Role of calcium in the modulation of ornithine decarboxylase activity in isolated pig granulosa cells *in vitro*

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(Received 23 December 1980/Accepted 4 March 1981)

We examined the role of Ca^{2+} in the control of basal and hormone-stimulated ornithine decarboxylase activity in isolated pig granulosa cells maintained under chemically defined conditions *in vitro*. Omission of Ca^{2+} from the incubation medium (measured Ca^{2+} concentration $5\ \mu\text{M}$) decreased basal enzymic activity, and significantly ($P < 0.01$) impaired the response to maximally stimulating doses of either lutropin or follitropin. No significant alteration occurred in the concentration of either gonadotropin required to elicit half-maximal effects. The addition of EGTA ($1.27\text{--}2.0\text{ mM}$) to chelate residual extracellular Ca^{2+} further decreased hormone-induced rises in ornithine decarboxylase activity. Despite the presence of 1.27 mM concentrations of extracellular Ca^{2+} , the administration of presumptive Ca^{2+} antagonists, believed to impair trans-membrane Ca^{2+} influx [verapamil ($10\text{--}100\ \mu\text{M}$), nifedipine ($1\text{--}100\ \mu\text{M}$) or CoCl_2 (1 mM)] suppressed hormone-stimulated ornithine decarboxylase activity. The inhibitory effects of verapamil or of Ca^{2+} omission from the medium were not overcome by the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (0.25 mM), or by cholera toxin, or by an exogenously supplied cyclic AMP analogue, 8-bromo cyclic AMP. Conversely, micromolar concentrations of a putative bivalent-cation ionophore, A23187, increased significantly the stimulation of ornithine decarboxylase activity by saturating concentrations of lutropin or 8-bromo cyclic AMP. Thus the present observations implicate Ca^{2+} ions in the modulation of hormone action and cellular function in normal ovarian cells.

Bivalent metal ions significantly modulate hormone action in several endocrine tissues (Rasmussen & Goodman, 1977). For example, Ca^{2+} participates in the control of stimulus–secretion coupling in pituitary cells (Conn *et al.*, 1980), the adrenal gland (Garcia *et al.*, 1975; Podesta *et al.*, 1980), the ovary (Higuchi *et al.*, 1976) or testes (Janszen *et al.*, 1976), and pancreatic islets (Borowitz & Matthews, 1980). Ca^{2+} ions have also been implicated in the mediation of certain hormonal effects on membrane permeability (Putney, 1979), protein synthesis (Cheung, 1980), cell replication (Cuprak *et al.*, 1979; Whitfield *et al.*, 1979) and intracellular enzymic activity (Geisow, 1978; Means *et al.*, 1979; Betteridge, 1980; Murphy *et al.*, 1980).

In the current work, we have investigated the role of Ca^{2+} in the action of lutropin and follitropin in isolated ovarian cells maintained under chemically defined conditions *in vitro*. We studied activity of a cytosolic enzyme, ornithine decarboxylase (L-orni-

thine carboxy-lyase, EC 4.1.1.17), which is rate-limiting in eukaryotic polyamine biosynthesis (Jänne *et al.*, 1978), since the responsiveness of this enzyme to gonadotropins has been well characterized in granulosa cells *in vitro* (Osterman & Hammond, 1977; Osterman *et al.*, 1978). Furthermore, we have defined changes in ornithine decarboxylase activity in relation to ovarian steroidogenesis and cell replication in this system (Veldhuis & Hammond, 1979). Notably, Ca^{2+} ions are also believed to participate in the regulation of both cell proliferation and steroidogenesis in several differentiated tissues (Higuchi *et al.*, 1976; Janszen *et al.*, 1976; Whitfield *et al.*, 1979; Cuprak *et al.*, 1979; Cheung, 1980; Hennings *et al.*, 1980; Podesta *et al.*, 1980). However, the discrete molecular mechanisms by which such complex regulatory effects of Ca^{2+} ions are exerted remain incompletely understood. In the present study, we have explored the role of Ca^{2+} ions in the control of ornithine decarboxylase activity, in

an effort to define a more proximate and a specific enzymic locus at which Ca^{2+} ions may regulate cellular function.

