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Opening of glibenclamide-sensitive K^+ channels in follicular cells promotes *Xenopus* oocyte maturation

(cAMP/electrophysiology/gonadotropins/pinacidil/K^+ efflux)

FLEMMING WIBRAND*, ERIC HONORÉ†, AND MICHEL LAZDUNSKI

Institut de Pharmacologie Moleculaire et Cellulaire, Centre National de la Recherche Scientifique, Université de Nice-Sophia Antipolis, 660 route des Lucioles, Sophia Antipolis, F-06560 Valbonne, France

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ABSTRACT **The vasorelaxing K^+ channel opener P1060 (a pinacidil analog), gonadotropins, and cAMP were shown to activate a glibenclamide-sensitive $^{86}Rb^+$ efflux from fully grown follicle-enclosed *Xenopus* oocytes. Glibenclamide-sensitive K^+ channels are located in follicular cells. Glibenclamide (*i*) depressed the gonadotropin- but not the progesterone-induced maturation and (*ii*) did not significantly modify progesterone production in oocytes exposed to *Xenopus* gonadotropin. In follicle-enclosed oocytes, the opener P1060 very significantly enhanced the oocyte sensitivity to progesterone. This increased sensitivity to the hormone induced by the K^+ channel opener was reversed by glibenclamide. Thus these results suggest that the opening of glibenclamide-sensitive K^+ channels in follicular cells by gonadotropins (and other activators of this channel) induces a hyperpolarization in the oocyte that greatly facilitates maturation by increasing the oocyte sensitivity to progesterone.**

Potassium channels are involved in a wide variety of biological functions (1). Therefore, it is not surprising that their pharmacology has attracted considerable interest (2–4). A number of series of K^+ channel effectors are now known. Two classes of such agents are antidiabetic sulfonylureas, which block ATP-sensitive K^+ channels in pancreatic β cells (3–6) and provoke insulin release (6, 7), and K^+ channel openers (KCOs), which hyperpolarize smooth muscle cells (3, 4, 8–12) and other cell types including pancreatic β cells (6), cardiac cells (13, 14), neurons (15, 16), and skeletal muscle cells (17). The relaxant effects of KCOs are antagonized by sulfonylureas (3, 4, 8, 12), and both families of drugs have the same target channel (3, 4, 8, 12, 14).

We have demonstrated that follicle-enclosed oocytes also have a K^+ channel that is activated by KCOs and blocked by antidiabetic sulfonylureas (18, 19). This channel was shown to be the major ionic pathway involved in cAMP-mediated K^+ currents (18) that are induced by gonadotropins (20) and by a large variety of other hormones and transmitters, such as catecholamines (21), adenosine (22), vasoactive intestinal peptide (23), the E series of prostaglandins, atrial natriuretic factor, ocytocin (24), corticotropin-releasing factor, arginine-vasopressin (25), and growth hormone-releasing hormone (26). The increase in K^+ conductance caused by activation of the channel by KCOs or by intracellular cAMP was found to be suppressed by treatments that stimulate protein kinase C (e.g., muscarinic effectors and phorbol esters) (18). The report that defolliculation of oocytes abolished K^+ currents elicited by KCOs and hormones and neurotransmitters (18, 19) suggests that these K^+ channels are situated on follicular cells.

The functional expression of the KCOs and glibenclamide-sensitive K^+ channels in follicle-enclosed oocyte and their modulation by gonadotropins (18) raise the question of whether these particular K^+ channels act as potential modulators of the gonadotropin-induced progesterone secretion and oocyte maturation. The present paper demonstrates that opening of these K^+ channels in follicular cells, which hyperpolarizes the oocyte, directly controls its progesterone sensitivity and, thereby, modulates indirectly the process of meiosis.

