Vol. 104, No. 2, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 29, 1982 Pages 603-610

ROLE OF CALCIUM IONS IN THE STIMULATORY ACTIONS OF LUTEINIZING HORMONE IN ISOLATED OVARIAN CELLS: STUDIES WITH DIVALENT-CATION IONOPHORES

Johannes D. Veldhuis and Patricia A. Klase

Department of Medicine Division of Endocrinology
The Milton S. Hershey Medical Center
The Pennsylvania State University
Hershey, Pennsylvania 17033

Current and Corresponding Address:
University of Virginia Medical Center
Charlottesville, Virginia 22908

Received December 9, 1981

SUMMARY: The divalent-cation ionophores A23187 and ionomycin exert dose-dependent suppressive effects on the stimulatory actions of luteinizing hormone in ovarian cells *in vitro*. Micromolar concentrations of both A23187 and ionomycin can inhibit the production of progesterone and the stimulation of ornithine decarboxylase activity. Inhibitory concentrations of these ionophores deplete total cell content of calcium, and also seem to suppress protein synthesis.

INTRODUCTION

Calcium ions participate in stimulus-secretion coupling in many exocrine glands (1), and in certain endocrine tissues (2-5). In the adrenal gland, calcium also modulates hormonal stimulation of steroid-hormone biosynthesis *per se*(3,4). In this regard, our studies in isolated granulosa cells have also implicated calcium ions in steroidogenic processes in these cells (6). In addition, calcium modulates the hormonal regulation of a discrete cytosolic protein, ornithine decarboxylase (ODC), [(L-ornithine carboxy)lyase, E.C. 4.1.1.17)] in ovarian cells (7).

In most studies, manipulation of extracellular concentrations of calcium ions has been used to discern the influence of calcium on cellular processes under observation. However, extracellular perturbations may alter intracellular content or distribution of divalent cations secondarily. In an attempt to modify intracellular levels of calcium more directly, investigators have employed divalent-cation ionophores, such as A23187, which facilitates the

0006-291X/82/020603-08$01.00/0
Copyright © 1982 by Academic Press, Inc.
All rights of reproduction in any form reserved.

transmembrane transport of calcium or magnesium ions (8). More recently, the more calcium-selective ionophore, ionomycin, has been utilized to explore the role of calcium ions in various intracellular processes (9,10). We have applied this strategy to explore further the nature of hormone action in isolated ovarian cells in vitro. The present studies disclose major effects of the divalent cation ionophores, A23187 and ionomycin, on the hormonal stimulation of progesterone production and cytosolic ODC activity in swine granulosa cells. Importantly, these studies also indicate that certain significant effects of micromolar concentrations of either A23187 or ionomycin are associated with depletion of cellular calcium content, and suppression of protein synthesis in this isolated cell system.

MATERIALS AND METHODS

Materials: Ovine LH (NIH-LH-S20) was obtained from the Hormone Distribution Office, NIAMDD, NIH, Bethesda, MD. L-[^{14}C(U)]-leucine, L-1-[^{14}C]-ornithine monohydrochloride and [1,2-^{3}H(N)] progesterone were purchased from New England Nuclear Corp.; 8-bromo-cyclic AMP, and L-ornithine hydrochloride from Sigma Chemical Corp., St. Louis, MO; and Eagle's minimum essential medium, cycloheximide, from Grand Island Biological Corp., New York. A23187 and ionomycin were provided by Eli Lilly (Indianapolis, IN) and E.R. Squibb and Sons, Inc., (Princeton, NJ), respectively.

Isolation of Ovarian Cells: Immature granulosa cells used in the studies of ornithine decarboxylase were harvested as previously described by fine-needle aspiration of 1-2 mm follicles from porcine ovaries (7,11). Approximately 3 x 10^7 viable cells were incubated per culture flask at 37°C in room air for 4 hr in a final volume of 5 ml Eagle's medium with Hank's salts (Flow Laboratories), 25 mM Hepes buffer, pH 7.4, and antibiotics (7). After incubation with relevant hormones, cells were washed, pelleted, and lysed by freeze-thawing. The 20,000 x g supernatant cytosol was assayed for ODC activity as previously characterized (7,12). Cytosolic protein concentrations were determined by the method of Bradford (13). ODC activity is expressed as pmol CO_2/mg cytosol protein/30 min.

