrane in comparison with those of Ca^{2+} (Ohmori & Yoshii, 1977). The maximum inward currents in the *I–V* relationships showed no marked difference between Ca^{2+} and Ba^{2+} nor between Ca^{2+} and Sr^{2+}, at least at 10 mM ($n = 2$).

One of the characteristics of the inward currents is that they decayed during maintained voltage pulses in standard saline (Figs 2 and 3*A*). Similar decay was observed in Ba^{2+} and Sr^{2+} salines (Fig. 3*B* and *C*). The decay was not affected when CsCl saline was used as the internal saline ($n = 3$). Cs^+ might have suppressed any K^+

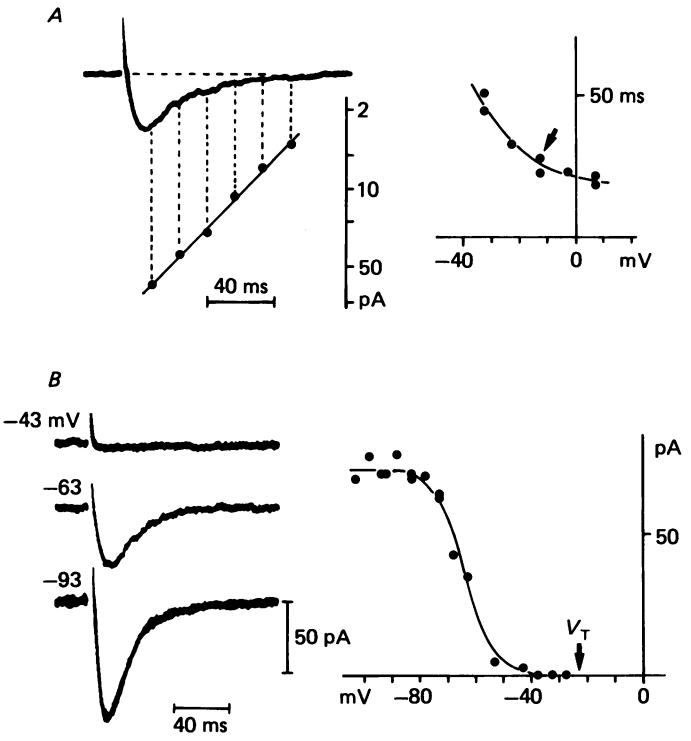

Fig. 4. Voltage-dependent inactivation of Ca^{2+} currents. *A*, left, specimen record. Inward current inactivated during maintained depolarization at −13 mV (130 ms). Holding potential, −83 mV. Time course of decay was replotted below after current amplitudes had been transformed into logarithm. Time constant of the decay was estimated by the slope of an approximated straight line. Right, dependence of time constant on membrane potential. Arrow indicates the value shown on left. *B*, effects of holding potential on peak inward current. Left, specimen records obtained with holding potentials of −43 mV (upper), −63 mV (middle) and −93 mV (lower), respectively. Applied voltage pulses, −23 mV, 130 ms. Right, dependence of inward current on holding potential. Peak inward currents were plotted against holding potentials. V_T indicates applied voltage pulse (to −23 mV) for evoking currents.

current if present (Blatz & Magleby, 1984; Hille, 1984). Thus, the decay was probably due to voltage-dependent inactivation rather than the development of Ca^{2+}-induced K^+ currents or Ca^{2+}-induced inactivation (for review, see Eckert & Chad, 1984). The time course of decay was mostly fitted by a single-exponential curve (Fig. 4*A*). Although the curve fitting should be considered as a phenomenological approximation showing occasional deviations, it revealed that the time course of decay became substantially faster as the depolarization of the membrane increased (Fig. 4*A*, right). In Fig. 4*B*, the effect of holding potential on the amplitude of inward currents is shown. The Ca^{2+} current started to be inactivated

around −80 mV and was almost completely inactivated at −40 mV. This means that the degree of Ca^{2+} influx or the magnitude of active membrane responses may be steeply dependent on the membrane potential at rest between −80 and −40 mV. This type of voltage-dependent inactivation of Ca^{2+} currents has been reported in

