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Calcium and Potassium Currents in Porcine Granulosa Cells Maintained in Follicular or Monolayer Tissue Culture

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Abstract. We studied membrane currents in granulosa cells (GC), immediately after collection or after variable culture time in the everted-follicle wall or in the monolayer.

GC in both systems express an inward calcium current (I_{Ca}) with T-type kinetics and voltage dependence. GC in the everted-follicle culture express an outward potassium current (I_K) kinetics, which remains unchanged during three days in culture. I_K has delayed-rectifier kinetics, but is insensitive to TEA, 4-AP and apamine. GC in monolayer culture develop a new, inactivating delayed-rectifier potassium current (I_{nK}), which progressively dominates as cells advance from day one to day three in culture. A similar I_{nK} was recorded in large luteal cells. A possible link between luteinization and the appearance of I_{nK} is hypothesized.

Key words: Pig follicular cells—Tissue culture—Whole-cell recording—Ca current—K current—Luteinization

Introduction

Granulosa cells play a pivotal role in the reproductive process. They provide support for oocyte growth and maturation, contribute to the endocrine milieu leading to ovulation and, successively, they differentiate into luteal cells.

The function of these cells is under the control of several paracrine and endocrine factors, and complex signal transduction mechanisms involving internal calcium and cyclic AMP have been described.

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In this context, we have recently shown that LH-induced increase of intracellular calcium clearly modifies the electrical properties of the plasma membrane of these cells, effecting a specific K current (Mattioli, Barboni & Seren, 1991). Although modifications in the physical properties of the membrane have been observed in many cell types, a precise relationship with cell function is still largely unknown. Changes at the level of the plasma membrane may participate in the transduction mechanism for different endocrine signals or, as a result of the hormonal stimulation, may set the cell into a new functional status. A persistent modification of membrane permeability may, in fact, represent a means of changing the interaction with the extracellular environment. This is consistent with modifications in the membrane currents which accompany the differentiation process of different cell types such as lymphocytes and monocytes.

Little information is presently available on the electrical properties of granulosa cell membranes. Previous electrophysiological studies (Asem et al., 1988; Schwartz et al., 1989) reported both calcium and potassium channels on the membrane of chicken granulosa cells, and preliminary experiments described the presence of potassium and calcium currents in swine granulosa cells. To acquire more information on the biophysical properties of granulosa cells, we studied electrical currents through their plasma membrane, using the patch-clamp technique in the whole-cell configuration. The study was conducted both in freshly isolated swine granulosa cells and in cells maintained in culture under different conditions. We also analyzed membrane currents in luteal cells as they are the final differentiation step of granulosa cells.

