Calcium ions modulate hormonally stimulated progesterone production in isolated ovarian cells

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Swine granulosa-luteal cells incubated in Ca^{2+} -deficient medium ($5\text{ }\mu\text{M}$ final Ca^{2+} concentration) for short time periods produced diminished quantities of progesterone in response to lutropin. Maximally stimulating effects of prostaglandin E_2 and L-adrenaline were also impaired significantly. Diminished progesterone production could not be attributed to alterations in protein synthesis or cell viability. Under Ca^{2+} -deprived conditions, the stimulatory actions of cholera toxin, 3-isobutyl-1-methylxanthine and 8-bromo cyclic AMP were also significantly impeded. Administration of a presumptive antagonist of transmembrane Ca^{2+} influx (verapamil) or of EGTA to chelate extracellular Ca^{2+} , significantly decreased the total cellular content of Ca^{2+} , and antagonized the actions of lutropin. Micromolar concentrations of trifluoperazine mimicked the suppressive effects of Ca^{2+} deprivation. Conversely, the bivalent-cation ionophore, ionophore A23187, significantly augmented the stimulation of progesterone production by lutropin. Thus the present observations implicate Ca^{2+} in the modulation of hormonally stimulated progesterone production in isolated ovarian cells, and suggest that Ca^{2+} may influence one or more processes distal to, or independent of, cyclic AMP generation. In addition, the susceptibility of progesterone biosynthesis to inhibition by trifluoperazine suggests a possible role for calmodulin in the ovary.

Ca^{2+} participates in stimulus–secretion coupling in several exocrine and endocrine glands (Birmingham *et al.*, 1953; Jaanus *et al.*, 1970; Sayers *et al.*, 1972; Bowyer & Kitabchi, 1974; Janszen *et al.*, 1976; Higuchi *et al.*, 1976; Putney, 1979; Podesta *et al.*, 1980). In certain steroidogenic tissues, such as the adrenal and testis, Ca^{2+} also modulates steroid-hormone biosynthesis (Birmingham *et al.*, 1953; Péron & McCarthy, 1968; Jaanus *et al.*, 1970; Sayers *et al.*, 1972; Bowyer & Kitabchi, 1974; Arthur & Boyd, 1976; Janszen *et al.*, 1976; Podesta *et al.*, 1980). The ovary is a major secretory and steroid-biosynthetic organ. In addition, the ovary has been shown to contain a Ca^{2+} -stimulated Mg^{2+} -dependent ATPase (Verma & Penniston, 1981). However, the role of Ca^{2+} in either secretory or steroidogenic processes in ovarian cells remains unknown. The present work describes significant regulatory actions of Ca^{2+} on both the secretion and biosynthesis of progesterone by isolated ovarian cells *in vitro*.

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Materials and methods

Materials

Ovine lutropin (NIH-LH-S19) was obtained from the Hormone Distribution Office (N.I.A.M.D.D., N.I.H., Bethesda, MD, U.S.A.). [$1,2(n)^3\text{H}$]Progesterone was from New England Nuclear Corp., Boston, MA, U.S.A. Ionophore A23187, 8-bromo cyclic AMP and 3-isobutyl-1-methylxanthine were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Verapamil hydrochloride was kindly supplied by Knoll Pharmaceutical Co. (Whippany, NJ, U.S.A.). Other chemicals and reagents were of the best quality commercially available.

Cell preparation

Granulosa cells were harvested by collagenase dispersion of the membrana granulosa of $>6\text{ mm}$ follicles of ovaries of sexually mature pigs (Veldhuis & Sweinberg, 1981). Cells were washed free of follicular fluid by centrifugation in culture medium, and approx. $(3\text{--}5) \times 10^6$ viable cells (determined by Trypan Blue exclusion) were incubated per culture flask, as described previously by Veldhuis &

Sweinberg (1981) and Veldhuis *et al.* (1981). The incubation medium comprised 2 ml of Eagle's minimal essential medium with Earle's salts (Grand Island Biological Corp., Grand Island, NY, U.S.A.; cat. no. 320-1380) with or without added CaCl_2 (1.27 mM, unless otherwise specified), MgCl_2 (0.84 mM), 25 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer (pH 7.4) (with the addition of equimolar concentrations of NaCl to control cultures whenever salt concentrations were manipulated). Granulosa cells were incubated for various intervals or for 4 h at 37°C in a metabolic shaker, and recovered by centrifugation. The cellular content of progesterone (measured after homogenization of cells in 100% ethanol) and the medium content of progesterone were determined by a previously validated radioimmunoassay, for which the sensitivity, specificity and precision have been characterized (Veldhuis *et al.*, 1981). Protein concentrations were determined by the method of Bradford (1976), with crystalline bovine serum albumin (fraction V) as standard.