Mature granulosa cells used in the studies of progesterone production were harvested by enzymatic dispersion of the extirpated follicle wall of large (>8 mm) follicles associated with regressing 2-4 mm corpora lutea, as reported earlier (14). Cells were washed 3 times in Eagle's medium and approximately 5 x 10^6 viable granulosa cells were incubated in Hepes-buffered culture medium in a metabolic shaker at 37°C. Cultures were administered designated doses of inhibitors 1/2 hr before treatment with hormone (or control solvent). Cultures were then incubated for an additional 4 hr. After incubation, cells and medium were separated by centrifugation (100 x g x 5 min). The cell pellet was washed twice, and prepared for the subsequent determination of proges- terone content. The content of progesterone in the medium was measured after hexane extraction, employing a radioimmunoassay with specific antisera donated by Dr. David Watson (Worcester Foundation). The cellular content of progesterone was determined after homogenization in 100% ethanol. Data were corrected for radiolabeled steroid recovery. The sensitivity and precision of this assay have been reported previously (11).

TABLE I. Effects of A23187 on Progesterone Production by Isolated, Swine Granulosa Cells In Vitro

Condition	Progesterone Production ($ng/10^6$ cells·4 hr)
Basal	1.62 ± 0.47
Luteinizing Hormone (LH)	8.41 ± 0.83
LH + A23187 (0.10µg/ml)	13.70 ± 0.65*
LH + A23187 (0.30µg/ml)	12.60 ± 0.72*
LH + A23187 (1.0 µg/ml)	9.10 ± 0.44
LH + A23187 (10.0µg/ml)	1.85 ± 0.31*

*$p < 0.01$ vs. LH alone by 1-way analysis of variance. Data are means ± SEM (n=3 cultures). Mature granulosa cells were incubated for 4 hr in Eagle's medium with the indicated final concentrations of A23187 (a maximally stimulating concentration of LH was used). The medium was assayed for progesterone content.

To assess the effects of A23187 and ionomycin on protein synthesis, granulosa cells were incubated in leucine-free medium in the presence of 0.1 uCi L-^{14}C(U)-leucine (294 mCi/mmol) for 4 hr. After incubation, the supernatant was discarded and the cell pellet washed 4 times in ice-cold phosphate-buffered saline, before precipitation of macromolecules with 5% ice-cold trichloroacetic acid. The residue was spotted in triplicate on filter-paper discs, as described by Bollum (15), and then subjected to serial washing procedures before measurement of radioactivity by liquid scintillation counting.

The total cellular content of calcium was estimated after washing granulosa cells three times in 20 volumes of ice-cold calcium-free medium containing 0.5 mM $LaCl_3$. Cell pellets were dissolved in 3 N HCl and the ashed calcium content determined by atomic absorption spectrometry as previously described (7).

Statistical analyses employed unpaired, two-tailed Student's t testing of untransformed data, or anaysis of varianace when multiple comparisons among means were required (16).

RESULTS AND DISCUSSION

The divalent-cation ionophore, A23187, significantly enhanced progesterone production in response to maximally stimulating concentrations of luteinizing hormone (Table I). This observation is similar to that reported earlier by Higuchi et al. in ovine luteal cells (17). In contrast, slightly higher concentrations of A23187 (similar to those employed in numerous other systems), markedly suppressed progesterone production. This suppressive effect of A23187 on steroid-hormone production in isolated ovarian cells is concordant with the recently recognized inhibitory actions of A23187 on steroidogenesis in adrenal cells (18). The biphasic pattern of action of A23187 also occurred in relation to stimulation of ODC activity by luteinizing hormone (see Fig. 1), although