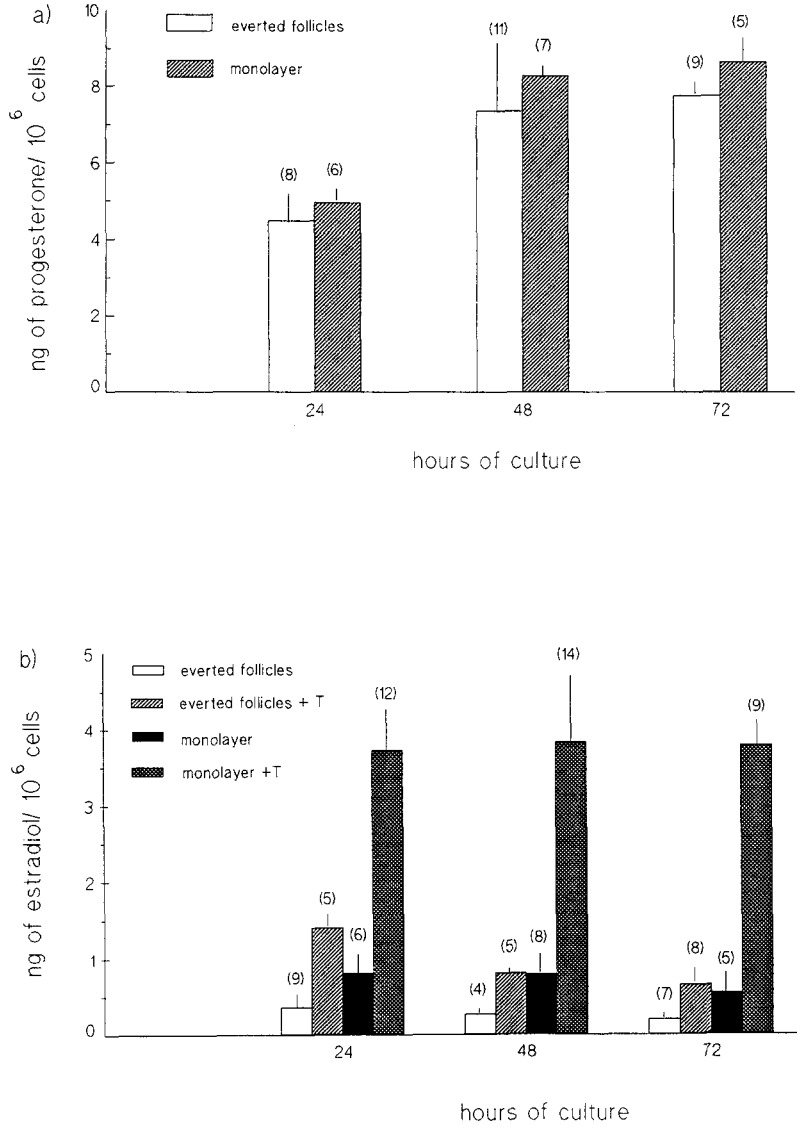

Fig. 1. Steroidogenic activity of granulosa cells cultured in the wall of the everted follicles or in monolayer. (*a*) Progesterone production in granulosa cells cultured in monolayer (shaded bars) or maintained in the everted follicle wall (open bars). The values are mean ± SEM. The number of replicates per group is given in parentheses. (*b*) Estradiol production in granulosa cells cultured with or without testosterone (1 μg/ml) in monolayer or maintained in the everted follicles.

Materials and Methods

Isolation and Culture of Granulosa Cells

Individual follicles were dissected from pig ovaries collected at a local slaughterhouse. We prepared the everted follicles following a previously described procedure (Mattioli et al., 1989) and cultured them in modified TCM 199, supplemented with 10% fetal calf serum, 100 μg/ml streptomycin, and 100 IU/ml penicillin. Under these conditions, granulosa cells maintained their natural localization in the mural granulosa for 2–3 days. Longer cultures were unachievable because the follicular wall gradually lost its structure. The everted follicle has been used successfully to mature pig oocytes in vitro (Mattioli et al., 1989). Granulosa cells for the monolayer cultures were obtained from healthy follicles by blowing dissection medium (Dulbecco's phosphate buffered saline containing bovine serum albumin 0.4%, w/v, pyruvate 0.36 mM, glucose 5.5 mM and kanamycin 70 μg/ml) onto the granulosa layer of the opened follicle. The medium was collected and centrifuged at 800 rpm for 8 min. Pelleted cells were resuspended in culture medium, seeded in 35 mm petri dishes, and incubated at 39°C in an atmosphere of 5% CO_2. After 12 hr of culture, unplated dead cells and cellular debris were removed by renewing the medium. Monolayered culture experiments were performed with approximately 10^6 viable cells/cm^2 in plastic, multiwell plates. At this density, most of the cells were not in contact with each other, enabling study of the single cell.

Isolation of Luteal Cells

Membrane currents were also studied in large luteal cells that are believed to derive from granulosa cells (Hansel & Dowd, 1986). Swine corpora lutea of day 8–12 of the estrous cycle (day 0 = estrus) were collected immediately after slaughter, minced and initially dissociated in 0.1% collagenase-hyaluronidase

(Sigma, St. Louis, MO) in dissection medium for 40 min at 39°C. Tissues were fully dissociated into single cell suspension by 0.3% trypsin, 1 mM ethyleneglycol-bis(β-amino-ethylether) N,N,N′,N′-tetraacetic acid (EGTA, GIBCO) treatment. The incubation was terminated by adding 0.3% lima bean trypsin inhibitor (Sigma). The cellular suspension was then incubated for 4 hr in culture medium for the cells to recover from the enzymes. Finally, the cells were placed in the recording chamber and only large luteal cells (>30 μm of diameter) were recorded.

Measurement of Estradiol and Progesterone Secretion

Steroidogenic activity of granulosa cells was evaluated after 24, 48 and 72 hr of culture. At these times the cells maintained in the everted follicle wall were mechanically removed by a flow of dissection medium and cultured for 2 hr in culture medium at a final concentration of 2–3 × 10^6 cells/ml. Cell suspensions were then centrifuged at 800 rpm for 10 min, and the supernatant was collected and frozen until assayed for its steroid content. Steroid production by plated cells was evaluated at the same times by replacing with fresh medium and collecting it 2 hr later. The medium samples were then stored at −20°C until assayed. To determine whether the culture conditions could affect aromatase activity, the production of estradiol was evaluated in cells cultured during the test period, either in medium alone or in medium supplemented with 1 μg/ml of testosterone. Progesterone and estradiol 17β in the medium were evaluated by validated RIAs.

