Volume 241, number 1,2, 169–172 FEB 06548 December 1988

Ionic currents in avian granulosa cells

Jean-Louis Schwartz, Geoffrey A.R. Mealing, Elikplimi K. Asem*, James F. Whitfield and Benjamin K. Tsang*

Cellular Oncology Group, Division of Biological Sciences, National Research Council of Canada, Ottawa, Ontario K1A 0R6 and **Reproductive Biology Unit, Department of Obstetrics and Gynecology, University of Ottawa, Ottawa Civic Hospital, Ottawa, Ontario K1Y 4E9, Canada*

Received 9 August 1988; revised version received 12 September 1988

Transmembrane ionic currents have been recorded in single granulosa cells from the laying hen using the whole-cell patch-clamp technique. Under voltage-clamp conditions, depolarizing voltage steps evoked currents composed of a fast inactivating inward component and a delayed outward component. The former was activated at voltages more positive than – 50 mV and was fully inactivated within 500 ms. It was blocked by D600 (methoxyverapamil) and by cobalt, suggesting that it is a calcium current. The latter displayed inward rectification and did not inactivate during long duration pulses. It was blocked by tetraethylammonium indicating that it is a potassium current. This is the first evidence of the existence of potassium and calcium transmembrane currents in granulosa cells.

K^+ current; Ca^{2+} current; Whole-cell patch-clamp; (Granulosa cell)

1. INTRODUCTION

Extracellular ions play important roles in granulosa cell steroidogenesis. In particular, removing calcium from the incubation medium significantly reduces basal and gonadotropin-induced progestin synthesis in swine [1], rat [2] and chicken [3] granulosa cells. However, the routes by which extracellular ions enter granulosa cells to trigger biochemical processes are unknown. Moreover, very little is known about the electrical properties of this type of cell. The present work was initiated to study transmembrane ionic currents in granulosa cells using the whole-cell patch-clamp technique [4].

2. MATERIALS AND METHODS

Granulosa cells were isolated from the largest preovulatory follicles of one-year-old, actively laying, white leghorn hens using a collagenase digestion technique [5]. They were plated on 35 mm plastic Petri dishes (Nunc, Rokskilde, Denmark) and cultured in a humidified air incubator at 37°C for 4–72 h in medium consisting of 90% (v/v) RPMI 1640 (Gibco, Grand Island, NY) and 10% (v/v) fetal calf serum buffered with 10 mM Hepes (pH 7.4).

Patch-clamp experiments were performed at room temperature (23°C) on single cells or on cells in small clusters (3–5 cells) in the whole-cell recording configuration using a Dagan 8900 amplifier (Dagan Corp, Minneapolis, MN). Patch pipettes (Pyrex 7740, 1.5 mm O.D., Corning Glass, Corning, NY) had tip diameters of approx. 2 mm and resistances of 2–5 MΩ in a high potassium pipette solution. The seal resistances were in excess of 2 GΩ.

Voltage-clamp pulses were generated by a custom made stimulation unit. Transmembrane currents were stored on FM magnetic tape (TEAC SR30, 5 kHz bandwidth, Teac Corp of America, Montebello, CA). Data were played back for analysis on a DP6000 (Analogic Corp., Danvers, MA) digital waveform analyzer.

The cells were perfused at approx. 1.5 ml/min. Switching from one solution to another took less than 1 min. For experiments in which potassium ions were present, the bath solution (solution B1) contained 130 mM NaCl, 5.4 mM KCl, 2.2 mM $CaCl_2$, 0.4 mM $MgCl_2$, 5.6 mM glucose, 5 mM Hepes (pH 7.4) and the pipette solution (solution P1) contained 130 mM KCl, 20 mM NaCl, 5 mM EGTA, 5 mM Hepes (pH 7.3). For experiments in which potassium ions were excluded, the bath solution (solution B2) contained 130 mM NaCl,

Correspondence address: J.L. Schwartz, Division of Biological Sciences, National Research Council of Canada, M-54, Ottawa, Ontario K1A 0R6, Canada

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

5.4 mM CsCl, 10 mM $CaCl_2$, 0.4 mM $MgCl_2$, 5.6 mM glucose, 5 mM Hepes (pH 7.4) and the pipette solution (solution P2) contained 130 mM CsCl, 20 mM NaCl, 3 mM ATP-Na, 5 mM EGTA (pH 7.3). In some experiments, $CoCl_2$ (5 mM) or D600 (50–100 μM) was added to the B1 and B2 bath solutions and tetraethylammonium (TEA, 3 mM) was added to solutions B1 and P1.