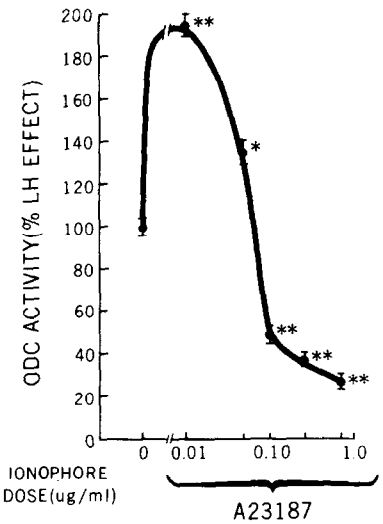

Figure 1 Bipotential actions of A23187 on luteinizing-hormone's stimulation of ornithine decarboxylase (ODC) activity in immature pig granulosa cells in vitro. Granulosa cells were maintained in serum-free Eagle's medium (see Methods) for 4hr in the presence of maximally stimulating concentrations of luteinizing hormone, with control solvent, or increasing concentrations of A23187 (given on the abscissa). ODC activity was 1460 ± 52 pmol CO_2/mg protein 30 min (100% LH effect). *$p < 0.05$ vs. LH alone. **$p < 0.01$ vs. LH alone. Data are means ± SEM (n=3 cultures).

significant inhibition of ODC activity occurred at somewhat lower concentrations of A23187. The inhibitory effects of A23187 were not attributable to the presence of extracellular calcium, since they could be observed in the absence of free calcium ions (e.g. in incubations containing 0.5 mM excess EDTA). In addition, our earlier studies have indicated that A23187 is devoid of any direct effect on ODC activity in cell-free (cytosolic) preparations (7).

The putatively more calcium-selective ionophore ionomycin (9,10) also inhibited progesterone production in response to luteinizing hormone (Fig. 2). Ionomycin concentrations >0.1 ug/ml markedly suppressed the accumulation of progesterone in both cells and medium, i.e. ionomycin inhibited both the production and secretion of progesterone in response to hormone stimulation. A similar inhibitory action of ionomycin was observed for the stimulation of ODC activity (Fig. 3). However, unlike the biphasic pattern of effect for A23187, we have been unable to discern stimulatory effects of ionomycin at lower concentrations (i.e., 0.10 to 0.0001 ug/ml ionomycin).

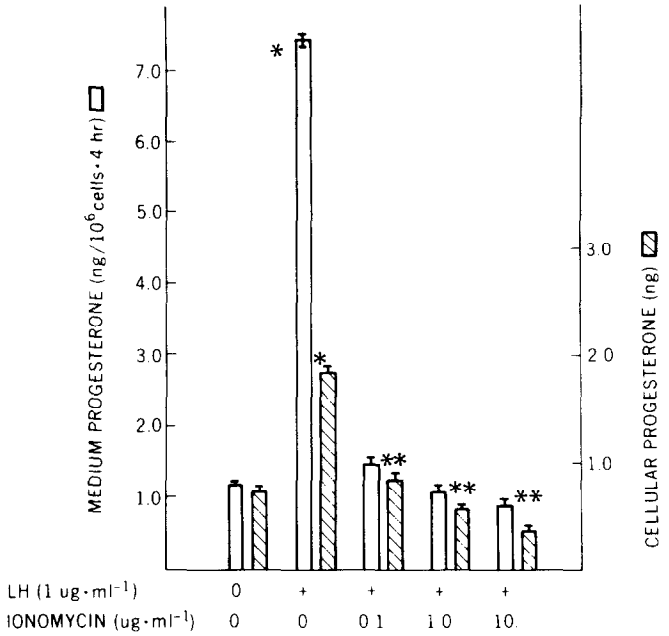

Figure 2 Inhibitory actions of ionomycin on progesterone production by mature porcine granulosa cells stimulated with luteinizing hormone. Cells were incubated as defined in Table I, except that progesterone concentrations were determined in both cells and medium (Methods). *$p < 0.01$ vs. basal. **$p < 0.01$ vs. LH control. Data are means ± SEM, n=3 cultures.