Materials and methods

Materials

Sheep lutropin (NIH-LH-S19) was obtained from the Hormone Distribution Office (NIAMDD, NIH, Bethesda, MD, U.S.A.) Highly purified sheep follitropin (approx. 50-fold the potency of NIH-oFSH-S1) was provided by Dr. Harold Papkoff. L-[1- ^{14}C]Ornithine monohydrochloride (sp. radioactivity 45 Ci/mol) was from New England Nuclear Corp., Boston, MA, U.S.A., and L-ornithine hydrochloride, 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.) and nifedipine by Dr. F. C. Greenslade (Pfizer, New York, NY, U.S.A.). Other chemicals and reagents were of the best quality commercially available.

Cell preparations and enzymic assay

Granulosa cells were harvested by fine-needle aspiration of small and medium-sized follicles from ovaries of sexually immature pigs. Cells were washed free of follicular fluid by centrifugation in culture medium, and approx. 3×10^7 – 5×10^7 viable cells (determined by Trypan-Blue exclusion) were incubated per culture flask, as previously described by Osterman & Hammond (1977). The incubation medium comprised 5 ml of Eagle's minimal essential medium (Gibco, Grand Island, NY, U.S.A.) 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 4 h at 37°C in a metabolic shaker bath, recovered by centrifugation (5 min at 100 g), and washed twice in 25 mM-Tris/HCl buffer (pH 7.4) containing 0.25 M-sucrose, 0.1 mM-EDTA and 2.5 mM-dithiothreitol (Osterman & Hammond, 1977). Cells were lysed by two cycles of freeze-thawing in sucrose-free buffer. The 20000 g supernatant was assayed in duplicate for the determination of ornithine decarboxylase activity, by measuring the liberation of $^{14}\text{CO}_2$ from L-[1- ^{14}C]ornithine in the presence of saturating substrate as reported by Pegg *et al.* (1970), and modified by Osterman & Hammond (1977). Exhaustive dialysis of cytosol against Ca^{2+} -free assay buffer did not modify enzymic activity. Cytosolic protein concentrations were determined by the

method of Bradford (1976), with crystalline bovine serum albumin (fraction V) as standard.

Experimental effectors of calcium transport were added to incubations of dispersed granulosa cells 20 min before the administration of hormonal or non-hormone stimulators, except when noted differently in the time-course experiments. The activity of ornithine decarboxylase was computed as pmol of CO_2/h per mg of protein. Data are given as means $\pm$ S.E.M. for three replicate cultures. Since basal ornithine decarboxylase activity varies somewhat among batches of granulosa cells (Osterman & Hammond, 1977), all experimental comparisons were performed within a single pool of cells harvested from 100–200 ovaries. Each experiment was repeated at least once with cells derived from another set of ovaries. Statistical analyses of data utilized one-way analysis of variance, with the Newman-Keul's procedure for ascertainment of significant differences among means (Winer, 1971).

The calcium content of 'calcium-free' Eagle's medium was determined by atomic absorption spectroscopy (Perkin-Elmer model 305) (Murphy *et al.*, 1979), after evaporating 200 ml of Eagle's medium and reconstituting in LaCl_3 buffer (McDonald *et al.*, 1977). Absorption of standard buffers was linear over the range 0.08–5.0 μg of calcium/ml.

Results

As depicted in Fig. 1(a), omission of Ca^{2+} from the incubation medium ('calcium-deprived', 5 μM final Ca^{2+} content of medium) significantly impaired the stimulation of enzymic activity by maximally effective concentrations of highly purified follitropin ($P < 0.01$). However, the concentration of hormone required to elicit a half-maximal increase in ornithine decarboxylase activity was not altered (1.85 ng/ml, denoted by arrows). As discussed further below, basal enzymic activity was slightly but significantly decreased ($P < 0.05$). A similar pattern of enzymic response was also observed after stimulation with lutropin, in the presence or absence of extracellular Ca^{2+} (see Fig. 1b). This impaired responsiveness to hormonal stimulation did not reflect displacement of the time-course, since peak stimulation of ornithine decarboxylase activity by lutropin continued to occur at 4–6 h (Fig. 2). Omission of Ca^{2+} from cultures inhibited the stimulatory effects of gonadotropin at both early and late time points (Fig. 2).