3. RESULTS

Single granulosa cells were first patch-clamped in the cell-attached configuration. They displayed single-channel electrical activity and a null-current membrane potential around −50 mV with solutions B1 and P1. With the pipette voltage set at this value, a brief suction pulse was applied to establish the whole-cell configuration. A holding potential between −60 and −80 mV was then applied to the cell in the voltage-clamp mode and sequences of hyperpolarizing pulses (−10, −20, and −30 mV) and depolarizing pulses (10–100 mV) were applied at various repetition rates (0.067–0.2 Hz) and for various durations (500 ms to 4 s).

Under quasi-physiological conditions (solutions B1 and P1), small hyperpolarizing and depolarizing pulses of short duration evoked a small linear leakage current. For larger depolarizing pulses, a larger current was recorded that displayed an inward component and a delayed outward component. Fig.1A shows typical recordings from a granulosa cell maintained at −70 mV between the test pulses. Transient inward currents were activated when the membrane was made more positive than −50 mV (fig.1B). They reached their maximum amplitude at voltages around −20 mV. The time to the peak of the transient current decreased with increasing depolarizing voltages: from 50 ms at a membrane voltage of −30 mV to 12 ms at 10 mV. The inactivation of the current could be fitted by a single exponential with a time constant of 185 ms. Negative (hyperpolarizing) pulses did not evoke any appreciable currents. Delayed outward currents were also observed for depolarizing test pulses. They activated at voltages above −40 mV and displayed strong outward rectification. They did not show signs of inactivation, even for depolarizing pulses up to 4 s in length.

To establish which ionic species were carrying the transient inward current and the delayed outward current, experiments were performed using ion substitution and channel blockers in the media.

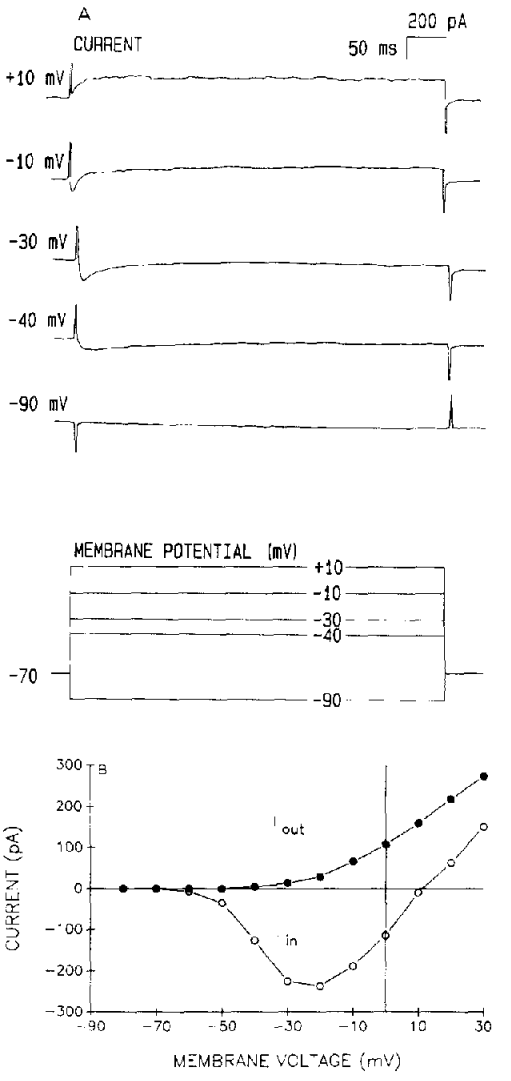

Fig.1. Characteristics of transmembrane currents in granulosa cells. (A) Current recordings under voltage-clamp conditions in the whole-cell patch-clamp configuration. The membrane holding potential was set at −70 mV. The voltages of the applied membrane pulses are indicated above the traces. Horizontal bar: 50 ms. Vertical bar: 200 pA. (B) Current-voltage relations of peak inward currents (I_{in}, ○) and maximum outward currents (I_{out}, ●) taken from the records in A. Data were corrected for leakage current.

When cesium was substituted for potassium (solutions B2 and P2), no voltage-dependent outward current was observed. This was also the case when TEA (3 mM) was added to solutions B1 and P1, which indicated that the outward current was carried mainly by potassium ions.

