J. steroid Biochem. Vol. 36, No. 6, pp. 687–693, 1990
Printed in Great Britain.

0022-4731/90 $3.00 + 0.00

DIFFERENTIAL EFFECTS OF CALCIUM ON PROGESTERONE PRODUCTION IN SMALL AND LARGE BOVINE LUTEAL CELLS

H. W. ALILA,* J. S. DAVIS,[1] J. P. DOWD, R. A. CORRADINO and W. HANSEL†
Department of Physiology, New York State College of Veterinary Medicine, Cornell University, Ithaca, NY 14853,
[1]Women's Research Institute, 2903 East Central, Wichita, KS 67214, U.S.A.

(*Received* 15 *January* 1990)

Summary—We studied the effects of calcium (Ca^{2+}) ions in progesterone (P) production by separated small and large luteal cells. Corpora lutea were collected from 31 heifers between days 10 and 12 of the estrous cycle. Purified small and large cells were obtained by unit gravity sedimentation and flow cytometry. P accumulation in cells plus media was determined after incubating 1×10^5 small and 5×10^3 large cells for 2 and 4 h respectively. Removal of Ca^{2+} from the medium did not influence basal P production in the small cells ($P > 0.05$). However, stimulation of P by luteinizing hormone (LH), prostaglandin E_2 (PGE_2), 8-bromo-cyclic 3′,5′ adenosine monophosphate (8-Br-cAMP) and prostaglandin $F_2\alpha$ ($PGF_2\alpha$) was impaired ($P < 0.05$) by low Ca^{2+} concentrations. LH and PGE_2-stimulated cAMP production was not altered by low extracellular Ca^{2+} concentrations, and $PGF_2\alpha$ had no effect on cAMP. In contrast, basal as well as LH and forskolin-stimulated P production were attenuated ($P < 0.05$) in Ca^{2+}-deficient medium in the large cells. However, P production stimulated by 8-Br-cAMP was not altered in Ca^{2+}-deficient medium. Steroidogenesis in large cells was also dependent on intracellular Ca^{2+}, since 8-*N*, *N*-diethylamineocytyl-3,4,5-trimethoxybenzoate (TMB-8), an inhibitor of intracellular Ca^{2+} release and/or action, suppressed ($P < 0.05$) basal, LH and 8-Br-cAMP stimulated P. In contrast, basal P in small cells was not altered by TMB-8; whereas LH-stimulated P was reduced 2-fold ($P < 0.05$). The calcium ionophore, A23187, inhibited LH-stimulated P in small cells and both basal and agonist-stimulated P in large cells. These studies show that basal P production in small cells does not require Ca^{2+} ions, while hormone-stimulated P production in small cells and both basal and hormone-stimulated P in large cells do require Ca^{2+}. The inhibitory effect of Ca^{2+} ion removal was exerted prior to the generation of cAMP in the large cells, but distal to cAMP generation in hormone-stimulated small cells. The calmodulin/protein kinase C antagonist, W-7, also inhibited both basal and hormone-stimulated P production in both small and large luteal cells, indicating that P production in luteal cells also involves Ca^{2+}-calmodulin/protein kinase C-dependent mechanisms.

INTRODUCTION

Recent studies have shown that steroidogenesis in the bovine corpus luteum (CL) may be regulated by calcium-mediated second messengers. Hormones such as luteinizing hormone (LH), prostaglandins $F_2\alpha$ ($PGF_2\alpha$) and E_2 (PGE_2) increase concentrations of intracellular calcium [1–3] as well as polyphosphoinositide metabolism [1, 4, 5] in isolated bovine luteal cells. Compounds that influence intracellular calcium also affect progesterone production in the luteal cells [6, 7].

Evidence for a role of calcium in steroidogenesis has also been demonstrated in ovarian cells from other species. Swine granulosa cells and rat luteal cells incubated in the absence of extracellular calcium showed a reduced progesterone biosynthesis in response to LH, PGE_2, epinephrine, and 8-Br-cAMP [8–11] indicating that calcium may play an important role in the actions of hormones that regulate progesterone biosynthesis in these ovarian cells.