Experimental effectors of Ca^{2+} transport were added to incubations of monodispersed granulosa cells 20 min before the administration of hormonal or non-hormonal stimulators. Data are means $\pm$ S.E.M. for replicate incubations. Each experiment was repeated at least once with cells derived from another set of ovaries. Statistical analyses of data was by one-way analysis of variance, with the Newman-Keul's procedure for ascertaining the significance of differences among means (Winer, 1971).

The Ca^{2+} content of granulosa cells was determined by atomic-absorption spectrometry on a Perkin-Elmer 305 spectrometer with a calcium/magnesium lamp (212 nm) as described previously (Veldhuis & Hammond, 1981). The cells were washed three times in cold 0.5 mM- LaCl_3 to remove loosely bound external Ca^{2+} and then dissolved in 3 M-HCl before ashing.

Results

Omission of Ca^{2+} from the incubation medium [final measured calcium concentration of 5 μM (Veldhuis & Hammond, 1981)] impaired significantly ($P < 0.01$) the time-dependent secretion of progesterone by swine granulosa cells *in vitro* in response to the administration of maximally stimulating concentrations of lutropin (see Fig. 1). In contrast, basal progesterone production (determined in the absence of lutropin) was not altered by omission of Ca^{2+} from the extracellular medium.

Omission of extracellular Ca^{2+} also significantly impeded the dose-dependent stimulation of progesterone production by prostaglandin E_2 (see Fig. 2a), and by L-adrenaline (Fig. 2b). In particular,

Fig. 1. Effect of cellular Ca^{2+} deprivation on the time-dependent stimulation of progesterone secretion by lutropin

Granulosa cells from >8 mm swine follicles were incubated in the presence of 0.84 mM- Mg^{2+} , and in the presence of 1.27 mM- Ca^{2+} (+ Ca^{2+}) or in the absence of added Ca^{2+} ($-\text{Ca}^{2+}$, final concentration 5 μM). Lutropin (LH, 1000 ng/ml) was added at time zero. In the absence of lutropin, progesterone secretion was unaffected by omission of Ca^{2+} (points denoted by *). Data are means $\pm$ S.E.M. ($n = \text{three cultures}$). Error bars encompass the symbol dimensions.

under Ca^{2+} -deficient conditions, the maximal secretion of progesterone in response to either prostaglandin E_2 or L-adrenaline was significantly impaired ($P < 0.01$). However, both hormonal effectors continued to elicit a discernible stimulatory response ($P < 0.02$), without any apparent shift in the hormonal concentrations required for half-maximal stimulation of progesterone production.

Since lutropin, prostaglandin E_2 and L-adrenaline are all believed to exert their actions via intracellular cyclic AMP in ovarian cells (Dufau & Catt, 1978; Veldhuis *et al.*, 1979), we examined the hypothesis that Ca^{2+} modulates the stimulatory effects of cyclic AMP itself. Stimulation of progesterone production by cholera toxin was also significantly impaired in Ca^{2+} -deficient medium. In addition, when granulosa cells were stimulated with maximally active concentrations of a stable analogue of cyclic AMP, in the form of 8-bromo cyclic AMP, omission of Ca^{2+} also significantly suppressed progesterone production (Table 1). Separate measurement of the cell and medium content of progesterone indicated that Ca^{2+} -deprived cells not only secreted less progesterone into the medium,

Fig. 2. Effect of Ca²⁺ deprivation on progesterone secretion in response to prostaglandin E₂ and L-adrenaline

Pig granulosa cells were incubated in serum-free suspension cultures for 4 h (see the Materials and methods section) in the presence (1.27 mM-Ca²⁺) or absence (5 μ M) of added Ca²⁺. Cells were treated with increasing concentration of prostaglandin E₂ (a) or L-adrenaline (in b). Results are means (with ranges indicated by the bars) for duplicate cultures.