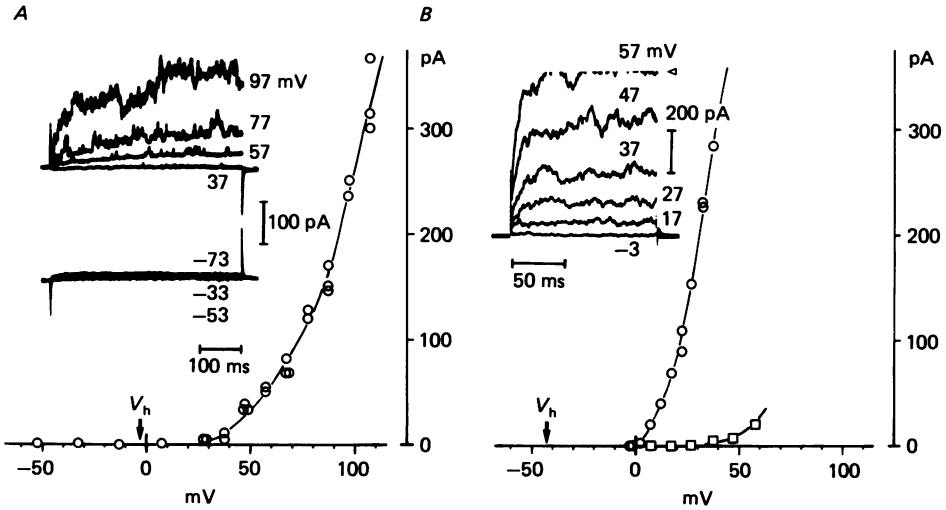

Fig. 5. Outward currents in a Leydig cell and their dependence on $[Ca^{2+}]_i$. *A*, outward currents were evoked from a holding potential of −3 mV. Cell was immersed in standard saline and the patch electrode contained internal KCl saline. Outward currents were evoked by depolarizing pulses (superimposed traces in upper panel), while no obvious currents were evoked by hyperpolarizing pulses (superimposed traces in middle panel). Figures (mV) on each trace indicate the membrane potential during the pulse (460 ms). Lower right, *I–V* relations at the peaks of evoked currents. V_h represents the holding potential. Cell diameter, 18 μm. Small outward current at −73 mV is probably an artifact due to improper subtraction of leakage current. *B*, records for another cell. Cell was immersed in standard saline and the patch electrode contained 10^{-6} M-Ca^{2+} saline. Immediately after the establishment of whole-cell configuration, outward currents were recorded. Their *I–V* relation is plotted with open squares in the lower panel. Amplitudes of outward currents then increased gradually and reached a steady state in 10–14 min. The steady *I–V* relation is plotted with open circles. V_h represents holding potential of −43 mV. Upper left, specimen record of superimposed current traces obtained after 14 min. Figures (mV) on each trace indicate the membrane potential during the pulse (140 ms). Upper part of trace is truncated (marked with open wedge). Cell diameter, 20 μm.

some excitable cells (Hagiwara & Byerly, 1981; Nowycky, Fox & Tsien, 1985; Bossu, Feltz & Thomann, 1985; Bean, 1985; Matteson & Armstrong, 1986; Narahashi, Tsunoo & Yoshii, 1987) and in other tissues (Fox, 1981; Hagiwara & Kawa, 1984; Fukushima & Hagiwara, 1985).

Membrane currents other than Ca^{2+} currents in Leydig cells

At membrane potentials ranging from −113 to 27 mV shifted from the holding potential of −83 mV, the only discernible currents were those of Ca^{2+}; outward currents were scarcely observed at the potentials examined.

The possibility remains, however, that some membrane currents in Leydig cells were undetected due to their linear *I–V* properties or their small amplitudes which might have been indistinguishable from those of subtracted leakage currents, thus possibly limiting the resolution of the present study.

It was, however, noted that clearly recognizable outward currents were evoked when the membrane potential was shifted to a value more positive than 30 mV (Fig. 5). The amplitudes of the currents showed prominent fluctuations during depolarization. The *I–V* relationship obtained from a typical experiment (Fig. 5*A*) indicated that the threshold was around 35 mV and that the currents gradually increased as the depolarization was strengthened. The amplitudes of the currents exceeded 100 pA at about 80 mV. Similar observations were obtained in four other cells.

Effects of internal Ca^{2+} on the outward currents. When the concentration of Ca^{2+} in the internal saline ($[Ca^{2+}]_i$) filling the patch electrode was increased 10-fold from $5{\cdot}8 \times 10^{-8}$ to $5{\cdot}4 \times 10^{-7}$ M (standard saline to 10^{-6} M-Ca^{2+} saline in Table 1), the outward current increased; the threshold potential shifted to around 0 mV and the amplitude of the outward currents evoked at +27 mV exceeded 100 pA ($n = 3$, Fig. 5*B*). In these experiments, patch electrodes with an orifice 2–3 μm in diameter (resistance, 2–5 MΩ) were used. The buffering capacity of Ca^{2+} in the internal saline was augmented with 20 mM-EGTA and a calculated amount of $CaCl_2$ (Table 1). The changes in current amplitude under these conditions required 10–14 min to reach a steady state, probably due to the time required for Ca^{2+} to diffuse onto the internal surface of the membrane. Thus, it is not unreasonable to assume that the outward currents recorded immediately after the start of the whole-cell configuration (open squares in Fig. 5*B*) represent an intact state of Leydig cells or a state in which the changes caused by internal perfusion are negligible. The threshold of the outward current in intact Leydig cells can be speculated to be around 35 mV.