However, the bovine CL is a heterogenous gland comprised of at least two morphologically and biochemically distinct cell types [12, 13]. These cell types, also known as small (theca derived) and large luteal cells, differ in their steroidogenic capabilities including their responses to hormonal agonists such as LH, $PGF_2\alpha$, and PGE_2 [14, 15]. The small, theca-derived cells are very responsive to LH and to agents that stimulate protein kinase C enzyme activity such as $PGF_2\alpha$. Conversely the large cells individually secrete

*Current address: Smith-Kline Beecham Animal Health, Applebrook Research Center, West Chester, PA 19380, U.S.A.
†To whom correspondence should be addressed: Dr William Hansel, 816 Veterinary Research Tower, NYS College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, U.S.A.

more basal progesterone than the small cells, but they respond only to very high levels of added LH.

In view of the difference in steroidogenic capacities between the small and large luteal cells, we hypothesized that calcium also has a differential role in regulating progesterone biosynthesis in these cells. Indeed, we recently showed that hormones such as LH, arachidonic acid and its metabolites $PGF_2\alpha$ and PGE_2 differed in their ability to increase intracellular calcium concentrations in the small and large luteal cells [2, 3]. In this study, we have examined the influence of agents that affect intracellular calcium regulation on progesterone production in highly purified populations of small and large bovine luteal cells from CL collected at known stages of the estrous cycle.

EXPERIMENTAL

Cell preparations, separations and incubations

Corpora lutea were obtained from normally cycling Holstein heifers between days 10 and 12 of the estrous cycle (day 0 = day of estrus). Luteal tissues were dissociated with collagenase, and the cells were separated by unit gravity sedimentation and flow cytometry (sort rates of 1000–2000 cells/s), and incubated as described earlier [14]. Cell viability was determined by trypan blue dye exclusion. Treatments (10 μl) were added and aliquots were removed after 10-min preincubation periods (0 h). Incubations were conducted with 1×10^5 viable small luteal cells in 0.5 ml Medium 199 for 2 h, while 5×10^3 large luteal cells (in 0.2 ml medium) were incubated for 2 or 4 h (3 replicates per treatment) at 37°C in 5% CO_2 and 95% air in a humidified incubator. Fewer large cells were needed because, on a per cell basis, these cells produce approximately 6 times more basal progesterone than the small cells [7, 14]. Calcium concentrations in the medium were determined by atomic spectrophotometry (Perkin Elmer, Norwalk, Conn.) using calibrated calcium standards (World Precision Instruments Inc., New Haven, Conn.). The final calcium concentration in the calcium-free medium was 5 μM. During the experiments, 0.84 mM $MgCl_2$ was added to calcium-free medium to restore divalent cation concentrations. Samples (cells plus medium) were collected and frozen at −20°C until assayed for progesterone by specific radioimmunoassay [14]. Progesterone accumulation was the difference between the concentration at 0 and 2 or 4 h.

cAMP measurement

cAMP content of cells plus medium was determined by radioimmunoassay after acetylation using succinyl-cAMP [^{125}I]tyrosine methyl ester as the tracer [1].

Reagents

Medium 199 was purchased from Grand Island Biological Co. (Gibco, Grand Island, N.Y.). Penicillin-streptomycin solution (Gibco) was added to the medium during incubations (100 U/ml penicillin and 100 μg/ml) streptomycin). Collagenase (CLS) was obtained from Worthington Biochemicals (Freehold, N.J.). $PGF_2\alpha$, PGE_2, 8-Br-cAMP, 8-*N*,*N*-diethylamineocytyl-3,4,5-trimethoxybenzoate (TMB-8), the calcium-ionophore A23187 and verapamil were purchased from Sigma Chemical Co. (St Louis, Mo.). TMB-8 was dissolved just before use in Medium 199. Forskolin, (Calbiochem, San Diego, Calif.) was prepared as a 1 mM stock solution in 6% aqueous dimethyl sulfoxide (DMSO) by first dissolving it in DMSO followed by adding distilled water to the solution. The final concentration of DMSO in medium containing cells was less than 0.05%. *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) was purchased from Seikagaku America Inc. (St Petersburg, Fla). Bovine LH (NIH-B9-LH) was supplied by NIH.