A more detailed examination of the dependence of basal ornithine decarboxylase activity on Ca^{2+} revealed significant effects of varying the extracellular Ca^{2+} concentration (Table 1). Maximal basal enzymic activity was observed at Ca^{2+} concentrations of approx. 1.2 mM. Enzymic activity was decreased significantly when cells were incubated for 4 h with Ca^{2+} concentrations either higher

Fig. 1. Inhibition of (a) follitropin-stimulated and (b) lutropin-stimulated ornithine decarboxylase activity by lowering the extracellular Ca²⁺ concentrations. Granulosa cells were incubated in the presence of (1.27 mM; ●) or 'absence' (5 μ M; ○) of CaCl₂, and exposed to increasing concentrations of highly purified sheep follitropin (a) or lutropin (b). Cultures were harvested after 4 h for the determination of cytosolic ornithine decarboxylase activity. Data are means (and range) for duplicate cultures at each hormone concentration (control cultures are triplicates, given as means $\pm$ S.E.M.). Ornithine decarboxylase activity is expressed as pmol of CO₂/h per mg of protein. Arrows indicate hormone concentrations giving half-maximal increase in enzyme activity.

or lower than 1.2 mM, and in the absence of any added Ca²⁺ basal ornithine decarboxylase activity fell further [but remained readily detectable (228 $\pm$ 12 c.p.m., versus assay blank of 25 $\pm$ 8 c.p.m.)]. Direct addition of Ca²⁺ or chelators to cytosol did not modify enzymic activity (results not shown; see also Costa & Nye, 1978).

To assess the dependence of ornithine decar-

Fig. 2. Time-course of lutropin action in the presence or absence of extracellular Ca²⁺ supplementation. Duplicate cultures of 3×10^7 viable granulosa cells per flask were maintained for various time intervals in the presence of maximally stimulated concentrations of lutropin (200 ng/ml), and in the presence (1.27 mM; ●) or 'absence' (5 μ M; ○) of CaCl₂. At the times indicated, cultures were prepared for the assay of cytosolic ornithine decarboxylase activity (data are expressed in pmol of CO₂/h per mg of protein). Data are given as the means and range of duplicate cultures.

Table 1. Dependence of basal ornithine decarboxylase activity on extracellular calcium concentrations

Various amounts of CaCl₂ (or equimolar NaCl) were added to Eagle's medium to achieve the final salt concentrations enumerated. For further details see the Materials and methods section. Ornithine decarboxylase activity is given as pmol of CO₂/h per mg of cytosol protein. Data are means $\pm$ S.E.M. for three cultures.

Concn. of Ca ²⁺ in incubation medium (mM)	Enzyme activity
4.0	639 $\pm$ 48†
2.0	829 $\pm$ 62
1.2	1010 $\pm$ 41
0.8	912 $\pm$ 96
0.4	649 $\pm$ 35†
'None'*	320 $\pm$ 51†

* The concentration of residual Ca²⁺ in Eagle's medium (prepared in the apparent absence of Ca²⁺) was 5 μ M as determined by atomic absorption spectrometry.

† $P < 0.01$ versus ornithine decarboxylase activity observed at 1.2 mM-CaCl₂.

boxylase activity on transmembrane Ca²⁺ uptake, putative inhibitors of cellular cation transport were employed. In the presence of 1.27 mM-Ca²⁺, verapamil (1–50 μ M), a presumptive inhibitor of Ca²⁺