Electrodes, Solutions and Recording Techniques

Granulosa cells were isolated from the follicle wall and placed in the registration chamber containing (mM): 130 NaCl, 3 KCl, 10 $CaCl_2$ and 4 $MgCl_2$ (pH 7.2). Monolayered cells were recorded in their original dish after replacing the medium with the recording solution. The 20 mM Ca bath solution contained 115 mM NaCl but was otherwise the same. The whole-cell electrode solution contained (mM): 140 KCl, 1 EGTA and 10 HEPES (pH 7.2). The electrodes were made from borosilicate glass with a horizontal puller (Sutter P87), and stored in a dry vacuum oven at 100°C for up to a week. The uncoated tips were fire-polished to 1–2 μm outside diameter just before using them. Filled pipettes had a resistance of 5–20 MΩ when dipped into the bath solution.

To evaluate the possible role of intracellular components, the nystatin perforated-patch method (Horn & Marty, 1988) was used in some of the cells studied. In brief, nystatin was first dissolved in methanol at the final concentration of 50 mg/ml. Just before recording, this stock solution was diluted with the electrode solution to obtain a working concentration of 50 μg/ml. Solubilization of the drug could be achieved only for short periods and after heating the solution at 70°C. To reduce problems in establishing high resistance patches when nystatin was present in the electrode solution, the electrode tips were dipped in nystatin-free solution and the electrodes were then backfilled with 50 mg/ml nystatin.

We used a List EPC-7 amplifier to measure the whole-cell current and an ITC 16 interface connected to an Atari Mega4 computer running a specific program (Patch Prg., Instrutech) to store and analyze data. Capacitive and leak currents were always subtracted.

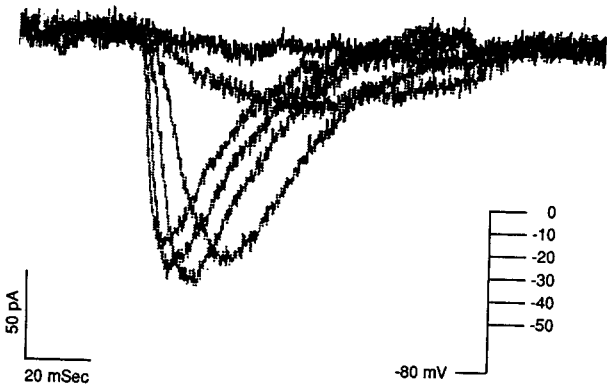

Fig. 2. Ca current from whole-cell recordings of granulosa cells at day one of an everted-follicle culture. Traces are corrected for leak and capacitive currents. The inset shows the voltage protocol. The time between test potentials is 3 sec; 70% of granulosa cells display such Ca currents (n = 195), independent of the culture system. The experiments on monolayer cultured cells are virtually identical (*data not shown*).

Results

Steroid Secretion

Culture conditions did not seem to substantially affect the amount of progesterone produced by granulosa cells. In both systems, progesterone output progressively increased throughout the culture as shown in Fig. 1*a* with similar values recorded in the two culture systems ($P > 0.1$, Student's t-test). By contrast, the basal production of estradiol was significantly higher ($P < 0.01$) in plated cells than in cells cultured in the follicle wall (Fig. 1*b*). Moreover, the addition of testosterone to the culture medium increased estradiol production 5–7 times in plated cells ($P < 0.01$) and only 3–4 times ($P < 0.01$) in follicle-wall-cultured cells.

Calcium Current

The Ca current is too small for detection in physiological concentrations of Ca. Holding the cells at −80 mV and placing them in the 20 mM Ca solution, we observed that 70% of granulosa cells (135 out of a total of 195 cells) monitored immediately after isolation or maintained in culture for one or more days have Ca currents. In other cells, too, it is difficult or impossible to study Ca currents because of the relatively small conductance in normal concentrations of Ca. More permeable ions, such as Ba, are often used for this reason. However, Ba changes not only the conductance but also the kinetics and pharmacology of Ca channels,

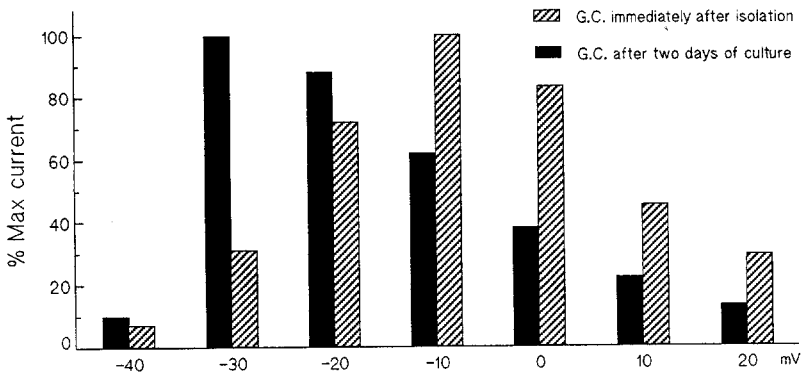

Fig. 3. Relative Ca current amplitude in granulosa cells recorded immediately after isolation or after two days of culture. The cells were held at −80 mV and stepped for 200 msec at the potentials indicated on the X axis.