Statistics

Paired *t*-test and analysis of variance were used to determine the significance of treatments. Differences between groups were evaluated with Duncan's multiple range test.

RESULTS

The purity of the small luteal cells after separation by unit gravity sedimentation was $96 \pm 1.6\%$ (mean $\pm$ SE, $n = 30$ CL) and their viability was $94 \pm 3\%$. The large luteal cell populations obtained by cell sorting as previously described [14] were $93 \pm 2\%$ pure ($n = 6$ CL) and their viability was $60 \pm 2\%$ ($n = 6$ CL). The number of cells and progesterone production reported are based on viable cells.

We first examined the requirement for calcium ions on the production of progesterone by the separated small and large cells. Removal of calcium from the medium had no effect on the viability of the cells ($94 \pm 2\%$ compared to $92 \pm 1.6\%$ for small cells in media containing 0 and 2 mM calcium respectively, $n = 10$ CL; and $60 \pm 2\%$ compared to $61 \pm 1.5\%$ for large cells in media containing 0 and 2 mM calcium respectively, $n = 6$ CL, after incubation).

As shown in Fig. 1(a–d). the dose-dependent stimulation of progesterone production in the small cells by LH, 8-Br-cAMP, PGE_2, and $PGF_2\alpha$ was significantly ($P < 0.05$) impaired in the calcium-free medium. However, the basal production of progesterone by the small cells was not changed in the absence of calcium in the medium. These results show that extracellular calcium ions are required for agonist-stimulated, but not for basal progesterone production in the small luteal cells.

The mechanism(s) of the action of calcium ions was also examined by measuring cAMP production after incubating the small luteal cells in calcium-free or calcium repleted medium in the presence or absence of increasing levels of LH, PGE_2 and $PGF_2\alpha$ (Table 1).