When the concentration of internal Ca^{2+} was decreased to one-tenth ($6{\cdot}2 \times 10^{-9}$ M), the amplitude of the current at a given membrane potential decreased; even at the maximal depolarization to 97 mV, the current amplitude was 60 pA or less ($n = 2$, not shown). The above results strongly suggest that the outward currents are dependent on the concentration of internal Ca^{2+} and that in intact Leydig cells the free-Ca^{2+} concentration is retained at around 5×10^{-7} M.

Effects of quinine, tetraethylammonium and K^+. Quinine is one of the blockers of so-called Ca^{2+}-dependent K^+ currents (Armando-Hardy, Ellory, Ferreira, Fleminger & Lew, 1975; Atwater, Dawson, Ribalet & Rojas, 1979; Burgess, Claret & Jenkinson, 1981; Schwarz & Passow, 1983), whereas at higher concentrations of more than 100–200 μM it may also block Na^+ and Ca^{2+} currents (Fishman & Spector, 1981; Ohmori, 1984). The outward currents in Leydig cells were suppressed by externally applied quinine in a dose-dependent manner (Fig. 6*A*). The suppression was almost completely reversible and the half-blocking concentration was about 110 μM. Tetraethylammonium (TEA) also suppressed the outward currents; when TEA (final concentration, 1 mM) was added to external standard saline the amplitude of outward currents was reduced to one-third of the control value (measured at 47 mV, $n = 3$). Although the effects of TEA are not specific (for review, see Stanfield, 1983; Marty & Neher, 1985), the above results are consistent with the notion that the outward currents are voltage- and Ca^{2+}-dependent K^+ currents which are blocked by TEA (Latorre, Vergara & Hidalgo, 1982; Blatz & Magleby, 1984).

Figure 6*B* shows the dependence of outward currents on the external concentration of K^+. The reversal potentials of evoked currents were determined from the reversal of tail currents which were induced by stepping back the membrane potential from