and in high concentrations, Ba is lethal to oocytes (Dale et al., 1989). For these reasons, we did the experiments in Ca instead of Ba. The elevated Ca appeared to have minimal side effects, and the calcium currents could be analyzed in concentrations as low as 20 mM Ca. Under these conditions the current had rapid activation and inactivation phases. The peak Ca current was U shaped as a function of test voltage (Fig. 2), and on day one the maximum amplitude ranged from 20 to 200 pA. There was no apparent relationship between cell size and current amplitude. Holding the cells at −40 mV abolishes this current. I_{Ca} is totally blocked by 30 mM Co and 1 mM Cd, but it is insensitive to nifedipine up to 100 μM. Current kinetics and its sensitivity to Co, Cd and nifedipine were similar when the cells were recorded by the nystatin perforated-patch method. Thus, intracellular components do not seem to influence characteristics of this Ca current which are typical of T-type Ca channel kinetics.

Immediately after isolation, granulosa cells had a peak I_{Ca} at a test potential near −30 mV. After keeping the cells in both kinds of culture for one or more days, we observed that, although the range of current amplitudes was unchanged, the voltage where the peak occurred shifted to the right, between −10 and 0 mV (Fig. 3).

Delayed-Rectifier Potassium Current

Holding the cells at −80 mV and placing them in normal bath solution, we observed that 68% of the granulosa cells (57 out of 85 cells), monitored immediately after isolation, had K currents (Fig. 4, I_K). The current had a slow, somewhat delayed activation phase but no inactivation during 1,000 msec. Tail currents reverse between −90 and −100 mV; they are approximately exponential for inward currents but nearly flat for outward currents (Fig. 5). The plateau current varied linearly with test voltage,

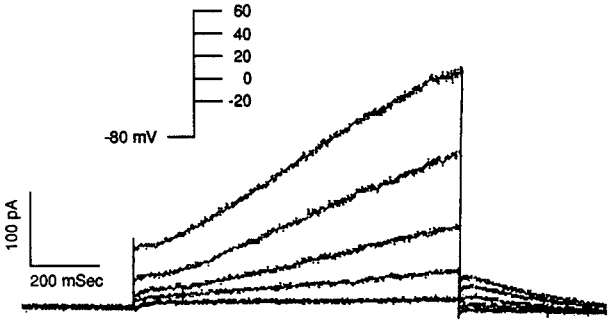

Fig. 4. Delayed potassium current recorded in granulosa cells immediately after isolation.

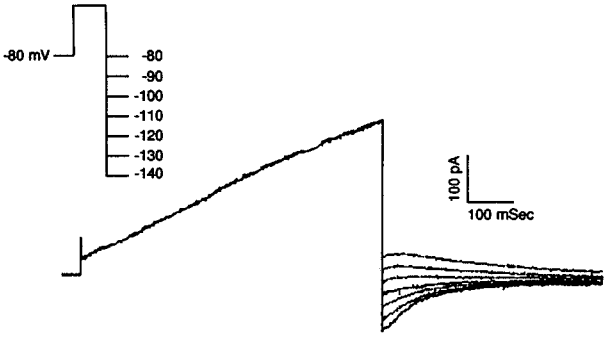

Fig. 5. Tail current analysis of I_K. The inset shows the voltage protocol; the time between test potentials is 12 sec, but it may be as short as 200 msec without effect. The current reverses between −90 and −100 mV.

and ranged from 100 to 500 pA at a test potential of 60 mV with no apparent relation to cell size. Holding the cells at −40 mV had no effect on this current. These characteristics are typical of the delayed rectifier. In none of the cells recorded did TEA (tetraethylammonium, 8 mM; n = 16 cells), 4-AP (4-aminopyridine, 1 mM; 23 cells), apamine (20 μM; 11 cells), Cd (1 mM; 15 cells) or Co (30 mM; 17 cells) inhibit I_K. This current is maintained unchanged in

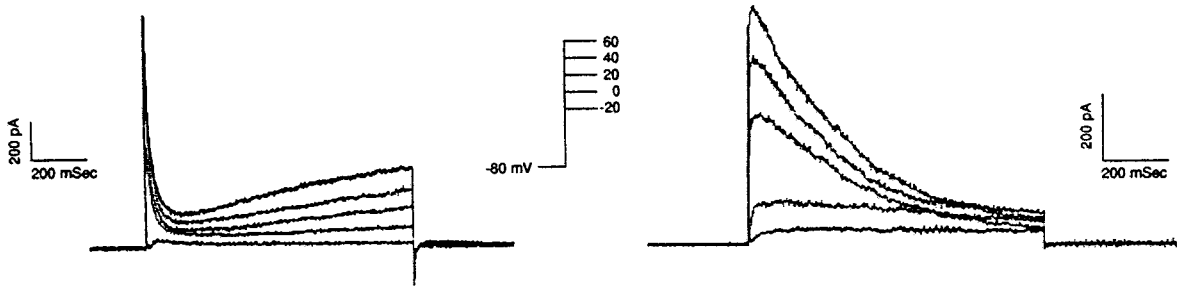

Fig. 6. Outward K currents from whole-cell recordings on a granulosa cell in the monolayer culture on day one (left) and day three (right). The inset shows the voltage protocol. The time between test potentials is 12 sec, and there is a marked effect if this time is reduced (*see text*).