The capacity of both divalent-cation ionophores to suppress the stimulatory actions of luteinizing hormone suggested to us that these compounds might deplete intracellular stores of calcium. Previous studies have shown that extracellular calcium ions are required for the stimulatory actions of luteinizing hormone in ovarian cells (6,7,17). Measurement of the cell calcium content (after washing cells in lanthanum-containing buffers to remove loosely bound extracellular calcium) revealed that both A23187 and ionomycin can deplete cellular stores of calcium. Thus, control cells contained 56 ± 14 nmol $Ca^{++}/4.10^6$ cells, while cells incubated with 10 ug/ml A23187 or ionomycin contained (respectively) 29 ± 4 and 25 ± 6 nmol $Ca^{++}/4.10^6$ cells ($p<0.01$ by analysis of variance). To our knowledge, this is the first demonstration that ionomycin can deplete total cellular stores of calcium in isolated cells. The effects of A23187 in ovarian cells are not dissimilar to those recently noted in dispersed hepatic parenchymal cells (19).

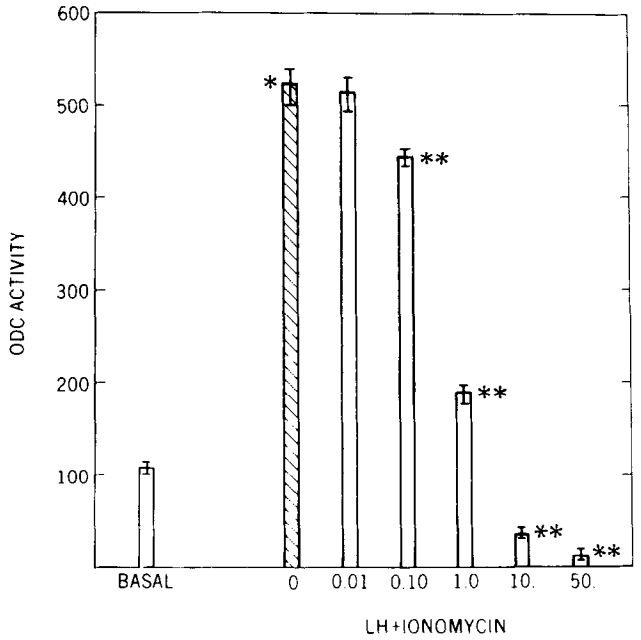

Figure 3 Ionomycin inhibits the stimulation of ODC activity by LH in immature porcine granulosa cells maintained in vitro (as defined in Fig. 1). 100% ODC activity was 353 ± 22 pmol CO_2/mg protein•30 min. *$p < 0.01$ vs. basal ODC activity. **$p < 0.05$ for 0.10µg/ml ionomycin and $p < 0.01$ for 1.0µg/ml ionomycin effects vs. LH alone.
Data are means ± SEM (n=3 cultures). Ionomycin was devoid of effect on ODC activity in cell-free (cytosolic) preparations.

The divalent-cation ionophores inhibit both progesterone production and ODC activity. This concordance in suppression of two processes is consistent with the hypothesis of some investigators that ODC may be functionally coupled to steroidogenesis in the ovary (20). However, concordant suppression could also be accounted for by effects of these ionophores upon one or more subcellular processes common to progesterone production and ODC activation. As depicted in Table II, the present study suggests that one common subcellular process affected by both ionophores is protein synthesis. Although we have not measured absolute rates of protein synthesis, both ionophores profoundly suppressed C^{14}-leucine incorporation into acid-precipitable macromolecules in isolated granulosa cells. The suppressive effect exerted by A23187 or ionomycin is nearly as great as that of cycloheximide. During preparation of this manuscript, a study in adrenal tissues (21) also indicated that A23187 inhibited radiolabeled leucine incorporation. In the adrenal, calcium

TABLE II. C^{14}-Leucine Incorporation into Acid-Precipitable Material in Swine Granulosa Cells

Condition	C^{14}-Leucine Incorporation (cpm/mg protein)
Basal	1375 ± 46
Luteinizing Hormone (LH)	1474 ± 37
LH + ionomycin (0.1µg/ml)	708 ± 27*
LH + ionomycin (1.0µg/ml)	303 ± 41*
LH + ionomycin (10 µg/ml)	52 ± 5*
LH + A23187 (10.0µg/ml)	68 ± 7*
LH + cycloheximide (10µg/ml)	23 ± 2*
C^{14}-leucine Blank	15 ± 2*

Data are means and range for duplicate cultures, each subjected to triplicate determinations. The C^{14}-leucine blank was determined by adding radiolabel to cells 1 minutes prior to acid precipitation.