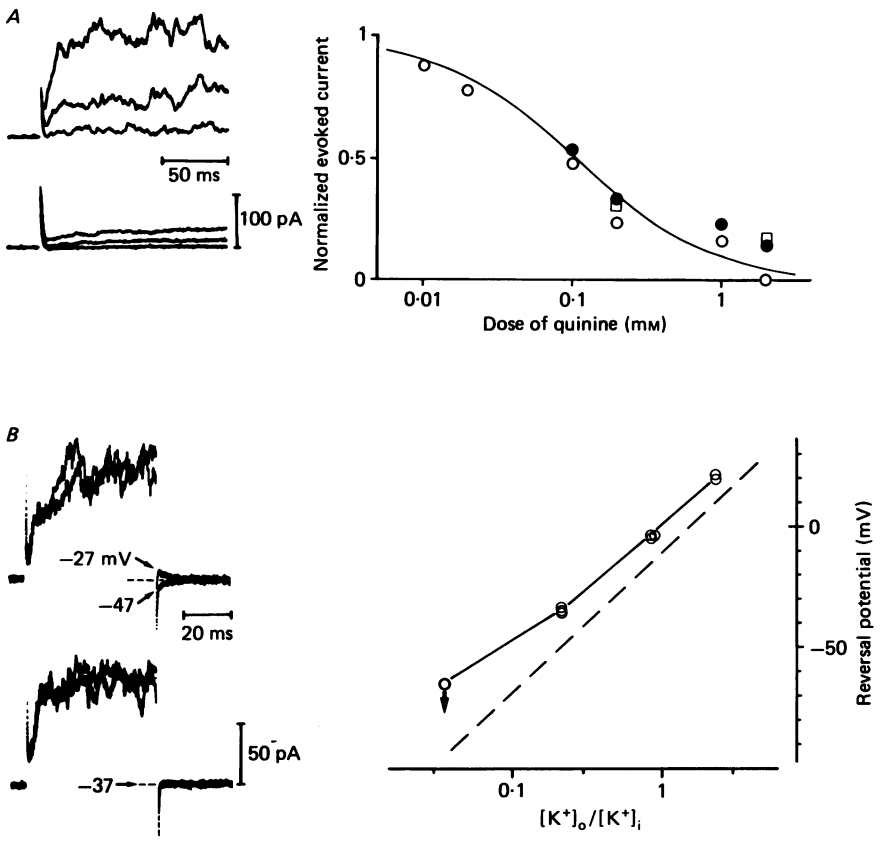

Fig. 6. Characterization of outward currents. *A*, suppression of outward currents by quinine. Left, specimen records of evoked currents in standard saline (upper traces) and in the presence of 2 mM-quinine (lower traces). Both traces were superimposed records evoked by pulses to 67 mV (upper), to 47 mV (middle) and to 27 mV (lower), respectively. Holding potential, −3 mV. Quinine was added to standard saline. Patch electrode contained 10^{-6} M-Ca^{2+} saline. Right, dose–effect relation for quinine. Amplitudes of evoked currents in the presence of quinine (final concentration, 0·01, 0·02, 0·1, 0·2, 1 or 2 mM) were normalized to those obtained in standard saline. Different symbols indicate the different membrane potentials examined (open circles, at 27 mV; filled circles, at 47 mV; open squares, at 67 mV). Holding potential, −3 mV. Curve was drawn by eye assuming one-to-one binding with dissociation constant, $K_D = 110\ \mu$M. *B*, reversal potential of outward current determined by tail currents and its dependence on K^+ concentration. Left, specimen records obtained in 30 mM-K^+ saline. From a holding potential of −43 mV conditioning depolarization to 17 mV (duration, 55 ms) was applied to activate outward currents; the depolarization was followed by test pulses to obtain tail currents. Tail currents were outward and inward at test pulses of −27 and −47 mV, respectively (upper superimposed traces). Tail current was almost flat at −37 mV (lower trace). Patch electrode contained 10^{-6} M-Ca^{2+} saline. Right, relationship between reversal potential and $[K^+]_o/[K^+]_i$ expressed on a logarithmic scale. Reversal potentials were measured by tail currents as shown in the left-hand panel. External media were standard saline, 30 mM-K^+ saline, 120 mM-K^+ saline and 120 mM-K^+ saline for the left to right plots, respectively. Internal medium was 10^{-6} M-Ca^{2+} saline except for the extreme right-hand plot where it was 10 mM-K^+ saline. The dashed line represents a slope of 58 mV for a 10-fold change in $[K^+]_o/[K^+]_i$. For further details see text.

the depolarization (17 mV or more positive potential) to various levels. In the standard saline (containing 5 mM-KCl) the tail currents were outward at potentials more positive than −65 mV ($n = 3$); below this membrane potential, the amplitude became so small that measurements were unreliable. When the external K^+

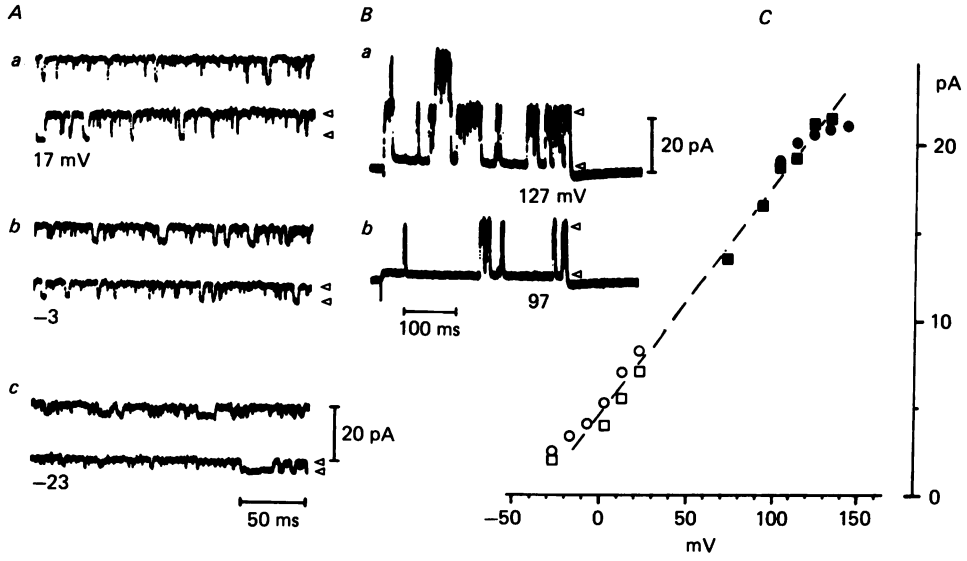

Fig. 7. Unitary currents in excised patch membrane under outside-out configuration and their dependence on membrane potential and $[Ca^{2+}]_i$. *A*, unitary outward currents obtained in the excised patch, where electrode contained 10^{-6} M-Ca^{2+} saline. At steady depolarizations of 17 mV (*a*), −3 mV (*b*) and −23 mV (*c*), the frequent occurrence of unitary outward currents was observed. External medium, standard saline. *B*, specimen records in excised patch, where electrode contained KCl saline. No observable unitary currents were recorded at depolarization pulses (duration, 350 ms) less positive than 60 mV. At higher depolarization, unitary currents were recorded as shown in *a* (at 127 mV) and *b* (at 97 mV). External medium, standard saline. Amplitudes of unitary current are indicated with open wedges in both *A* and *B*. *C*, amplitudes of unitary current plotted against membrane potential. Different symbols indicate different patches. Open symbols and filled symbols represent records obtained with internal media of 10^{-6} M-Ca^{2+} saline and KCl saline, respectively. *I–V* relations of unitary currents were approximately recapitulated by a straight line with a slope of 130 pS (interrupted line).