uptake (Eto *et al.*, 1974), produced a dose-related decrease in basal ornithine decarboxylase activity, and 50 μM -verapamil significantly ($P < 0.01$) impaired the absolute response to lutropin (Table 2). However, verapamil (100 μM) did not modify hormone-stimulated progesterone secretion, or alter cell viability even in longer-term (12–24 h) cultures (J. Veldhuis & J. Hammond, unpublished work). The suppressive effect of verapamil on ornithine decarboxylase activity was not prevented by the administration of a phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (0.25 mM), in the presence of 1.27 mM- Ca^{2+} . Similarly, 3-isobutyl-1-methylxanthine was incapable of ameliorating the inhibitory effects of 'calcium-free' incubations on lutropin stimulation of enzymic activity (Table 3). The putative Ca^{2+} antagonist nifedipine hydrochloride (Malaisse & Boscher, 1977) also decreased the stimulatory effect of lutropin in a dose-related fashion, i.e. by $22 \pm 7\%$ (1 μM -nifedipine), by $53 \pm 8\%$ (10 μM drug), and by $88 \pm 3\%$ (100 μM drug). CoCl_2 (1.0 mM), a selective blocker of slow Ca^{2+} channels in excitable tissues (Baker *et al.*, 1973), impaired the effect of maximally stimulating concentrations of lutropin by $75 \pm 4\%$ ($P < 0.01$).

EGTA was used to chelate extracellular Ca^{2+} in preference to Mg^{2+} (Schmid & Reilley, 1957). The addition of EGTA (2 mM) to Eagle's medium (1.27 mM- Ca^{2+} and 0.84 mM- Mg^{2+}), coincidentally with (or in 1 h or 2 h preincubations before) lutropin suppressed the effect of saturating concentrations of lutropin by $85 \pm 3\%$. The inhibitory effects of EGTA were overcome by the presence of excess Ca^{2+} (3.54 mM), but not by similar concentrations of Mg^{2+}

(results not shown). EGTA also decreased the stimulatory effect of cholera toxin, from 2076 ± 48 to 464 ± 18 pmol of CO_2/h per mg of protein. To circumvent the requirement for cyclic AMP generation, we examined the effect of EGTA on enzymic activity stimulated by exogenously supplied 8-bromo cyclic AMP. As shown in Table 4, chelation of extracellular Ca^{2+} also significantly decreased the

Table 3. *Effect of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine on lutropin-stimulated ornithine decarboxylase activity*

For details see the Materials and methods section. Data are means $\pm$ S.E.M. for three cultures. LH denotes presence or absence of maximally stimulating concentrations of lutropin (200 ng/ml). Cultures receiving Ca^{2+} are denoted by '+' (final extracellular Ca^{2+} concentration 1.27 mM). Other cultures '-' received equimolar NaCl without Ca^{2+} . IBMX denotes presence or absence of 3-isobutyl-1-methylxanthine (0.25 mM). Ornithine decarboxylase activity is given as pmol of CO_2/h per mg of protein. * $P < 0.01$ for LH effect versus basal; ** $P < 0.05$ for IBMX effect. Omission of Ca^{2+} from the medium produced a significant ($P < 0.02$) decrease in LH-stimulated enzymic activity in the presence (+) or absence (–) of IBMX.

LH	Ca^{2+}	IBMX	Ornithine decarboxylase activity
–	+	–	666 $\pm$ 12
+	+	–	1600 $\pm$ 95*
+	+	+	2200 $\pm$ 140**
+	–	–	540 $\pm$ 14
+	–	+	880 $\pm$ 84

Table 2. *Inhibition of basal and lutropin-stimulated ornithine decarboxylase activity by the calcium antagonist, verapamil hydrochloride*

Verapamil hydrochloride was added 10 min before maximally stimulating concentrations of lutropin (LH), or LH + 3-isobutyl-1-methylxanthine, IBMX; 0.25 mM), or control solvent (basal). The concentration of lutropin was 200 ng/ml. For further details see the Materials and methods section. Ornithine decarboxylase activity is given as pmol of CO_2/h per mg of protein. Data are means $\pm$ S.E.M. (for three cultures. * $P < 0.01$ for verapamil effect versus corresponding control cultures in each column (zero verapamil); ** $P < 0.05$ for inhibition by verapamil of the effect of lutropin + IBMX.