MATERIALS AND METHODS

Adult female *Xenopus* laevis were purchased from the Centre de Recherches de Biochimie Macromoléculaire (Montpellier, France). The animals were anesthetized on ice and pieces of the ovary were surgically removed and placed in ND 96 medium (96 mM NaCl/2 mM KCl/1.8 mM $CaCl_2$/2 mM $MgCl_2$/5 mM Hepes, adjusted to pH 7.4 with NaOH). Stage 5 and 6 follicle-enclosed oocytes (27) were freed from the ovary by manual dissection and stored at 19°C in ND 96 medium supplemented with penicillin (10 μg/ml) and streptomycin (20 μg/ml). To obtain defolliculated oocytes, follicles were treated for 3 h with collagenase (type Ia, Sigma; 1 mg/ml) in ND 96 medium, and the follicular layers were subsequently manually removed with fine forceps. Frog pituitary homogenate (FPH) was prepared from a pool of *Xenopus* pituitary glands essentially as described (28). All experiments were performed at room temperature (20–22°C). The chemicals used were from Sigma unless otherwise stated. Solvents (ethanol and/or dimethyl sulfoxide) were added in parallel in control experiments to ensure the effectiveness of the compounds tested at the concentrations used. The variability of the results was expressed as the SEM.

$^{86}Rb^+$ Efflux Experiments. Follicle-enclosed oocytes and defolliculated oocytes were incubated for 30 min in ND 96 medium containing $^{86}Rb^+$ (Amersham; 100 μCi/ml; 1 Ci = 37 GBq). After $^{86}Rb^+$ loading, oocytes were washed thoroughly and placed in individual chambers of a 24-well culture plate containing nylon-mesh bottom fittings. Isolated follicular cells were obtained by carefully bissecting $^{86}Rb^+$-loaded follicle-enclosed oocyte in the chamber. In all cases, a 100-min wash with ND 96 solution was carried out before the experiment was started. Then, the amount of $^{86}Rb^+$ efflux from gently shaken oocytes (1 ml of ND 96 medium per well) was measured every 5 min for 50 min. Activators were added for 10 min as shown in the figures. Inhibitors such as glibenclamide and phorbol 12-myristate 13-acetate were added 5 min and 30 min, respectively, before the activators and kept in the medium during the activation period. At the end of the experiment, oocytes were homogenized to deter-

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Abbreviations: FPH, frog pituitary homogenate; KCO, potassium channel opener.

*Present address: The August Krogh Institute, Universitetsparken 13, DK-2100 Copenhagen, Denmark.
†To whom reprint requests should be addressed.