Increased activity of phosphodiesterase in Ca²⁺-deficient cells seems unlikely to account for the suppressive effects of Ca²⁺ deprivation, since 8-bromo cyclic AMP is a sparsely hydrolysed analogue of cyclic AMP (Yagura & Miller, 1980). In addition, 3-isobutyl-1-methylxanthine (0.25 mM), an inhibitor of phosphodiesterase activity, failed to overcome the suppressive effects of Ca²⁺ depletion on the stimulatory actions of lutropin (Table 1).

Complete omission of Mg²⁺ also suppressed progesterone production significantly ($P < 0.01$) (Table 2). However, the effects of Ca²⁺ omission could not be superseded by the addition of physiological concentrations of Mg²⁺. Maximal progesterone production required the presence of both bivalent cations. In contrast, addition of LaCl₃ (1 mM) significantly suppressed the stimulation of progesterone production by lutropin (results not shown). Similarly, verapamil, an organic inhibitor of transmembrane Ca²⁺ uptake by cells (Mannhold *et al.*, 1978), and EGTA, a preferential chelator of Ca²⁺ (Schmid & Reilley, 1957), also impaired the stimulatory actions of lutropin (Table 2).

The total cellular content of Ca²⁺ was decreased significantly under incubation conditions designed to restrict Ca²⁺ uptake by cells; for example, 56 ± 14 nmol of Ca²⁺/4 × 10⁷ cells in control (1.27 mM-Ca²⁺) incubations compared with 36 ± 7 nmol in Ca²⁺-poor (5 μ M Ca²⁺) conditions ($P = 0.08$), 33 ± 7 nmol in verapamil-treated cells ($P < 0.05$) and 21 ± 4 nmol of Ca²⁺/4 × 10⁷ cells in EGTA (2 mM)-treated cultures ($P < 0.02$) (results are means $\pm$ S.D. for four experiments with triplicate incubations in each experiment).

To assess whether alterations in intracellular Ca²⁺ modulated steroidogenesis, we used ionophore A23187, a bivalent-cation ionophore, which increases Ca²⁺ uptake in a variety of cells (Pressman, 1975). Ionophore A23187 significantly ($P < 0.01$) increased progesterone production in response to maximally stimulating concentrations of lutropin [basal progesterone production, 2.94 ± 0.08 ; lutropin-stimulated cultures, 28.1 ± 0.92 ; lutropin + ionophore A23187 (0.1 μ g/ml) treatment, 34.7 ± 0.66 ng of progesterone/10⁶ cells per 4 h]. Since certain actions of intracellular Ca²⁺ are mediated by binding to specific proteins, we used trifluoperazine as a high-affinity inhibitor of Ca²⁺ binding (Cheung, 1979). Micromolar concentrations of trifluoperazine significantly inhibited the stimulatory effects of lutropin (Fig. 3). Trifluoperazine was as effective as depletion of intracellular Ca²⁺ with EGTA in suppressing progesterone production. In addition, trifluoperazine was capable of inhibiting the stimulatory actions of exogenously supplied cyclic AMP, in the form of 8-bromo cyclic AMP (Fig. 4). The inhibitory effect of trifluoperazine could not be overcome by maximally stimulat-

but also accumulated lower concentrations of progesterone within cells. Thus reduction in the amount of progesterone secreted into the medium could not be accounted for by simple retention of progesterone within cells under Ca²⁺-deficient conditions.

Table 1. Ca^{2+} modulates progesterone production in pig ovarian cells

Granulosa cells from swine follicles were incubated for 4 h in the presence (+) of Ca^{2+} (1.27 mM) or absence (–) of Ca^{2+} (final Ca^{2+} concentration 5 μM). Various stimulators of progesterone production were added at the outset of culture at the final concentrations denoted. Abbreviation used: IBMX, 3-isobutyl-1-methylxanthine. Progesterone concentrations were assayed in the medium. * $P < 0.01$ versus + Ca^{2+} cultures in the same row. Data are means $\pm$ S.E.M. for three cultures. In Experiment B, after 8-bromo cyclic AMP (2.0 mM), the cellular content of progesterone (ng/ 10^6 cells) was 8.21 ± 0.31 in the presence of Ca^{2+} and 4.05 ± 0.22 in the absence of added Ca^{2+} (basal value 1.26 ± 0.07 ng/ 10^6 cells).