Verapamil concn. (μM)	Ornithine decarboxylase activity		
	Basal	LH	LH + IBMX
0	734 $\pm$ 48	3160 $\pm$ 67	4230 $\pm$ 187
1	624 $\pm$ 69	3130 $\pm$ 112	—
10	273 $\pm$ 44*	2900 $\pm$ 121	—
50	198 $\pm$ 45*	2560 $\pm$ 62*	3010 $\pm$ 140**

Table 4. *Effect of extracellular Ca^{2+} depletion on 8-bromo-cyclic AMP-stimulated ornithine decarboxylase activity*

For details see the Materials and methods section. Data are means $\pm$ S.E.M. for three cultures. '8-Br-cAMP' denotes presence or absence of 8-bromo cyclic AMP in maximally stimulating concentrations (0.5 mM). Cultures received (+) or did not receive (–) CaCl_2 (1.27 mM), in the presence (+) or absence (0) of 1.27 mM-EGTA. Ornithine decarboxylase activity is in pmol of CO_2/h per mg of protein. * $P < 0.01$ versus basal activity (620 $\pm$ 18); ** $P < 0.01$ for effects of Ca^{2+} depletion on 8-Br-cAMP-stimulated enzymic activity.

8-Br-cAMP	Ca^{2+}	EGTA	Enzyme activity
0	+	0	620 $\pm$ 18
0	–	0	361 $\pm$ 20*
0	–	+	270 $\pm$ 26*
+	+	0	2040 $\pm$ 50*
+	–	0	848 $\pm$ 40**
+	–	+	462 $\pm$ 14**

Table 5. *Effect of delayed addition of verapamil on lutropin-stimulated ornithine decarboxylase activity*
Verapamil hydrochloride (100 μ M) was administered coincidentally with, or at various intervals (min) after, cells were exposed to maximally stimulating concentrations of lutropin (LH) (200 mg/ml). For further details see the Materials and methods section. Ornithine decarboxylase activity is pmol of CO₂/h per mg of protein (means $\pm$ s.e.m. for three cultures). * $P < 0.001$ for LH effect; ** $P < 0.01$ for verapamil effect.

Verapamil addition: time after lutropin (min)	Presence of LH	Enzyme activity
None	—	956 $\pm$ 41
None	+	2727 $\pm$ 123*
Simultaneous	+	485 $\pm$ 20**
+10	+	667 $\pm$ 101**
+30	+	708 $\pm$ 23**
+90	+	975 $\pm$ 64**

stimulatory action of 8-bromo cyclic AMP, which is believed to activate cyclic AMP-dependent isoenzymes of protein kinase directly (Yagura & Miller, 1980).

The degree of inhibition exerted by EGTA was similar to or greater than that achieved by simple omission of Ca²⁺ from the medium or by treatment with verapamil hydrochloride (Table 5) (100 μ M produced 82 $\pm$ 6% inhibition). When the addition of verapamil was progressively delayed after exposure of cells to lutropin in the presence of 1.27 mM-Ca²⁺, its suppressive effects could still be observed (Table 5).

The potential relevance of alterations in intracellular Ca²⁺ concentrations in modulating basal and gonadotropin-stimulated ornithine decarboxylase activity was examined further by treatment of incubated cells with the bivalent-cation ionophore, A23187 (Pressman, 1975). At low concentrations of ionophore A23187 (0.010 and 0.075 μ g/ml), the effect of maximally stimulating concentrations of lutropin could be increased further, by respectively 1.96 $\pm$ 0.08- and 1.43 $\pm$ 0.07-fold ($P < 0.01$ and $P < 0.05$ respectively). Ionophore A23187 (0.25 μ g/ml) also increased significantly the effect of maximally stimulating concentrations of 8-bromo cyclic AMP (0.05 mM), from 4100 $\pm$ 85 to 5830 $\pm$ 180 pmol of CO₂/h per mg of protein ($P < 0.02$).

Discussion

With untransformed pig granulosa cells maintained under chemically defined conditions *in vitro*, the current studies define a specific dependence of cytosolic ornithine decarboxylase activity on Ca²⁺ ions. Since ornithine decarboxylase activity is subject

to rapid regulation in most tissues (Maudsley, 1979; Russell, 1980), we could discern significant effects of altered Ca²⁺ status during short-term incubation of cultured ovarian cells. The present experiments demonstrate that perturbations of Ca²⁺ concentrations in the incubation medium, or manipulations of putative cellular uptake of Ca²⁺, were capable of modulating both basal and maximally stimulated enzymic activity. Changes in enzymic activity could not be attributed to simple alterations in extracellular osmolality [which also stimulate ornithine decarboxylase activity (Perry & Oka, 1980)], since osmolality was experimentally controlled, and enzymic activity decreased with either high (>1.2 mM) or low (<1.2 mM) extracellular Ca²⁺ concentrations. Furthermore, the addition of EGTA, to achieve high-affinity chelation of extracellular Ca²⁺ in preference to chelation of Mg²⁺ (Schmid & Reilley, 1957), also decreased ornithine decarboxylase activity further, both basally and after hormonal stimulation.