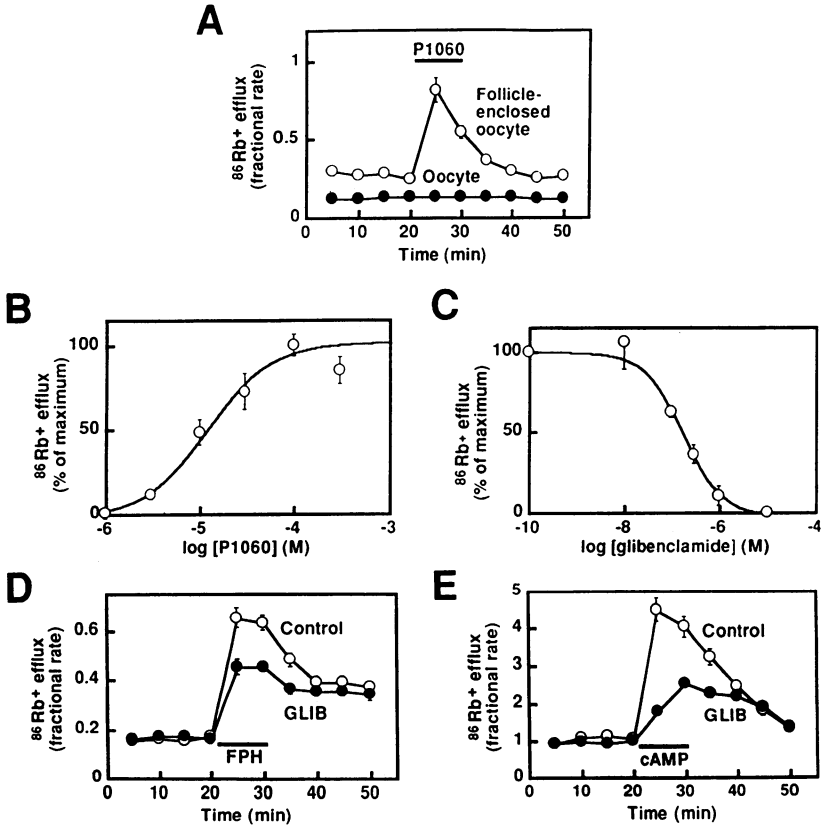

FIG. 1. (*A*) $^{86}Rb^+$ efflux from a follicle-enclosed oocyte (○) and a defolliculated oocyte (●) induced by the K^+ channel opener P1060 (30 μM). The horizontal bar represents the period of stimulation with P1060. The curves are data from 8 and 6 oocytes (mean ± SEM), respectively, from one donor. (*B*) Dose–response curve of the P1060 effect on follicle-enclosed oocytes. Responses were calculated as the $^{86}Rb^+$ efflux above the baseline after 5 min of P1060 stimulation and expressed as the percentage of the maximum. Data points are from at least 5 oocytes from one frog (mean ± SEM). (*C*) Dose–response curve for the inhibition by glibenclamide of $^{86}Rb^+$ efflux induced by 30 μM P1060 in follicle-enclosed oocytes. Data points are from at least 3 oocytes from one donor (mean ± SEM). (*D*) $^{86}Rb^+$ efflux from follicle-enclosed oocytes elicited by FPH (0.01 pituitary equivalent per ml) in the presence (●) or absence (○) of 10 μM glibenclamide (GLIB). The horizontal bar represents the period of stimulation by FPH. Data are from 9 or 10 oocytes from one donor (mean ± SEM). (*E*) $^{86}Rb^+$ efflux from follicle-enclosed oocytes elicited by 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate (1 mM; CPT-cAMP) in the presence (●) or absence (○) of 10 μM glibenclamide (GLIB). The horizontal bar represents the period of stimulation by CPT-cAMP. Data are from 6 and 7 oocytes from one donor (mean ± SEM).

mine the residual content of radioactivity. Fractional rates of efflux represented $^{86}Rb^+$ efflux during each 5-min interval and were expressed as the percentage of the $^{86}Rb^+$ content in the oocyte at the beginning of the respective intervals. The extent of $^{86}Rb^+$ loading and of the activation of $^{86}Rb^+$ efflux varied among frogs and from day to day.

Maturation Experiments. On the first day after dissection, follicle-enclosed oocytes in groups of 50–100 were incubated in 2 ml of ND 96 medium containing the required hormones and/or K^+ channel effectors. Oocytes were inspected every 30 min under a binocular microscope for the appearance of a white spot at the animal pole to indicate germinal vesicle breakdown, a process related to meiotic maturation (for a review, see ref. 29). In some cases, a histological examination was performed to reveal the presence of the metaphasic plate.

Progesterone Measurements. In parallel with the maturation experiments, groups of 40 follicle-enclosed oocytes were incubated in 1 ml of ND 96 medium containing FPH (0.02 pituitary equivalent per ml) in the presence or the absence of 10 μM glibenclamide. Incubation mixtures contained 0.1% dimethyl sulfoxide (final concentration). At the designated times, the medium was collected and oocytes were treated with 1 ml of methanol for 15 min with shaking to extract progesterone (30). Methanol extracts and medium samples were then stored frozen at −20°C until they were assayed. Samples of the methanol extract were lyophilized and all samples were assayed for progesterone by radioimmunoassay (Progesterone Direct Radioimmunoassay kit, CIS Biointernational, Gif-sur-Yvette, France).

Electrophysiological Experiments. Electrophysiological experiments (current-clamp and voltage-clamp recordings) were performed as described (18, 19).

RESULTS AND DISCUSSION

Since K^+ channels are permeable to Rb^+, $^{86}Rb^+$ efflux experiments have been used to confirm results obtained with electrophysiological techniques (18). Fig. 1*A* shows that $^{86}Rb^+$ efflux is activated in follicle-enclosed oocytes by the pinacidil analog P1060 [*N-tert*-butyl-*N′*-cyano-*N″*-(3-pyridinyl)guanidine]. This activation was not observed in defolliculated oocytes (Fig. 1*A*). The dose–response curve of