concentration was increased to 30 mM (30 mM-K^+ saline) and to 120 mM (120 mM-K^+ saline), the tail currents reversed at −35 mV and at −3 mV, respectively (mean of three cells). In these experiments, patch electrodes contained 10^{-6} M-Ca^{2+} saline (Table 1). When the electrode contained 10 mM-K^+ saline, the reversal of tail current in cells immersed in 120 mM-K^+ saline shifted to 19 mV (mean of two cells). When the changes of reversal potential were plotted against the ratio of external and internal K^+ concentrations ($[K^+]_o/[K^+]_i$) on a logarithmic scale, the observed value fitted the value expected from the Nernst equation (Fig. 6*B*, right).

Properties of single-channel current. As shown in Figs 5 and 6, the outward current showed prominent fluctuations in amplitude during depolarization pulses. This suggested that the currents may consist of unitary events having a large amplitude

(Marty, 1981, 1983; Blatz & Magleby, 1984; Marty & Neher, 1985). To explore this possibility, single-channel recordings from an outside-out membrane patch (Hamill *et al.* 1981) were attempted after observing whole-cell currents in cells immersed in standard saline. When the patch electrode was pulled back from the cell to obtain an outside-out patch, pulse-like outward currents were recorded from patches with a high-resistance seal (> 5 GΩ). The occurrence of pulse-like currents strongly depended on the concentration of free Ca^{2+} in the pipette. When the saline in the pipette contained 10^{-6} M-Ca^{2+} saline (Fig. 7*A*), membrane depolarization around 0 mV induced frequent occurrence of unitary currents (Fig. 7*Ab*). Although detailed analysis was not carried out, it was obvious that the probability of channel opening increased with greater depolarization (Fig. 7*Aa*) and decreased with less depolarization (Fig. 7*Ac*). In contrast, when the pipette contained $5{\cdot}8 \times 10^{-8}$ M-Ca^{2+} (KCl saline in Table 1), the pulse-like current was rarely observed even at depolarization around 60 mV. When the potential inside the patch was made more positive, the occurrence of pulse-like currents increased and currents with multiple values of the unitary amplitude appeared (Fig. 7*B*). The currents in the specimen records showed triple steps of current amplitude at 147 mV. The rapid flickering of rectangular currents was recognized (Fig. 7*A* and *B*). In Fig. 7*C*, the unitary amplitude of the rectangular currents from four typical recordings are plotted against membrane potential. Each slope of the *I–V* relation in individual patches showed some variation (110–145 pS; $n = 6$) probably due to the limited range of reliable measurements on the abscissa and/or inaccurate amplitude determination in the presence of rapid flickering. As shown in Fig. 7*C*, it was possible to approximately fit the *I–V* relation of unitary currents by a straight line with a slope of 130 pS. When the line was extrapolated, it crossed the voltage axis at around -40 mV. However, as the *I–V* relation may presumably show positive curvature as it approaches the voltage axis (Pallotta, Magleby & Barrett, 1981; Benham, Bolton, Lang & Takewaki, 1986), the actual reversal potential may have been less than -60 mV.

These characteristics of outward currents in Leydig cells suggest that the currents are a type of 'Ca^{2+}-dependent K^+ current' with large unitary conductance. The special feature of the channel is that the range of membrane potential for activation of the channel seems to be beyond the physiological potential (more positive than 40 mV), at least in the cases examined with an internal saline containing $5{\cdot}8 \times 10^{-8}$ M-Ca^{2+}.

We were unable to consistently observe the other type of unitary current presumed to correspond to macroscopic Ca^{2+} currents recorded in the whole-cell configuration. This was probably due to the fragile nature of the functional Ca^{2+} channels or 'wash-out' of the channels (Fenwick, Marty & Neher, 1982).

Intercellular coupling

As mentioned above, some of the Leydig cells present in the chamber following isolation from each animal using a mild procedure appeared in clusters. For the characterization of this endocrine system, it is pertinent to study the existence of junctional intercellular communication among the cells (Loewenstein, 1981). We applied two patch electrodes to a pair of neighbouring cells in a cluster and recorded their respective membrane potentials (Fig. 8). In all of seven pairs of Leydig cells

examined in different clusters, obvious electrical couplings were revealed. The observed couplings were unlikely to have been artifacts of the recording system. Even when the tips of the two patch electrodes were located close to each other (around 10 μm) in the external medium, no measurable interference between the two recording systems was observed when maximum current pulses were applied (100 pA, 150 ms). From the plots shown in Fig. 8 (right), the average coupling ratio was found to be $0{\cdot}84 \pm 0{\cdot}08$ (S.D., $n = 7$).