	Progesterone produced (ng/ 10^6 cells per 4 h)	
	+ Ca^{2+}	– Ca^{2+}
Experiment A		
Basal	2.54 ± 0.14	2.61 ± 0.23
IBMX (0.25 mM)	8.79 ± 0.04	$6.90 \pm 0.14^*$
Lutropin (1000 ng/ml)	24.2 ± 0.22	$11.8 \pm 0.59^*$
Lutropin + IBMX (0.25 mM)	29.4 ± 0.83	$16.4 \pm 0.55^*$
Cholera toxin (1 $\mu\text{g}/\text{ml}$)	12.6 ± 0.08	$8.09 \pm 0.69^*$
Experiment B		
Basal	2.05 ± 0.10	2.13 ± 0.14
8-Bromo cyclic AMP (0.25 mM)	26.2 ± 1.40	$10.0 \pm 1.20^*$
8-Bromo cyclic AMP (2.0 mM)	25.5 ± 1.30	$11.2 \pm 0.80^*$

Table 2. Regulatory actions of Ca^{2+} on progesterone production by isolated swine granulosa cells

Granulosa cells from swine follicle were maintained *in vitro* in the absence (–) or presence (+) of Mg^{2+} (0.84 mM) and in the presence (+, 1.27 mM) of Ca^{2+} or absence (–, 5 μM) of Ca^{2+} . Effectors were added at the outset of the 4-h incubation. See the Results section for further discussion. * $P < 0.01$ versus control (basal) values; ** $P < 0.01$ versus lutropin (+ Ca^{2+} , + Mg^{2+}) values. Data are means $\pm$ S.E.M. for three cultures.

Condition	Mg^{2+}	Ca^{2+}	Progesterone production (ng/ 10^6 cells per 4 h)
Basal	+	+	3.05 ± 0.56
Basal	–	–	3.18 ± 0.09
Lutropin (200 ng/ml)	–	–	3.95 ± 0.18
Lutropin	+	–	4.24 ± 0.32
Lutropin	–	+	$7.85 \pm 0.37^*$
Lutropin	+	+	$17.66 \pm 0.53^*$
Lutropin + EGTA (1.27 mM)	+	+	$4.37 \pm 0.26^{**}$
Lutropin + EGTA (2.0 mM)	+	+	$2.89 \pm 0.41^{**}$
Lutropin + Verapamil (50 μM)	+	+	$8.31 \pm 0.52^{**}$

ing concentrations of 8-bromo cyclic AMP. Micro-molar concentration of trifluoperazine do not inhibit [^{14}C]leucine incorporation into acid-precipitable protein, or suppress the stimulation of 6-oxoprostaglandin $\text{F}_{1\alpha}$ production in response to lutropin administration (J. D. Veldhuis & P. A. Klase, unpublished work).

Discussion

The present studies delineate major modulatory effects of Ca^{2+} on progesterone production by isolated ovarian cells. Under conditions *in vitro* capable of depleting the total cellular content of Ca^{2+} in granulosa cells, the stimulatory actions of

lutropin are impaired significantly. Thus incubation in Ca^{2+} -deficient medium, in medium containing EGTA to chelate extracellular Ca^{2+} or incubation in the presence of verapamil, an organic antagonist of cellular Ca^{2+} uptake (Mannhold *et al.*, 1978), all effectively inhibit the stimulatory actions of lutropin. In contrast, ionophore A23187, which is believed to augment intracellular pools of Ca^{2+} (Pressman, 1975), significantly enhances progesterone production in response to lutropin. These observations suggest that alterations in intracellular pools of Ca^{2+} may modulate progesterone production in ovarian cells.

In addition to suppressing the stimulatory actions of lutropin, Ca^{2+} -deficient conditions also impede the

Fig. 3. *Trifluoperazine inhibits lutropin-stimulated progesterone secretion*

Fig granulosa cells were incubated in suspension culture for 4 h in the presence or absence of lutropin (LH), with or without 25 μ M-trifluoperazine (TFP), or EGTA (1.27 mM) in medium containing 0.84 mM-Mg²⁺ and 1.27 mM-Ca²⁺. **P* < 0.01 versus basal value; ***P* < 0.01 versus value for LH alone. Data are means $\pm$ S.E.M. (represented by the bars; *n* = three cultures).