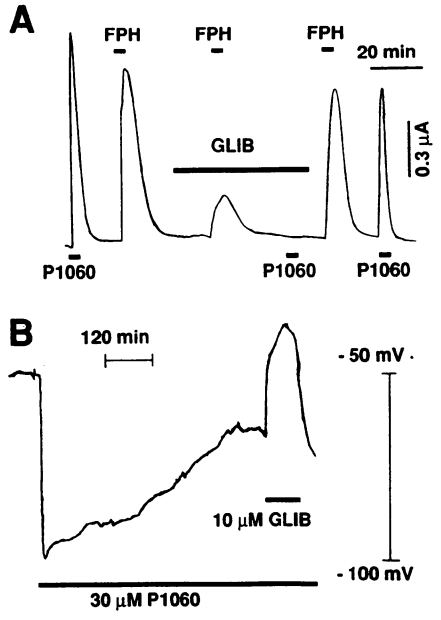

FIG. 2. (*A*) Activation of glibenclamide-sensitive outward K^+ currents in follicle-enclosed oocytes by 30 μM P1060 and FPH (0.01 pituitary equivalent per ml of superfusion buffer). Additions of P1060, FPH, and glibenclamide (10 μM) are indicated by horizontal bars. In this experiment the holding potential was −20 mV. (*B*) Continuous recording of membrane potential in a follicle-enclosed oocyte. The 30 μM P1060 treatment is indicated by a horizontal bar. During P1060 treatment, 10 μM glibenclamide was added to the medium (indicated by a horizontal bar).

the P1060 effect indicates a $K_{0.5}$ value of 12 μM (Fig. 1*B*). The P1060-activated $^{86}Rb^+$ efflux is inhibited by glibenclamide with a $K_{0.5}$ value of 150 nM (Fig. 1*C*). P1060 also activates $^{86}Rb^+$ efflux from isolated follicular cells (not shown).

Since human chorionic gonadotropin activates the glibenclamide-sensitive K^+ channel in follicular *Xenopus* oocytes (18), it was important to show that the same was true with the corresponding hormone from *Xenopus*. A *Xenopus* pituitary homogenate (FPH) also activates a $^{86}Rb^+$ efflux and this effect is depressed by glibenclamide (Fig. 1*D*). The same type of effect is observed with cAMP, which is the mediator for FPH activity (20) (Fig. 1*E*). cAMP-activated K^+ channels have been shown (18) to be extensively inhibited by glibenclamide and nearly totally inhibited by phorbol esters. The same observation has been made with isolated follicular cells (data not shown).

FPH clearly activates K^+ channels as does P1060, and this activation is largely inhibited by glibenclamide (Fig. 2*A*).

Fig. 2*B* shows a typical experiment in which P1060 application to follicle-enclosed oocytes immediately caused a hyperpolarization of about 50 mV to reach a new resting membrane potential of −98 mV. This latter value is close to the expected reversal potential for K^+ ions in the *Xenopus* oocyte (for a review, see ref. 31). It was observed that the P1060-induced hyperpolarization was maintained for long periods of time after a single application of P1060 without washing. The membrane potential of the P1060-treated oocyte remained below −80 mV for 6 h. A substantial glibenclamide-sensitive hyperpolarization was still observed after a 10-h P1060 treatment (Fig. 2*B*). To determine whether the decrease in the P1060 effect was due to a reduction in membrane K^+ conductance or to an eventual accumulation/depletion process, we measured in a voltage-ramp-like manner, the current–voltage relationships during P1060 treatment. The amplitude of the outward current decreased as a function of time without any significant variation in the value of the reversal potential, which was stable at a value of −103 mV for 10 h of P1060 treatment. Similar results were obtained with FPH (data not shown).

FPH is known to induce maturation in fully grown follicle-enclosed *Xenopus* oocytes (32, 33). The hormonal action is believed to be associated with a response of follicular cells to gonadotropins and involves synthesis and release of progesterone (32–34), which, in turn, induces resumption of meiosis by acting at the oocyte surface (35). Are KCOs and sulfonylurea-sensitive K^+ channels involved in the maturation process? Fig. 3*A* shows typical kinetics of the FPH-induced maturation in follicle-enclosed oocytes. The amount of follicle-enclosed oocytes that underwent germinal vesicle breakdown within 8 h in this typical experiment was 59 ± 4% (with 17 female frogs). Glibenclamide drastically reduced the percentage of oocytes undergoing meiosis in the presence of FPH. Tolbutamide, an antidiabetic sulfonylurea of the first generation, which is about 1000 times less active than glibenclamide on pancreatic β cells ($K_{0.5}$ = 4–20 nM for glibenclamide and $K_{0.5}$ = 1–17 μM for tolbutamide) (5), was not active on glibenclamide-sensitive K^+ channels in follicular oocytes at 10 μM and was also ineffective on maturation.