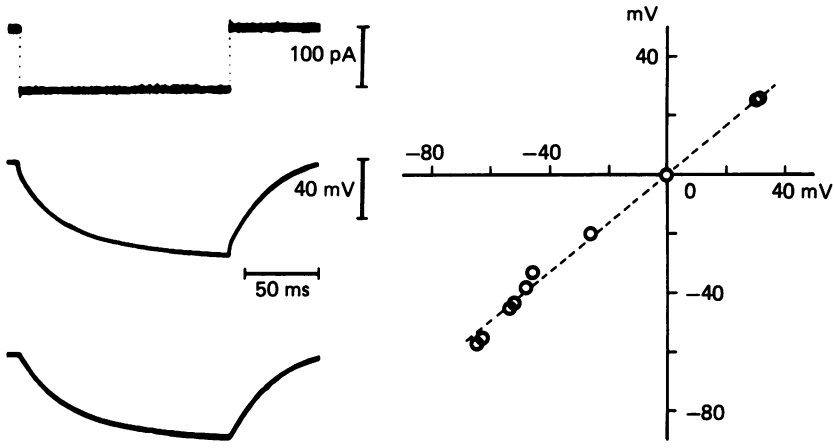

Fig. 8. Electrical coupling between Leydig cells. Two separate patch electrodes were applied to each of the two neighbouring cells in a cluster. Left, specimen records. When a hyperpolarizing current pulse (upper trace) was injected into a cell, its membrane potentials shifted (middle trace). Resistance of the electrode was 5 MΩ. Fast on-and-off relaxations are a transient artifact due to improper frequency compensation and are negligible in steady-state shift of the membrane potential. In an adjacent cell, a similar but slightly smaller shift of membrane potential was observed (lower trace). Right, relations between magnitudes of potential shift in a current-injected cell (ordinate) and those in the adjacent cell (abscissa). Slope of dashed line indicates a coupling ratio of 0·82.

To demonstrate the passage of dye molecules into the neighbouring cells in a cluster, the fluorescent dye Lucifer Yellow was injected into one of the cells in a cluster using a patch electrode. Seven injections of Lucifer Yellow were made into each Leydig cell in seven clusters; the number of cells constituting each cluster ranged from two to seven. The dyes were seen to diffuse into the neighbouring cells even by phase-contrast microscopy. When observed by fluorescence microscopy, all the cells in each cluster appeared well filled (Plate 1); the density of fluorescence in a particular cell, however, tended to decrease as its distance from the injected cell increased. These data obtained from electrophysiological and fluorescent-dye experiments thus indicated that intense intercellular couplings exist among testosterone-secreting cells in the mouse. These observations are consistent with electron-microscopic findings of characteristic intercellular junctional coupling structures in mammalian Leydig cells *in vivo* (Christensen, 1975; Nagano & Suzuki, 1976). Thus, it is unlikely that the couplings were artificially induced as a result of the isolation procedure.

DISCUSSION

The electrical properties of the plasma membrane in the testosterone-secreting cells (i.e. Leydig cells) of the mouse were studied using patch-electrode voltage clamp. The Leydig cells were shown to generate action potential-like responses when the cells were electrically stimulated. In the Leydig cell membranes at least two kinds of voltage-dependent ion channels existed. One was a Ca^{2+} channel, the activation of which led to the generation of action potential-like responses, while the other was a Ca^{2+}-dependent K^+ channel with a high threshold potential and a large unitary conductance (130 pS). Furthermore, the Leydig cells occurring in cluster were shown to be coupled to one another in terms of passage of ionic currents and transfer of injected fluorescent dyes.