The suppressive effects of simple extracellular Ca²⁺ depletion, or of chelation of Ca²⁺ with EGTA, could be mimicked by several presumptive antagonists of transmembrane Ca²⁺ influx, specifically verapamil hydrochloride (Eto *et al.*, 1974), nifedipine (Malaisse & Boscher, 1977) or CoCl₂ (Baker *et al.*, 1973). These observations suggested that extracellular Ca²⁺ ions are required for maximal enzymic stimulation, and for maintenance of basal activity. Conversely, treatment with a dicarboxylic ionophore, A23187, which is capable of enhancing intracellular accumulation of Ca²⁺ in many tissues (Pressman, 1975; Malaisse *et al.*, 1979; Kurzinger *et al.*, 1980; Weissmann *et al.*, 1980), evoked a significant increase in the stimulatory effects of saturating concentrations of either lutropin or a stable cyclic AMP analogue, 8-bromo cyclic AMP. These observations permit the inference that modulation of ornithine decarboxylase activity by Ca²⁺ ions may occur at the level of intracellular Ca²⁺ pools. However, pharmacological manipulation of extracellular Ca²⁺ concentrations or of transmembrane Ca²⁺ uptake could also induce equilibrium changes in intracellular Ca²⁺ content or distribution (Steinhardt & Epel, 1974; Chen *et al.*, 1978; Brostrom *et al.*, 1979; Murphy *et al.*, 1979; Kneer *et al.*, 1979; Kurzinger *et al.*, 1980). Thus the nature of relevant intracellular pools of Ca²⁺ cannot be defined precisely without the direct measurements of subcellular Ca²⁺ distribution in relation to hormonal stimulation. The capability to accomplish such determinations in non-excitabile cells has been suggested by Murphy *et al.* (1980), who utilized calcium-sensitive metallochromic indicators to describe a rapid increase in apparent cytosolic free Ca²⁺ concentrations in isolated hepatocytes after hormonal stimulation.

The mechanisms by which alterations in intracellular Ca^{2+} concentrations might regulate ornithine decarboxylase activity are unknown. Preliminary reports of extracellular calcium requirements for stimulation of ornithine decarboxylase activity have also been set forth for Chinese-hamster ovary cells, a rat astrocytoma cell line, and BALB 3T3 cells maintained in chemically defined medium (Costa & Nye, 1978; Costa, 1979; Gibbs *et al.*, 1980). In order to understand further the mechanisms subserving calcium-dependence, in the current work we have focused initially on Ca^{2+} interactions with the hormone-stimulated cyclic AMP system. Previous studies from our laboratory have suggested an intermediate role for intracellular cyclic AMP in the hormonal stimulation of ornithine decarboxylase activity in pig granulosa cells *in vitro* (Osterman *et al.*, 1978). Our present results indicate further that the inhibitory effects of Ca^{2+} depletion are unlikely to occur through stimulation of the cyclic AMP phosphodiesterase system. In particular, the suppressive effects of verapamil, or of extracellular Ca^{2+} depletion, could not be overcome by treatment with 3-isobutyl-1-methylxanthine to inhibit phosphodiesterase activity in granulosa cells. Furthermore, in many tissues (Rasmussen & Goodman, 1977), depletion of Ca^{2+} ions is associated with a decline rather than a stimulation of phosphodiesterase activity (Cheung, 1971). On the other hand, our data suggest that the effects of Ca^{2+} may be mediated in part at loci distal to intracellular cyclic AMP generation. In particular, maximal enzymic stimulation by a stable analogue of cyclic AMP (8-bromo cyclic AMP) was decreased significantly in the absence of extracellular Ca^{2+} , and was increased significantly in the presence of low concentrations of the bivalent-cation ionophore A23187. In this context, we would note that in the study of steroidogenesis in adrenal and Leydig cells, Ca^{2+} was inferred to exert effects distal to cyclic nucleotide generation, without direct alteration in cyclic AMP-dependent protein kinase activity (Perchellet & Sharma, 1979; Janszen *et al.*, 1976). The current observations suggest that the regulatory effects of Ca^{2+} on ornithine decarboxylase activity may also be exerted in part at loci distal to intracellular cyclic AMP production. However, we have not yet excluded additional effects of Ca^{2+} in ovarian cells at other loci of hormone action, such as at the level of hormone binding, hormone-receptor coupling to adenylate cyclase, or direct stimulation of cyclic AMP synthesis. In other hormonally responsive isolated cell systems, involvement of Ca^{2+} has been suggested at these sites (Lefkowitz *et al.*, 1970; Marian & Conn, 1980; Jackowski *et al.*, 1980; Mahaffee & Ontjes, 1980; Podesta *et al.*, 1980).