cells cultured in the everted follicles for 24–48 hr (Fig. 8).

Inactivating Delayed-Rectifier Current

After one day in monolayer culture, I_K declined and a second K current emerged in a proportion of cells (6 out of 48 cells). During second day in monolayer culture, both I_K and I_{nK} were clearly visible, while after three days only I_{nK} was detectable (45 out of 49 cells, *see* Fig. 8). Maximum current amplitude (measured at a test potential of 60 mV) ranged between 100 and 500 pA, occasionally reaching values up to 1 nA after three days of culture. As with Ca and K currents, there was no apparent relationship between the amplitude of I_{nK} and the size of the cell. Holding the cells at −80 mV and placing them in the normal bath solution, we observed that virtually all granulosa cells (48 out of 56) monitored after two days in culture have I_{nK} currents. This current had a rapid activation followed by a slower inactivation phase, which shifted from a 10 to a 300 msec time constant depending on the day of culture (Fig. 6). Full recovery from inactivation required an interpulse interval of 10–30 sec. Half recovery occurred in 2.5 sec on day one of culture but in 15 sec on day three. The peak current increased monotonically with a level test voltage. Holding the cells at −40 mV (57 cells) or adding 4-AP (1 mM, 26 out of 28 cells) to the bath abolishes I_{nK} (Fig. 7). These characteristics are typical of the inactivating delayed-rectifier current.

Large luteal cells displayed I_{nK} similar to that observed in granulosa cells in monolayer culture. Long-lasting inhibition followed each stimulation step, and maximum current amplitude was achieved with interpulse intervals > 40 sec (Fig. 9). As observed in monolayered cells, this current inactivated completely from holding potentials > −40 mV, and was blocked by 1 mM 4-AP.

Discussion

Cell function of granulosa cells is controlled by a variety of endocrine factors such as LH, FSH, etc. The transduction of these hormonal signals may involve changes in the ionic compartments of the cells. Studying the electrophysiological properties of granulosa cells may help us understand the mechanisms that control their function, their influence on oocyte maturation and, possibly, their differentiation into luteal cells. Granulosa cells are nonexcitable. They contain Ca channels like those found in other nonexcitable cells, such as chromaffin cells (Fenwick, Marty & Neher, 1982), pituitary cells (Cota, 1986; Matteson & Armstrong, 1986) and glomerulosa cells (Durroux, Gallo-Payet & Payet, 1988).

These Ca channels are also similar to those found in excitable cells, such as cardiac myocytes and neurons. Generally, Ca currents have at least two components: (i) L-type, a slow component activated at relatively large depolarizations from a holding potential of −80 mV, and (ii) T-type, a faster transient component activated at smaller depolarizations (Hagiwara & Najaima, 1966; Nilius et al., 1985; Hagiwara, Irisawa & Kameyana, 1988). L-type currents are more likely to be involved in control of cytosolic background levels of Ca, whereas T-type currents influence transitory levels of Ca. Cells may have only L- or T-type, or they may have both in various proportions. For example, seven-day embryonic chick ventricle cells have L-type currents (Mazzanti, De Felice & Liu, 1991), whereas canine atrial cells have L- and T-type (Bean, 1985).

A previous study reported both T- and L-type currents in granulosa cells obtained by dissection of large, preovulatory chicken follicles (Schwartz, et al. 1989). The classification was based on single channel records with 20 mM Ba in the bath solution and 110 mM Ba in the cell-attached patch solution.