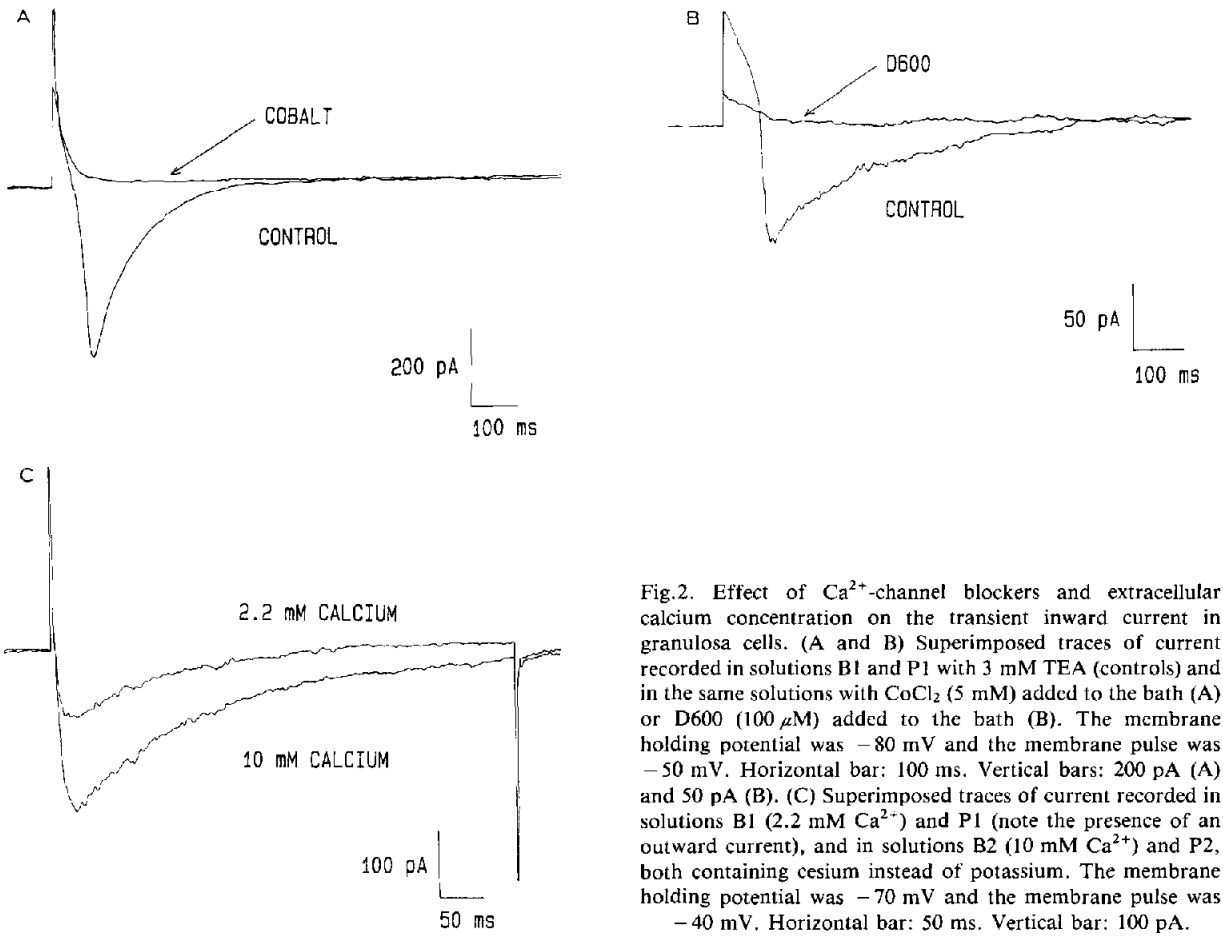

Fig.2. Effect of Ca^{2+}-channel blockers and extracellular calcium concentration on the transient inward current in granulosa cells. (A and B) Superimposed traces of current recorded in solutions B1 and P1 with 3 mM TEA (controls) and in the same solutions with $CoCl_2$ (5 mM) added to the bath (A) or D600 (100 μM) added to the bath (B). The membrane holding potential was −80 mV and the membrane pulse was −50 mV. Horizontal bar: 100 ms. Vertical bars: 200 pA (A) and 50 pA (B). (C) Superimposed traces of current recorded in solutions B1 (2.2 mM Ca^{2+}) and P1 (note the presence of an outward current), and in solutions B2 (10 mM Ca^{2+}) and P2, both containing cesium instead of potassium. The membrane holding potential was −70 mV and the membrane pulse was −40 mV. Horizontal bar: 50 ms. Vertical bar: 100 pA.

The transient inward current was completely abolished when cobalt (5 mM $CoCl_2$) was added to the B1 or B2 bath solution (fig.2A). However, when potassium was present in the pipette, the outward current was also reduced by cobalt. D600, a specific Ca^{2+}-channel blocker, significantly reduced the transient inward current when added to the B1 solution at a dose of 50 μM, and at a dose of 100 μM maximally reduced it to a small residual current (fig.2B). The effects of cobalt or D600 were reversible within minutes following removal of the blocker. Therefore, it appeared that this inward current was carried mainly by calcium ions. This was further supported by the fact that raising the extracellular calcium concentration from 2.2 mM (solution B1) to 10 mM (solution B2) increased significantly the maximum amplitude of the peak inward current (fig.2C).