As pointed out by Hagiwara & Byerly (1981) and by Hille (1984), the presence of voltage-dependent Ca^{2+} channels seems to be more scattered than previously considered. Some endocrine cells are reported to possess voltage-dependent Ca^{2+} channels in the membrane (adrenal medullary cells, Fenwick, Marty & Neher, 1982; clonal pituitary cells, Hagiwara & Ohmori, 1982; pancreatic β-cells, Rorsman & Trube, 1986) which probably participate in controlling the secretion of hormones (Williams, 1981; Ozawa & Sand, 1986). It is relevant to note that in adrenal cortical cells in the rabbit, Matthews & Saffran (1973) have described spontaneous electrical activity in the presence of adrenocorticotrophic hormone (ACTH) and the absence of external K^+, which is resistant to tetrodotoxin. Similar action potentials have also been reported in the cells of the adrenal cortex of the cat which may secrete mineral corticoids (Natke & Kabela, 1979), or in an adrenocortical cell line (Y-1 cells, Tabares & López-Barneo, 1986). The presence of voltage-dependent Ca^{2+} channels in dissociated zona glomerulosa cells from the rat adrenal cortex was recently confirmed using the whole-cell patch-clamp technique by Maruyama, Matsunaga & Hoshi (1986). The gating properties of Ca^{2+} channels in endocrine cells, however, seem to differ among tissues. The Ca^{2+} currents in adrenal medullary cells, clonal pituitary cells and pancreatic β-cells show negligible or slight inactivation during maintained depolarization, while those in Leydig cells and in zona glomerulosa cells show remarkable inactivation (Fig. 4). The existence of more than one kind of Ca^{2+} channel and the functional significance of this have been described in neurones and heart cells (Nowycky, Fox & Tsien, 1985; Bossu *et al.* 1985; Bean, 1985; Nilius, Hess, Landsman & Tsien, 1985; Matteson & Armstrong, 1986; Narahashi *et al.* 1987). This is also probably true for endocrine cells. At present, we have no definite idea about the functional role of the transient-type Ca^{2+} channels in Leydig cells. In dissociated Leydig cells from the mouse, the secretion of testosterone from the cell in response to stimulation with luteinizing hormone has been shown to be suppressed in the presence of the Ca^{2+} channel blocker, Co^{2+}, in a dose-dependent manner (Moger, 1983). Reduction of extracellular Ca^{2+} concentration is also known to decrease this luteinizing hormone-stimulated testosterone production (Janszen, Cooke, van Driel & van der Molen, 1976). Although the process of hormone production and the mode of secretion in endocrine cells which secrete steroid hormones may be different from those aminergic or peptidergic cells (Christensen, 1975), the participation of Ca^{2+} ions in regulating the production of steroid hormones in the cells may also occur via

voltage-gated Ca^{2+} channels (Lymangrover, Matthews & Saffran, 1982; Aguilera & Catt, 1986).

The presence of Ca^{2+}-dependent K^+ channels has recently been described in a variety of tissues including exocrine cells (Petersen & Maruyama, 1984; Findlay, 1984) and endocrine cells (Atwater *et al.* 1979; Cook, Ikeuchi & Fujimoto, 1984; Tabares *et al.* 1985). In dissociated Leydig cells from the rat, Ca^{2+}-activated K^+ permeability has also been described (Joffre, Mollard, Régondaud & Gargouil, 1984). The large unitary conductance of the present preparation (130 pS, Fig. 7) is consistent with the observation of Ca^{2+}-activated K^+ channels in other tissues (Marty, 1981, 1983; Pallotta *et al.* 1981; Blatz & Magleby, 1984; Marty & Neher, 1985; Benham *et al.* 1986). One interesting feature of the K^+ channel in Leydig cells is that the threshold potential for activation of the channel seemed to have a value more positive than 30 mV when investigated using a patch electrode containing $5{\cdot}8 \times 10^{-8}$ M-Ca^{2+} (Figs 5 and 7). At present, it is difficult to explain the mechanisms and the physiological role of such a high threshold, but it is unlikely to be the result of an artifact of internal cell perfusion since a comparable threshold potential was observed immediately after the establishment of a whole-cell configuration (Fig. 5*B*). Although we were unable to exclude the possibility that the sensitivity of the K^+ channels to internal Ca^{2+} may change according to the metabolic state of the cell or to the animal species used, the observed conductance of Ca^{2+}-activated K^+ channels in rat Leydig cells at a membrane potential of between -25 and -35 mV (Joffre *et al.* 1984) was probably due to the activation of the channels by increased internal Ca^{2+} during or after impalement of the cells.

It has been documented that intercellular couplings may be present in a variety of tissues (Loewenstein, 1981; Verselis, White, Spray & Bennett, 1986), and the molecular mechanisms have recently been analysed using single-channel current recordings (Neyton & Trautmann, 1985; Veenstra & DeHaan, 1986). Among representative cells of the endocrine system, electrophysiological studies on the β-cells of pancreatic islets have revealed definite electrical couplings between the cells (Meissner, 1976). Also in adrenal cortical cells from the rabbit, Matthews & Saffran (1973) have described the phenomenon of fluctuation in resting membrane potentials which are suggestive of electrical couplings among the cells. The present study has provided direct evidence of intercellular couplings in the testosterone-secreting cells of the mouse (Fig. 8 and Plate 1). Ultrafine morphological studies using electron microscopy have revealed corresponding membrane structures of Leydig cells *in vivo* (Christensen, 1975; Nagano & Suzuki, 1976). The mean coupling ratio of 0·84 obtained above might be an underestimate; the actual ratio might have been greater if the leakage between the recorded cell and the tip of the applied patch electrode had been cancelled. The biological significance of such intense intercellular coupling has yet to be clarified. It may serve as a pathway among cells for the exchange of intracellular messenger molecules or metabolic substances for the control of synchronous or intensified endocrine activities (Loewenstein, 1981). It is, however, of interest to note that in the progesterone-secreting cells of ovine corpora lutea, intercellular couplings were not detected either by electrical measurement or by dye diffusion methods (Higuchi *et al.* 1976).