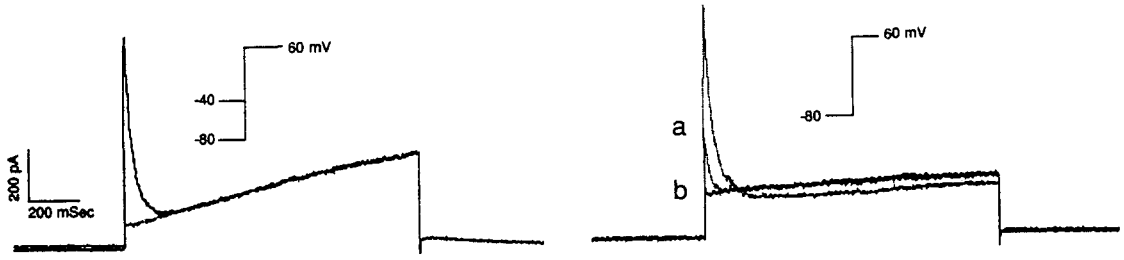

Fig. 7. Granulosa cells in monolayer culture. The graph on the left illustrates the sensitivity of I_{nK} to voltage. The graph on the right illustrates the sensitivity of I_{nK} to 4-AP perfusion. The insets show the voltage protocols used in these two experiments. On the right, currents were recorded at (*a*) 30 sec or (*b*) 90 sec after adding 1 mM 4-AP to the bath. Time between test potentials is 12 sec, with a marked effect if reduced.

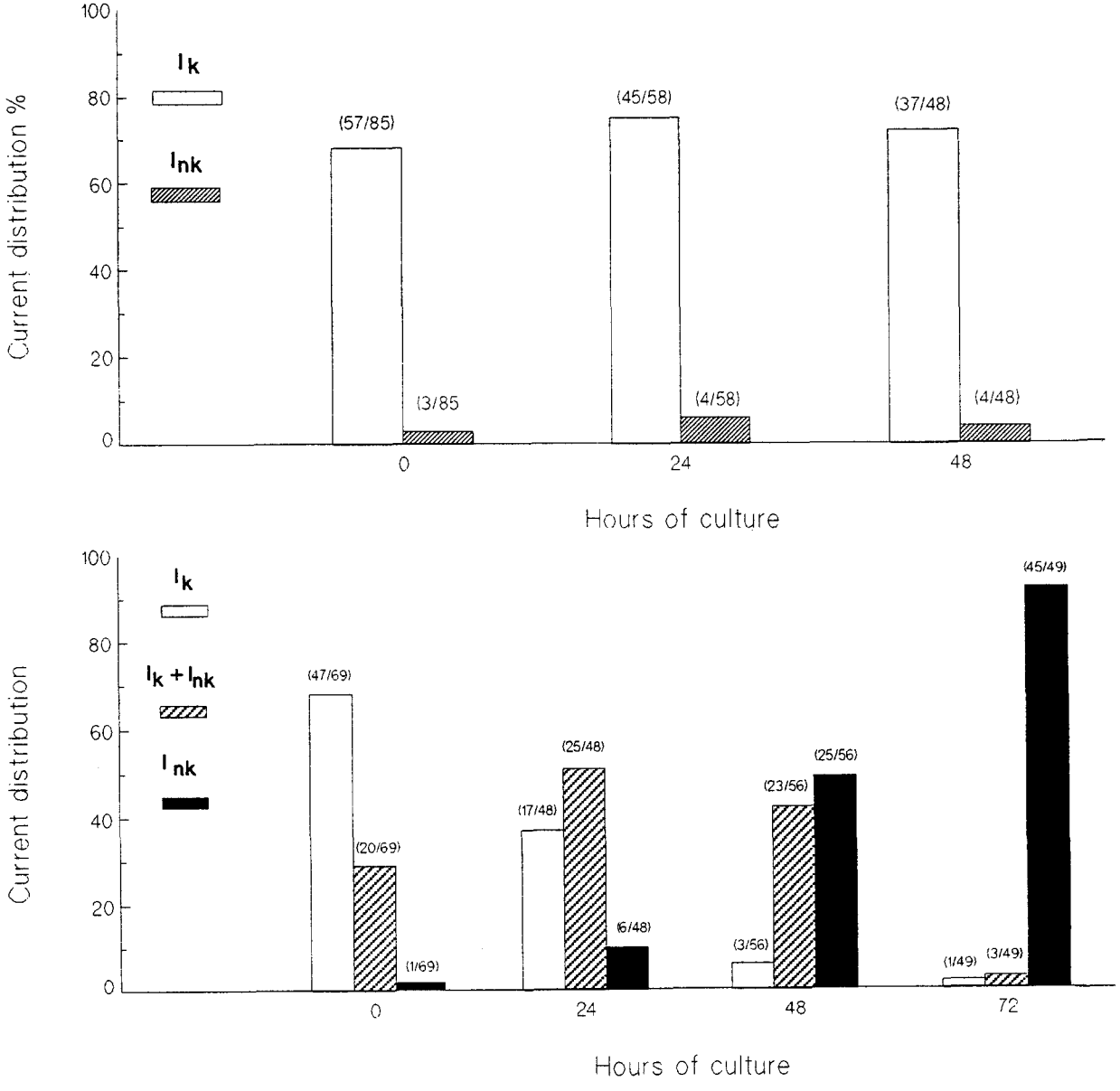

Fig. 8. Relationship between I_K and I_{nK} in granulosa cells maintained in culture in the everted-follicle wall (top panel) or in monolayer (bottom panel). In the monolayer I_K is progressively substituted by I_{nK}.