4. DISCUSSION

In the present study, we have investigated the transmembrane currents of chicken granulosa cells under voltage-clamp conditions using the whole-cell patch-clamp recording technique. This is the first direct demonstration of the existence of potassium and calcium currents crossing the depolarized granulosa cell membrane, implying that these granulosa cells possess potassium and calcium channels. In fact, we have recorded two types of potassium channels [6] in these cells at single-channel level using both the cell-attached and the inside-out configurations of the patch-clamp technique [7].

Calcium from both intracellular and extracellular sources is necessary for granulosa cell function. Indeed, exposure of granulosa cells to lu-

teinizing hormone (LH) promptly triggers a transient calcium surge as observed in fura-2-loaded cells (unpublished). The intracellular source is a non-mitochondrial pool [8] and is presumably released by Ins(1,4,5)P_3, a product of polyphosphoinositide hydrolysis [9,10].

Papaverin derivatives (D600 and verapamil), benzothiazepine derivatives (diltiazem), dihydropyridines (nifedipine), as well as cobalt suppress steroidogenesis and progesterone synthesis in granulosa cells [1–3]. The inhibition of granulosa-cell steroidogenesis by these Ca^{2+}-channel blockers seems likely to be due to the inhibition of the inward calcium current that we have described here and which is blocked by cobalt and D600. However, in an earlier study [11] it was shown that BAY-K8644 and CGP-28392, dihydropyridine activators of a specific type of calcium channel (referred as L-type calcium channel [12,13]), did not enhance basal or hormone-stimulated progesterone production in granulosa cells. Thus, it appears that the inward calcium current reported in this study crosses the plasma membrane through calcium channels that are not influenced by BAY-K8644 and CGP-28392, although their activation threshold and inactivation kinetics seem to indicate that they belong to the L-type family. Our findings provide the first evidence of the existence of these voltage-dependent calcium channels in membranes of ovarian cells. These channels may play a key role in steroidogenesis. It remains to be determined whether these channels are involved in the generation of the LH-induced calcium transient in granulosa cells.

Acknowledgements: White leghorn hens were generously provided by the Animal Diseases Research Institute (ADRI), Ottawa, Canada. We thank Mr G. Charette, Electronic Services Group, Biological Sciences, NRC, Ottawa, for the design and the construction of the voltage pulse generator used in the present work. This study was supported in part by the Medical Research Council of Canada (MRC grant MA-10369).

REFERENCES

[1] Veldhuis, J.D. and Klase, P.A. (1982) Endocrinology 111, 1–6.
[2] Tsang, B.K. and Carnegie, J.A. (1983) Endocrinology 113, 763–769.
[3] Asem, E.K. and Hertelendy, F. (1986) Gen. Comp. Endocrinol. 62, 120–128.
[4] Hamill, O.P., Marty, A., Neher, E., Sakman, B. and Sigworth, S.J. (1981) Pflügers Arch. 391, 85–100.
[5] Asem, E.K., Zakar, T., Biellier, H.V. and Hertelendy, F. (1984) Dom. Anim. Endocrinol. 1, 235–249.
[6] Asem, E.K., Schwartz, J.L., Mealing, G.A.R., Tsang, B.K. and Whitfield, J.F. (1988) Biochem. Biophys. Res. Commun., submitted.
[7] Neher, E., Sakman, B. and Steinbach, J.H. (1978) Pflügers Arch. 375, 219–228.
[8] Asem, E.K., Molnar, M. and Hertelendy, F. (1987) Endocrinology 120, 853–859.
[9] Davis, J.S., Weakland, L.L., Farese, R.V. and West, L.A. (1987) J. Biol. Chem. 262, 8515–8521.
[10] Dimino, M.J., Smitzer, J. and Brown, K.M. (1987) Biol. Reprod. 37, 1129–1134.
[11] Asem, E.K. and Tsang, B.K. (1987) Biochem. Biophys. Res. Commun. 146, 314–320.
[12] Nowycky, M.C., Fox, A.P. and Tsien, R.W. (1985) Nature 316, 440–443.
[13] Kass, R.S. and Kraffe, D.S. (1987) in: Receptor Biochemistry and Methodology (Veuter, J.C. and Harrison, L.C. eds) vol.9, pp.71–88, Alan R. Liss, New York.