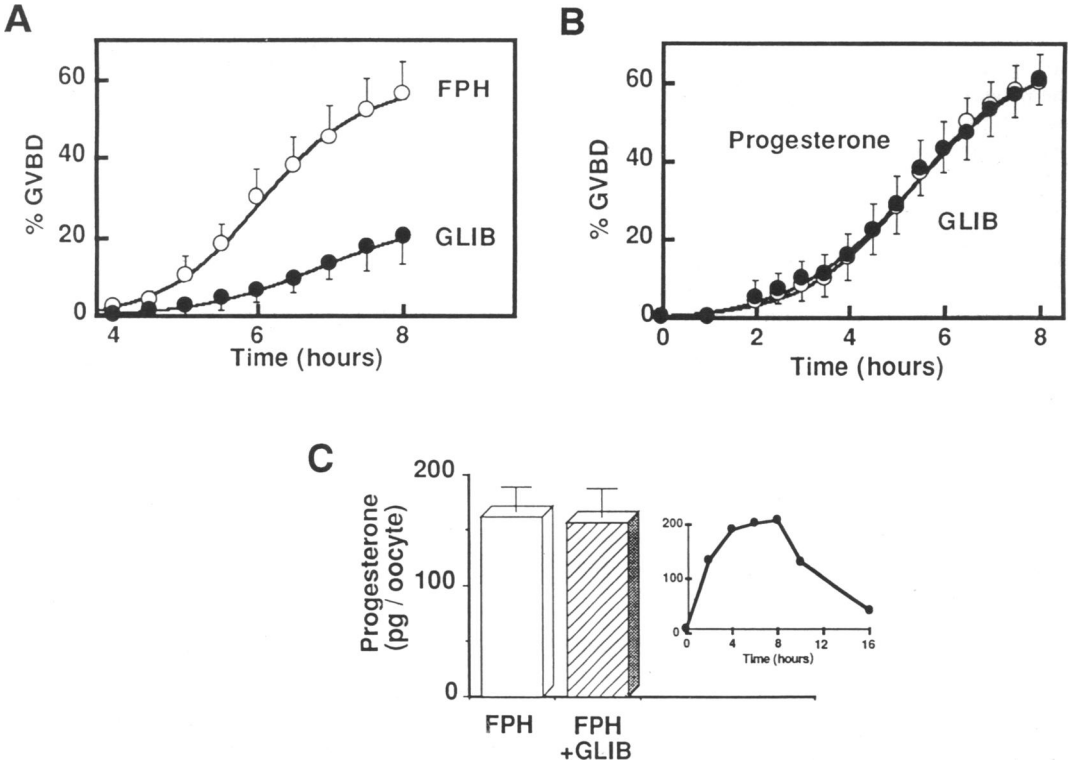

FIG. 3. (*A*) Effect of glibenclamide on FPH-induced maturation in follicle-enclosed oocytes. Kinetics of maturation was followed in oocytes continuously exposed to FPH (0.02 pituitary equivalent per ml) alone (○) or in the presence of 30 μM glibenclamide (●). Data are from more than 250 oocytes from five frogs (mean ± SEM). (*B*) Lack of effect of glibenclamide on progesterone-induced maturation in follicle-enclosed oocytes. The kinetics of maturation was assayed in oocytes exposed continuously to progesterone (10 μM) alone (○) or in the presence of 10 μM glibenclamide (●). Data are from more than 300 oocytes from six frogs (mean ± SEM). (*C*) Lack of effect of glibenclamide (10 μM) on the progesterone production measured after a 6-h FPH treatment (0.01 pituitary equivalent per ml). Duplicate samples on 40 oocytes from nine donors were assayed for progesterone production. Data are mean ± SEM. (*Inset*) Time course of FPH-induced progesterone production in follicle-enclosed oocytes. Groups of 40 oocytes were exposed continuously to FPH (0.02 pituitary equivalent per ml). At the designated times, the incubation medium was collected and the oocytes were extracted with methanol. The data points represent the total (medium and extract) mean progesterone content per oocyte from duplicate samples from one frog. The progesterone level reached at that time was 162 ± 22 pg of progesterone per oocyte (N = 9). We observed, as have other authors in previous studies (33, 35, 36), great variations of time course and of the maximal percentage of oocyte maturation among different donors. We therefore included an internal control in each experiment.