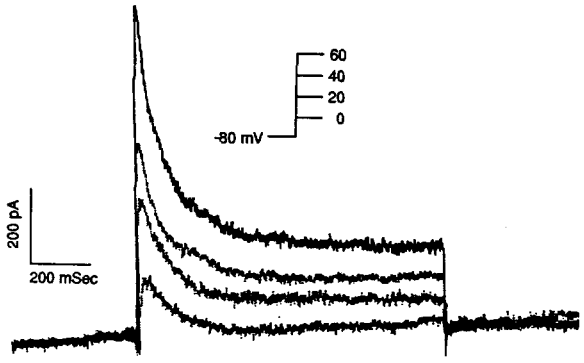

Fig. 9. I_{nK} recorded from large luteal cells (*see text* for details). The inset shows the voltage protocol. The time between test potentials is 12 sec, with a marked effect if reduced.

The T-type channels inactivated and had small conductances (4–5 pS).

The L-type channels had large conductances (20–30 pS). As noted, L-type currents most likely control cytosolic Ca. However, Schwartz et al. (1989) reported that the L-type channels rarely opened in the chicken granulosa cells unless the dihydropyridine agonist, Bay K-8644, was added. After increasing their activity in Bay K, Schwartz et al. showed that the channels could be blocked by the dihydropyridine antagonist, nifedipine, further identifying them as L-type channels.

Because dihydropyridine agonists are presumably not present under physiological conditions, their contribution to normal function is uncertain. However, these authors proposed that hormones activate the Ca channels by depolarization of the membrane or by second messenger pathways. The resulting surge of Ca, they suggest, may regulate granulosa cell function, such as the release of regulatory proteins or steroidogenesis.

The Ca current we observe in pig granulosa cells is most likely carried by T-type Ca channels. After 2–3 days of culture the potential required to elicit the maximum Ca current shifts to more positive values, and the absolute current becomes smaller. If the Ca entering by this pathway were involved in activating a particular cell function, several days in culture should render the cells less reactive in performing that function.

Delayed-Rectifier Channels

Asem et al. (1988) report two kinds of K channels in chicken granulosa cells. Their classification was based on single channel records with 5 mM K in the bath solution and 140 mM K in the cell-attached patch solution. In the inside-out patch configuration, the solutions were changed to 140 mM K on both sides of the membrane, and Ca was titrated in the bath. One of these K channels is called g_{K1} (not to be confused with the ubiquitous I_{K1}). It had a conductance of 15–30 pS, and its gating depended on neither voltage nor Ca. The second K channel, called g_{K2}, had a conductance of 160–195 pS. It was activated by a depolarizing voltage and by an elevation of Ca on the cytoplasmic side of the membrane. The physiological role of g_{K1} is unclear. Asem et al. suggest it is involved in maintaining the resting potential, but the evidence is scant. g_{K1} corresponds to no known channel. g_{K2} appears to be the Ca-activated K channel, $I_{K(Ca)}$. Asem et al. suggest that g_{K2} may repolarize granulosa cells after the increase in intracellular Ca associated with hormone-induced steroidogenesis. Repolarization would then turn off the Ca signal that had turned on K(Ca). Such feedback mechanisms are well established in other systems. For example, Sherman, Keizer and Rinzel (1990) have modeled the interaction between K(Ca) channels and Ca channels in cells. Unfortunately, g_{K2} is infrequently observed in chicken granulosa cells, and its contribution to their function remains uncertain.

The K currents we observe in pig granulosa cells are most likely carried by the delayed-rectifier, I_K, and the inactivating delayed-rectifier, I_{nK} (*discussed below*). The current we identify as I_K has reversal potential near −90 mV in normal solutions, activates slowly, is not saturated 1 sec after the test potential, and does not depend on holding potential. These characteristics are typical of the delayed-rectifier in cardiac cells (Simmons, Creazzo & Hartzell, 1986; Clapham & Logothesis, 1988). In spite of these similarities, I_K is resistant to the most widely used delayed-rectifier channel blockers, TEA and 4-AP. The delayed-rectifier belongs to a large class of K-selective channels, including the inactivating delayed-rectifier and the Shaker H4 channel. These K currents share many common features, and all are blocked by TEA from the inside. Block from the outside, however, is highly variable from cell type to cell type, and the blocking concentration varies over orders of magnitude. For example, rapidly activated neuronal delayed-rectifier channels block completely in the node of Ranvier in 6 mM TEA, whereas no block occurs in the squid giant axon up to 250 mM TEA (Armstrong & Hille, 1972). Furthermore, MacKinnon and Yellen (1990) show that, whereas wild-type Shaker K channels are sensitive to external TEA (K_D = 27 mM), mutants with one lysine substitution in the S5–S6 linker region have either reduced sensitivity (K_D = 66 mM, position 431) or abolished sensitivity (position 449). Thus, the nonblock of the pig follicular cell I_K in externally

applied concentrations of TEA up to 10 mM does not disqualify it as a delayed-rectifier current.