effects of other hormones such as prostaglandin E₂ and L-adrenaline, which are believed also to act via cyclic AMP (Marsh & LeMaire, 1974; Dufau & Catt, 1978; Veldhuis *et al.*, 1979). Ca²⁺ deprivation does not alter basal progesterone production or diminish cell viability or limit certain other cellular responses to hormonal stimulation that are believed to be dependent on protein synthesis (Veldhuis & Hammond, 1981). However, Ca²⁺ deprivation does significantly impair the stimulatory response of cells to either exogenously supplied cyclic AMP (e.g. 8-bromo cyclic AMP) or agents that maintain or generate cyclic AMP endogenously (e.g., in response to cholera toxin or 3-isobutyl-1-methylxanthine; Rasmussen & Goodman, 1977). Thus, at least one component of steroidogenesis that is

Fig. 4. *Trifluoperazine inhibits progesterone production in response to cyclic AMP*

Swine granulosa cells were incubated in short-term suspension cultures (defined in the Materials and methods section). Cultures were treated with control solvent or increasing concentrations of 8-bromo cyclic AMP, in the presence or absence of trifluoperazine (50 μ M). The medium was assayed for progesterone content. Data are means $\pm$ S.E.M. (represented by the bars) for triplicate cultures.

dependent on Ca²⁺ seems to reside distal to, or independent of, the actual generation of cyclic AMP by ovarian cells. This inference is similar to that by some investigators studying the adrenal gland (Bowyer & Kitabchi, 1974), but not to that of others, who have shown that Ca²⁺ influences cyclic AMP synthesis (Rubin *et al.*, 1972). However, Ca²⁺ has been shown not to affect the generation of cyclic AMP or the activation of cyclic AMP-dependent protein kinase by lutropin (Janszen *et al.*, 1976; Carnegie & Tsang, 1981) under some conditions.

Both the secretion of progesterone into the extracellular medium, and the total mass of progesterone accumulated within granulosa cells in response to lutropin, were impaired in Ca²⁺-deficient cells. Thus alterations in secretory processes alone are unlikely to account for the effects of Ca²⁺ deprivation on progesterone production. To explore further the mechanisms by which Ca²⁺ modulates total progesterone production in ovarian cells, we employed trifluoperazine, a phenothiazine compound with high-affinity binding to calmodulin (Cheung, 1979). Calmodulin is believed to mediate many actions of intracellular Ca²⁺ in a variety of cells (Means & Dedman, 1980). Our studies indicate that in isolated swine granulosa cells, low concentrations of trifluoperazine (25–50 μ M) effectively

suppress progesterone production in response to maximally stimulating concentrations of either lutropin or an exogenously supplied analogue of cyclic AMP. These observations suggest, but do not prove, an intermediate role for high-affinity Ca^{2+} -binding proteins in progesterone biosynthesis in ovarian cells. This novel inference in the ovary is congruent with recent preliminary observations in the adrenal gland, in which liposome-entrapped complexes of calmodulin and Ca^{2+} stimulate steroidogenesis (Hall & Osawa, 1981).

In many tissues, including the ovary, Ca^{2+} -dependent protein kinases may modify intracellular processes by phosphorylation of relevant protein substrate (Kuo *et al.*, 1980). In ovarian cells, the nature of such endogenous substrates has not yet been characterized. In addition, precise interrelationships among various protein-phosphorylation systems (including those stimulated by cyclic nucleotides and Ca^{2+} -calmodulin) have not yet been defined. Nonetheless, one general intracellular locus at which Ca^{2+} may regulate steroidogenesis is mitochondrial conversion of cholesterol into steroid hormones (Péron & McCarthy, 1968; Arthur & Boyd, 1976; Farese & Prudente, 1978).

In conclusion, although a role for Ca^{2+} in steroidogenesis has been implied previously by studies in the adrenal and in the testis (see the Introduction), the present work demonstrates significant modulatory actions of Ca^{2+} in another major steroidogenic organ, the ovary. Our observations also implicate one or more intracellular processes distal to, or independent of, cyclic AMP generation in the action of Ca^{2+} in ovarian cells. The susceptibility of progesterone production to inhibition by trifluoperazine further raises the important consideration that high-affinity Ca^{2+} -binding protein(s) in the ovary participate in steroidogenic processes. Thus the present model is likely to provide an opportunity to explore further the nature of the intracellular actions of Ca^{2+} in modulating the steroidogenic effects of reproductive hormones, and of cyclic AMP, in the ovary.

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