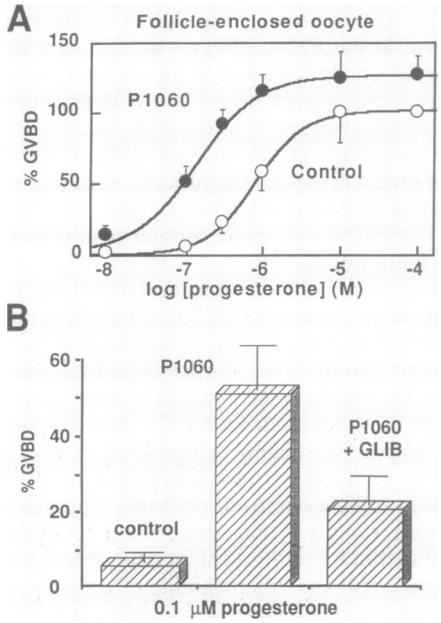

FIG. 4. (*A*) Effect of P1060 on progesterone-induced maturation in follicle-enclosed oocytes. Oocytes were exposed continuously to the indicated concentration of progesterone alone (○) or in the presence of 30 μM P1060 (●) and were examined for germinal vesicle breakdown (GVBD) after a 16-h exposure. Data are from 250 oocytes from four donors (mean ± SEM). (*B*) Glibenclamide at 10 μM reverses the stimulatory effect of 30 μM P1060. Experimental procedure is identical to that in *A*.

Conversely, no significant effect of glibenclamide could be observed on the progesterone-induced maturation (Fig. 3*B*).

Fig. 3*C* shows a typical profile of FPH-induced progesterone production. Progesterone production reached a stable level after 6 h of FPH treatment. Glibenclamide had no effect on the progesterone production (either in the external medium or in the tissue), although glibenclamide significantly depressed the maturation process in these particular oocytes.

Fig. 4*A* shows that, in control conditions, the progesterone concentration threshold for oocyte maturation was 0.1 μM and that the half-maximal maturation was obtained near 1 μM progesterone. In the presence of the K^+ channel opener P1060, the concentration threshold was shifted to 3 nM progesterone and half-maximal maturation was observed near 0.1 μM progesterone. The percentage of maturation observed with a maximum progesterone concentration (100 μM) was also increased by about 20% in the presence of P1060. The stimulatory effect of P1060 on oocyte maturation was largely reversed by 10 μM glibenclamide (Fig. 4*B*). Maturation of defolliculated oocytes with progesterone was not affected by 30 μM P1060 (data not shown).

Thus all these results strongly indicate (*i*) that the hormone FPH activates a K^+ channel that is sensitive to antidiabetic sulfonylureas such as glibenclamide, (*ii*) that this channel is the same as the channel activated by the K^+ channel opener of the pinacidil family P1060, and (*iii*) that opening of this channel is important for oocyte maturation since a large component of FPH-induced maturation is inhibited by glibenclamide and the K^+ channel opener P1060 accelerates maturation induced by progesterone with a stimulatory effect that is also inhibited by glibenclamide.

What is the mechanism by which K^+ channel opening is effective on maturation? A first possibility is that the hyperpolarization induced by FPH induces a change in the level of progesterone production by follicular cells. Fig. 3*C* indicates that this is not the case. A second possibility would be that the progesterone effect on maturation is, itself, a glibenclamide-sensitive event. Fig. 3*B* shows that this interpretation is also not correct. A third possibility is that K^+ channel opening favors the action of progesterone, and Fig. 4*A* clearly shows that this is the most likely interpretation. The probable sequence of events would then be as follows: (*i*) The pituitary hormone binds to follicular cells; this binding increases intracellular cAMP and activates sulfonylurea-sensitive K^+ channels. (*ii*) This opening produces a hyperpolarization that is transmitted to the oocyte itself through gap junctions and this hyperpolarization (or the K^+ efflux it probably induces) favors progesterone action.

Of course it remains to be seen whether mammalian granulosa cells that can now be easily studied (37) have glibenclamide-sensitive K^+ channels similar to those found in *Xenopus* follicular oocytes and whether the mechanism proposed here also holds for the mammalian system.