*$p < 0.01$ vs. control by 1-way analysis of variance. Incubations were conducted as defined in Methods.

depletion alone suppresses protein synthesis (22) and may provide a mechanism for the inhibitory actions of A23187. At present, comparable information regarding the effects of calcium depletion on protein synthesis in the ovary is not available.

The present observations in ovarian cells provide independent support for the participation of calcium ions in the hormonal stimulation of progesterone production and ODC activity in ovarian cells. Of major general importance, we have also demonstrated that A23187 and ionomycin are capable of depleting - rather than simply enhancing - total cellular calcium stores. Recognition of these actions of divalent-cation ionophores is required for an accurate interpretation of the significance of cellular responses observed after treatment of cells with these agents. In addition, the apparent capacity of both ionophores to suppress protein synthesis is of broad significance to many investigators employing these compounds in intact cell systems.

ACKNOWLEDGEMENTS

LH was generously supplied by the Hormone Distribution Office of NIAMDD. We thank the Shamokin Packing Corp. and Arbogast and Bastian for porcine ovaries, and Marlene Thompson and Judy Woodson for the preparation of this manuscript. This work was supported by a National Research Service Award (1F32HD05755) to J.D. Veldhuis from NICHD.

REFERENCES

1. Rasmussen, H., and Goodman, D.B.P. (1977) Physio. Rev. 57, 421-509.

2. Conn, P.M., Marian, J., McMillian, M. and Rogers, D. (1980) Cell Calcium 1, 7-20.

3. Garcia, A.G., Kirpekar, S.M. and Prat, J.C. (1975) J. Physiol (Lond) 244, 253-262.

4. Podesta, E.J., Milani, A., Steffen, H., and Neher, R. (1980) Biochem. J. 186: 391-397.

5. Janszen, F.H.A., Cooke, B.A., Van Driel, M.J.A., and van der Molen, H.J. (1976) Biochem. J. 160, 433-437.

6. Veldhuis, J.D. and Klase, P.A. (1981, in press) Biochem. J.

7. Veldhuis, J.D. and Hammond, J.M. (1981) Biochem. J. 196, 795-801.

8. Pressman, B.C. (1975) Fed. Proc. Fed. Am. Soc. Exp. Biol. 32, 1698-1703.

9. Beeler, T.J., Jona, I., and Martonosi, A. (1979) J. Biol. Chem. 254: 6229-6231.

10. Lin, C.-M. and Herman, T.E. (1979) J. Biol. Chem. 253, 5892-5894.

11. Veldhuis, J.D., Klase, P.A., and Hammond, J.M. (1980) Endocrinology 107, 42-46.

12. Osterman, J. and Hammond, J.M. (1977) Endocrinology 101, 1335-1338.

13. Bradford, M.M. (1976) Analyt. Biochem. 72, 248.

14. Veldhuis, J.D. and Sweinberg, S. (1981, in press) J. Cell. Physiol. 108, 213-220.

15. Bollum, J. (1966) Procedures in Nucleic Acid Research, Vol. 1, p. 296.

16. Winer, B.J. (1971) Statistical Principles in Experimental Design, McGraw-Hill, NY, p. 196.

17. Higuchi, T., Kaneko, A., Abel, J.H., Jr., and Niswender, G.D. (1976) Endocrinology 99, 1023-1032.

18. Warner, W., Carchman, R.A. (1978) Biochim. Biophys. Acta 528, 409-415.

19. Kneer, N.M., Wagner, M.J. and Lardy, H.A. (1979) J. Biol. Chem. 254, 12160-12168.

20. Guha, S.K., and Janne, J. (1977) Biochem. Biophys. Res. Commun. 75, 136.

21. Farese, R.V., Sabir, M.A., and Larson, R.E. (1981) Endocrinology 108, 1243-1246.

22. Farese, R.V. (1971) Endocrinology 89, 1057-1061.