In summary, the current work demonstrates a specific dependence on Ca^{2+} of both basal and

hormone-stimulated ornithine decarboxylase activity in normal pig granulosa cells maintained in serum-free conditions *in vitro*. Our data suggest that important regulatory effects of Ca^{2+} ions are exerted, at least in part, through intracellular pools of Ca^{2+} that operate at loci distal to cyclic AMP generation.

We thank the Shamokin Packing Corp. and Arbogast and Bastin, Inc., Allentown, PA, U.S.A., for the pig ovaries, Marlene Thompson for the skilled preparation of this manuscript, and Patricia Klase and Eileen Wolck for valuable technical assistance. We appreciate the gifts of verapamil and nifedipine from Knoll Pharmaceutical Co. and Pfizer, respectively. Dr. Thomas J. Rohner and Stephanie Garlick performed the atomic-absorption determination of calcium content. This work was supported by NIH grant HD10122 to J. M. H. and a National Research Service award F32HD0577 to J. D. V. from the National Institutes of Child Health and Human Development, Bethesda, MD, U.S.A.

References

- Baker, P. F., Meves, H. & Ridgway, E. B. (1973) *J. Physiol. (London)* **231**, 511–518
- Betteridge, A. (1980) *Biochem. J.* **186**, 987–992
- Borowitz, J. L. & Matthews, E. K. (1980) *J. Cell Sci.* **41**, 233–243
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Brostrom, M., Brostrom, C. O. & Wolff, D. J. (1979) *J. Biol. Chem.* **254**, 7548–7557
- Chen, J.-L. J., Babcock, D. F. & Lardy, H. A. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 2234–2238
- Cheung, W. Y. (1971) *J. Biol. Chem.* **246**, 2859–2869
- Cheung, W. Y. (1980) *Science* **207**, 19–27
- Conn, P. M., Marian, J., McMillian, M. & Rogers, D. (1980) *Cell Calcium* **1**, 7–20
- Costa, M. (1979) *Life Sci.* **25**, 2113–2124
- Costa, M. & Nye, J. S. (1978) *Biochem. Biophys. Res. Commun.* **85**, 1156–1164
- Cuprak, L. J., Lammi, C. J. & Crane, J. I. (1979) *In Vitro* **15**, 900–909
- Eto, S., Wood, J. M., Hutchins, M. & Fleischer, N. (1974) *Am. J. Physiol.* **226**, 1315–1320
- Garcia, A. G., Kirpekar, S. M. & Prat, J. C. (1975) *J. Physiol. (London)* **244**, 253–262
- Geisow, M. J. (1978) *Nature (London)* **276**, 211–212
- Gibbs, J. B., Hsu, C.-Y., Terasaki, W. L. & Brooker, G. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 995–999
- Hennings, H., Michael, D., Cheng, C., Steinert, P., Holbrook, K. & Yuspa, S. H. (1980) *Cell* **19**, 245–254
- Higuchi, T., Kaneko, A., Abel, J. H., Jr. & Niswender, G. D. (1976) *Endocrinology* **99**, 1023–1032
- Jackowski, M. M., Johnson, R. A. & Exton, J. H. (1980) *Biochim. Biophys. Acta* **630**, 497–510
- Jänne, J., Pösö, H. & Raina, A. (1978) *Biochim. Biophys. Acta* **473**, 241–293
- Janszen, F. H. A., Cooke, B. A., Van Driel, M. J. A. & van der Molen, H. J. (1976) *Biochem. J.* **160**, 433–437
- Kneer, N. M., Wagner, M. J. & Lardy, H. A. (1979) *J. Biol. Chem.* **254**, 12160–12168