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1. Rudy, B. (1988) *Neuroscience* **25**, 729–749.
2. Moczydlowski, E., Lucchesi, R. & Ravindran, A. (1988) *J. Membr. Biol.* **105**, 95–111.
3. Quast, U. & Cook, N. S. (1989) *Trends Pharmacol. Sci.* **10**, 431–435.
4. Cook, N. S. & Quast, U. (1989) in *Potassium Channels: Structure, Classification, Function and Therapeutic Potential*, ed. Cook, N. S. (Horwood, Chichester, U.K.), pp. 181–225.
5. Schmid-Antomarchi, H., De Weille, J. R., Fosset, M. & Lazdunski, M. (1987) *J. Biol. Chem.* **262**, 15840–15844.
6. Dunne, M. J. & Petersen, O. H. (1991) *Biochim. Biophys. Acta* **1071**, 67–82.
7. Petersen, O. H. & Findlay, I. (1987) *Physiol. Rev.* **67**, 1054–1116.
8. Edwards, G. & Weston, A. H. (1990) *Trends Pharmacol. Sci.* **11**, 417–422.
9. Beech, D. J. & Bolton, T. (1989) *Br. J. Pharmacol.* **98**, 851–864.
10. Gelband, C. H., Lodge, N. J. & Van Breemen, C. (1989) *Eur. J. Pharmacol.* **167**, 201–210.
11. Standen, N. B., Quayle, J. M., Davies, N. W., Brayden, J. E., Huang, Y. & Nelson, M. T. (1989) *Science* **245**, 177–180.
12. Nelson, M. T., Patlack, J. B., Worley, J. F. & Standen, N. B. (1990) *Am. J. Physiol.* **259**, C3–C18.
13. Arena, S. L. & Kass, R. S. (1989) *Circ. Res.* **65**, 436–445.
14. Escande, D., Thuringer, D., Leguern, S. & Cavero, I. (1988) *Biochem. Biophys. Res. Commun.* **154**, 620–625.
15. Häusser, M., De Weille, J. R. & Lazdunski, M. (1991) *Biochem. Biophys. Res. Commun.* **174**, 909–914.
16. Schmid-Antomarchi, H., Amoroso, S., Fosset, M. & Lazdunski, M. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 3489–3492.
17. Quasthoff, S., Franke, C., Hatt, H. & Richter-Turtur, M. (1990) *Neurosci. Lett.* **119**, 191–194.
18. Honoré, E. & Lazdunski, M. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 5438–5442.
19. Honoré, E. & Lazdunski, M. (1991) *FEBS Lett.* **287**, 75–79.
20. Woodward, R. M. & Miledi, R. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 4135–4139.
21. Kusano, K., Miledi, R. & Stinnakre, J. (1982) *J. Physiol. (London)* **328**, 143–170.
22. Lotan, I., Dascal, N., Cohen, S. & Lass, Y. (1982) *Nature (London)* **298**, 572–574.
23. Woodward, R. M. & Miledi, R. (1987) *Proc. R. Soc. London Ser. B* **231**, 489–497.
24. Miledi, R. & Woodward, R. M. (1989) *J. Physiol. (London)* **416**, 623–643.
25. Moriarty, T. M., Gillo, B., Sealfon, S. & Landau, E. M. (1988) *Mol. Brain Res.* **4**, 201–205.
26. Yoshida, S. & Plant, S. (1991) *J. Physiol. (London)* **443**, 651–667.

27. Dumont, J. N. (1972) *J. Morphol.* **136,** 153–180.
28. Lessman, C. A. & Schuetz, A. W. (1982) *Gamete Res.* **6,** 95–106.
29. Smith, L. D. (1989) *Development* **107,** 685–699.
30. Lin, Y.-W. L. & Schuetz, A. W. (1985) *Gen. Comp. Endocrinol.* **58,** 424–435.
31. Dascal, N. (1987) *CRC Crit. Rev. Biochem.* **22,** 317–387.
32. Fortune, J. E., Concannon, P. W. & Hansel, W. (1975) *Biol. Reprod.* **13,** 561–567.
33. Fortune, J. E. (1983) *Dev. Biol.* **99,** 502–509.
34. Thibier-Fouchet, C., Mulner, O. & Ozon, R. (1976) *Biol. Reprod.* **14,** 317–326.
35. Baulieu, E.-E., Godeau, F., Schorderet, M. & Schorderet-Slatkine, S. (1978) *Nature (London)* **275,** 593–598.
36. Mulner, O. & Ozon, R. (1981) *Gen. Comp. Endocrinol.* **44,** 335–343.
37. Sun Suh, B. & Amsterdam, A. (1990) *Endocrinology* **127,** 2489–2500.