The secretory activities of Leydig cells are remarkably influenced by tropic

hormones (Christensen, 1975; Ewing & Zirkin, 1983). An understanding of how the Ca^{2+} channels, the K^+ channels and the intercellular couplings participate in this process or how they are modulated by the changes in secretory activity may be the next key step for clarifying the function of the cell membrane in Leydig cells.

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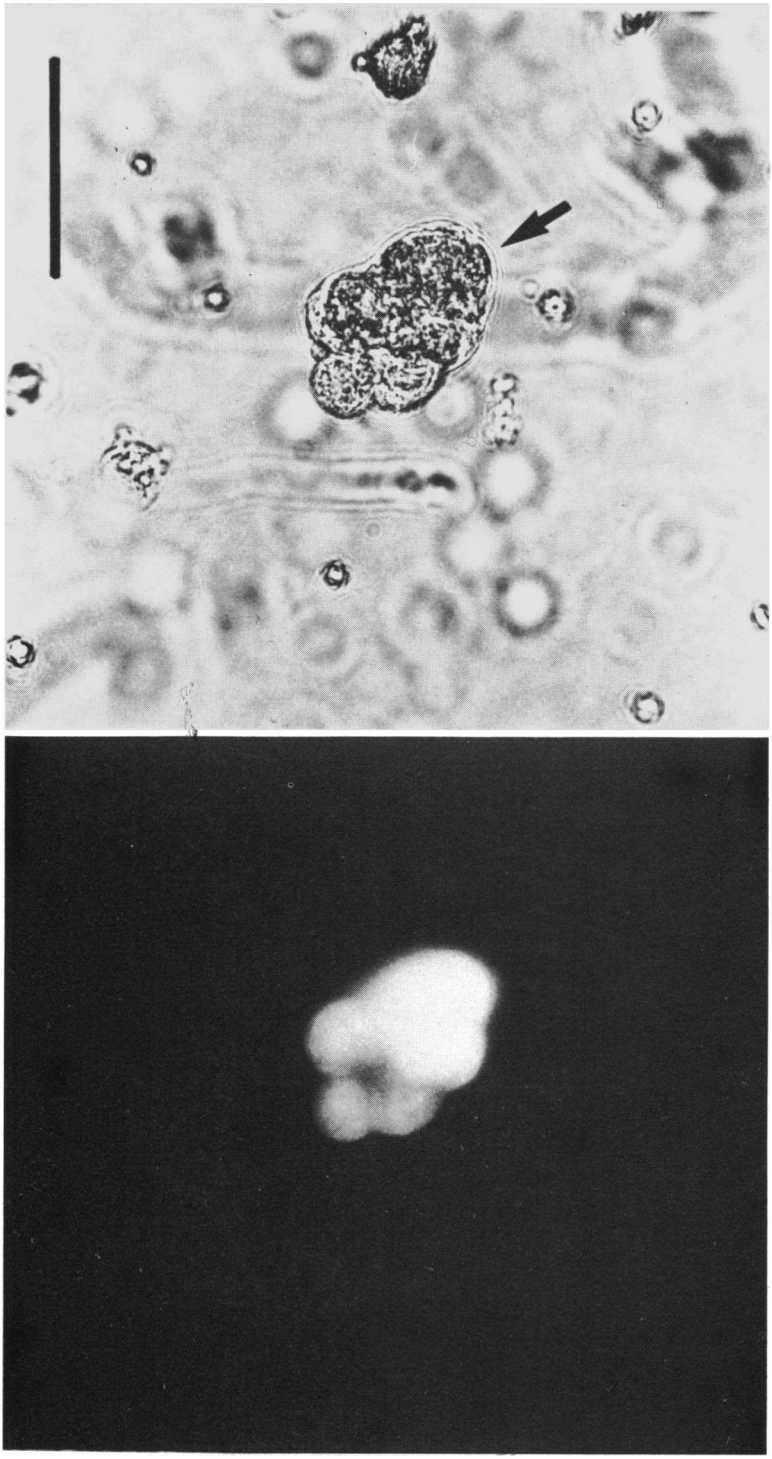
EXPLANATION OF PLATE

Intercellular couplings among Leydig cells revealed by injection of a fluorescent dye. Above, a cluster of Leydig cells photographed with transmission illumination. The cluster in the centre consists of seven cells. Arrow indicates the cell where a patch electrode containing Lucifer Yellow CH was applied. Below, epifluorescence view of the same field as above. The photograph was taken about 10 min after the internal application of Lucifer Yellow CH. Each cell in the cluster was stained, the intensity of staining decreasing as the distance from the injected cell increased. Scale bar, representing 50 μm, applies to both plates.



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