- Kurzinger, K., Stadtkus, C. & Hamprecht, B. (1980) *Eur. J. Biochem.* **103**, 597–611
- Lefkowitz, R. J., Roth, J. & Pastan, I. (1970) *Nature (London)* **228**, 864–866
- Mahaffee, D. D. & Ontjes, D. A. (1980) *J. Biol. Chem.* **255**, 1565–1571
- Malaisse, W. J. & Boschero, A. C. (1977) *Horm. Res.* **8**, 203–209
- Malaisse, W. J., Valverde, I., Devis, G., Somers, G. & Couturier, E. (1979) *Biochimie* **61**, 1185–1192
- Marian, J. & Conn, P. M. (1980) *Life Sci.* **27**, 87–92
- Maudsley, D. V. (1979) *Biochem. Pharmacol.* **28**, 153–161
- McDonald, J. M., Bruns, D. E., Jarett, L. & Davis, J. E. (1977) *Anal. Biochem.* **82**, 485–492
- Means, A. R., Dedman, J. R., Welsh, M. J., Marcum, M. & Brinkley, B. R. (1979) in *FSH and Calcium as Modulators of Sertoli-Cell Differentiation and Function* (Hamilton, T. H., Clark, J. H. & Sadler, W. A., eds.), pp. 207–224, Raven Press, New York
- Murphy, E., Coll, K., Viale, R. O., Tischler, M. E. & Williamson, J. R. (1979) *J. Biol. Chem.* **254**, 8369–8376
- Murphy, E., Coll, K., Rich, T. L. & Williamson, J. R. (1980) *J. Biol. Chem.* **255**, 6600–6607
- Osterman, J. & Hammond, J. M. (1977) *Endocrinology* **101**, 1335–1338
- Osterman, J., Demers, L. M. & Hammond, J. M. (1978) *Endocrinology* **103**, 1718–1724
- Pegg, A. E., Lockwood, D. H. & Williams-Ashman, H. G. (1970) *Biochem. J.* **117**, 17–22
- Perchellet, J.-P. & Sharma, R. K. (1979) *Science* **203**, 1259–1260
- Perry, J. W. & Oka, T. (1980) *Biochim. Biophys. Acta* **629**, 24–35
- Podesta, E. J., Milani, A., Steffen, H. & Neher, R. (1980) *Biochem. J.* **186**, 391–397
- Pressman, B. C. (1975) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **32**, 1698–1703
- Putney, J. W., Jr. (1979) *Pharmacol. Rev.* **30**, 209–245
- Rasmussen, H. & Goodman, D. B. P. (1977) *Physiol. Rev.* **57**, 421–509
- Russell, D. H. (1980) *Pharmacology* **20**, 117–129
- Schmid, R. W. & Reilley, C. N. (1957) *J. Anal. Chem.* **29**, 264–268
- Steinhardt, R. A. & Epel, D. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 1915–1919
- Veldhuis, J. D. & Hammond, J. M. (1979) *Biochem. Biophys. Res. Commun.* **91**, 770–777
- Weissmann, G., Anderson, P., Serhan, C., Samuelsson, E. & Goodman, E. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 1506–1510
- Whitfield, J. F., Boynton, A. L., MacManus, J. P., Sikorska, M. & Tsang, B. K. (1979) *Mol. Cell. Biochem.* **27**, 155–179
- Winer, B. J. (1971) *Statistical Principles in Experimental Design*, pp. 196–210, McGraw-Hill, New York
- Yagura, T. S. & Miller, J. P. (1980) *Biochim. Biophys. Acta* **630**, 463–467