In excitable cells the delayed-rectifier is crucial to the balance of current during the plateau phase and to the repolarization current during the action potential. In granulosa cells, I_K probably helps maintain the cell resting potential. It becomes secondary to another K current that appears only in monolayer cultures and in luteal cells.

Inactivating Delayed-Rectifier Current

If we remove granulosa cells from the follicle and culture them in monolayer, the delayed-rectifier current is overcome by another K current with completely different characteristics. This new component has similar kinetics to the inactivating delayed-rectifier, I_{nK}, so named because it activates like I_K but inactivates during sustained voltage. (I_K may also inactivate in some cells, but on a much longer time scale, and to a lesser degree, than I_{nK}. It never displays the slow recovery from inactivation of I_{nK}). I_{nK} has been described in a developmental study of K conductance in mouse macrophages (Ypey & Clapham, 1984) and in T lymphocytes (DeCoursey et al., 1984; Fukushima, Hagiwara & Henkart, 1984; Matteson & Deutsch, 1984; Cahalan et al., 1985). Recovery from inactivation, typically 30 sec at room temperature, requires a much longer time than either activation or inactivation. The distinguishing property of I_{nK} is diminution of peak current during repetitive depolarizations.

In T lymphocytes before the onset of immunological competence, the dominant K current is I_{nK}. Using epifluorescence to identify developing thymocytes, Lewis and Cahalan (1988) described three kinds of K current in the mouse, I_{nK}, $I_{n'K}$ and I_{1K}. The n- and n'-type are blocked by 5 nM CTX (charybdotoxin), but the 1-type is not. Functionally immature thymocytes have a high density of n-type channels (1–3/μm^2) and no n'- or 1-type channels. In thymocytes with mature phenotypes, K channels correlate with immunological function. Those destined to become helper T cells that recognize class II antigens express n-type channels at one-tenth to one-fifth the density of immature thymocytes. Those destined to become suppressor T cells that recognize class I antigens express n'- and 1-type in various proportions. (Expression was heterogenous, and roughly 10% of prospective suppressor cells had predominantly• n-type channels). This work suggests that n-type channels are required for T cells to proliferate in response to mitogens (Chandy et al., 1986; Deutsch, Krause & Lee, 1986). This interpretation is supported by the observation that, in the thymus, large, actively cycling cells have a greater proportion of n-type channels than do quiescent cells (Scollary, Barlett & Shortman, 1984). Exposing lymphocytes to K channel blockers represses thymidine uptake, further suggesting that n-type K channels are needed for proliferation.

The n-type inactivating delayed-rectifier is well characterized in a variety of other cells, including macrophages, Schwann cells, and Leydig cells (Ypey & Clapham, 1984; Gallin & Sheehy, 1985; Konishi, 1989; Duchatelle & Joffre, 1990). All are nonexcitable, pluripotential cells capable of further differentiation, and in some of them, a direct link between n-type K channels and cell proliferation has been established (Konishi, 1989). Plated granulosa cells exhibited a K current clearly distinguished from that observed in freshly isolated granulosa cells. Which factor triggers this transformation is, as yet, unclear. A possible cause of these modifications may be the disruption of all the intercellular connections between granulosa cells during preparation of cell suspension. A similar event occurs in vivo after the LH surge when the junctional area between granulosa cells undergoes a rapid and extensive reduction (Larsen, Wert & Brunner, 1986). Another possible factor triggering this transformation may be cell plating. Plating is known to markedly affect cell function, and granulosa cells under these conditions have been shown to undergo a progressive luteinization in vitro (McNatty & Sawers, 1975), even if adding gonadotropins to the culture is required to complete the process (Channing, 1970; Van Thiel, Bridson & Kohler, 1971). Although the analysis of steroid production during the three days of culture does not confirm any functional difference between the two culture systems, the evolution of currents recorded in plated cells might be an early sign of luteinization. In fact, even under ideal culture conditions, the modifications of the steroidogenic pathways associated with luteinization in vitro become clearly detectable after 5–7 days of culture (Henderson & McNatty, 1977). A possible relationship between I_{nK} and cellular differentiation is also suggested by the observation that freshly isolated granulosa cells exhibit I_K, whereas large, freshly isolated luteal cells, which are believed to derive from granulosa cells (Hansel & Dowd, 1986), exhibit I_{nK}. Any causal link between luteinization and the observed changes in the electrical properties of the granulosa cell membrane remains to be determined.

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