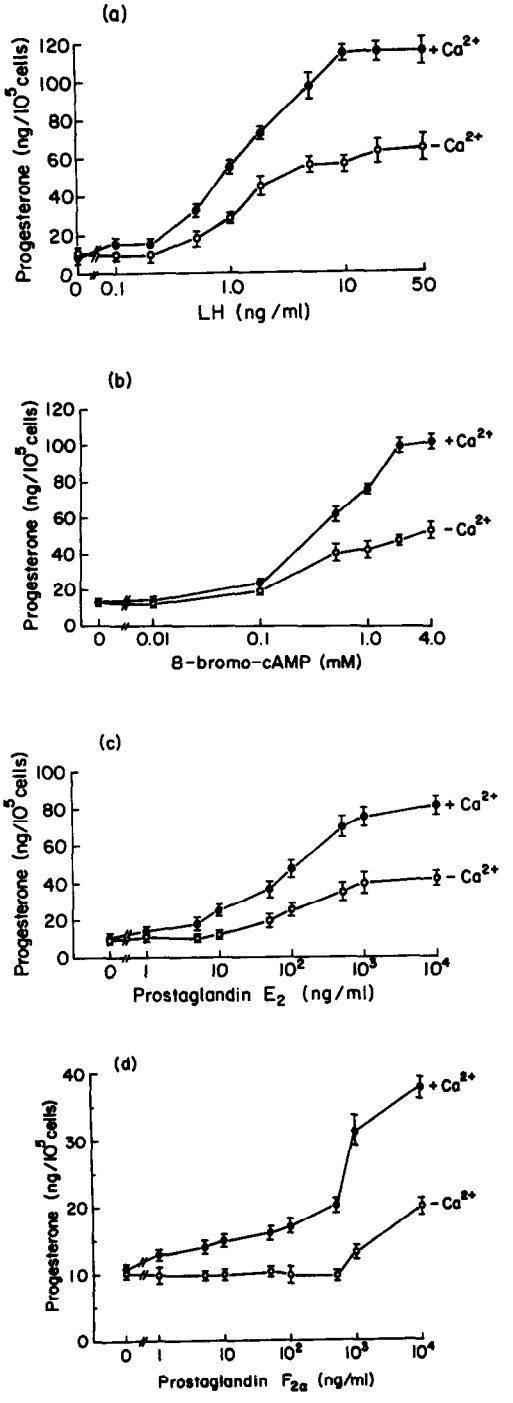

Fig. 1. The effect of calcium deprivation on progesterone production by small luteal cells in response to increasing concentrations of (a) LH, (b) 8-Br-cAMP, (c) prostaglandin E_2, and (d) prostaglandin $F_2\alpha$. The cells were incubated in 3 replicates per treatment per CL in the presence of 2.0 mM calcium (+ Ca^{2+}) or 5 μM calcium (− Ca^{2+}) in medium 199 for 2 h. Each data point represents the mean ± SE for 3 CL.

LH and PGE_2 increased ($P < 0.05$) cellular cAMP in a dose-dependent manner; and LH stimulated cAMP to a greater extent than PGE_2. PGE_2 stimulated cAMP only at levels higher than 1000 ng/ml. $PGF_2\alpha$ showed no effect on cAMP at any of the concentrations used. Moreover, the effects of LH or PGE_2 on cAMP did not differ ($P > 0.05$) between cells incubated with or without added calcium in the medium.

Table 1. cAMP production (pmol/10^5 cells) in small bovine luteal cells incubated for 2 h in the absence (controls) or presence of various concentrations of LH, PGE_2 and $PGF_2\alpha$ with (+Ca^{2+}, 2 mM) or without (−Ca^{2+}) added calcium in the medium

Treatment	−Ca^{2+}	+Ca^{2+}
Controls	0.7 ± 0.2[a]	0.3 ± 0.2[a]
LH (ng/ml)		
1	1.7 ± 2.2[a]	0.7 ± 0.4[a]
10	22.6 ± 11.7[d]	21.3 ± 11.0[d]
100	61.1 ± 16.8[e]	59.1 ± 6.5[e]
1000	87.8 ± 11.7[f]	107.3 ± 16.6[f]
PGE_2 (ng/ml)		
1	0.3 ± 0.1[a]	0.3 ± 0.1[a]
10	0.2 ± 0.1[a]	0.5 ± 0.1[a]
100	0.4 ± 0.1[a]	0.5 ± 0.1[a]
1000	1.5 ± 0.1[a]	2.3 ± 0.6[b]
10,000	4.3 ± 0.6[c]	6.4 ± 2.1[c]
$PGF_2\alpha$ (ng/ml)		
1	0.5 ± 0.1[a]	0.4 ± 0.1[a]
10	0.5 ± 0.2[a]	0.5 ± 0.2[a]
100	1.1 ± 0.8[a]	0.6 ± 0.2[a]
1000	0.4 ± 0.2[a]	1.1 ± 0.7[a]
10,000	0.9 ± 0.1[a]	0.6 ± 0.3[a]

Values are means ± SE for 3 corpora lutea (3 replicates per treatment). Numbers with different superscripts are significantly different ($P < 0.05$).

In contrast to the small cells, basal as well as LH, and forskolin-stimulated progesterone productions in the large cells was attenuated ($P < 0.05$) in calcium-deficient media (Fig. 2). However, progesterone stimulated by 8-Br-cAMP was not altered ($P > 0.05$) in calcium-deficient medium. The levels of LH and 8-Br-cAMP used were previously shown to produce maximal stimulations of progesterone production.

A comparison of the effects of calcium ionophore, A23187, at micromolar concentrations in small and large cells is shown in Fig. 3. Results show that the ionophore used at various concentrations had no effect on basal progesterone production in the small cells. However, the presence of this compound at 5 or 10 μM significantly ($P < 0.05$) inhibited LH-stimulated progesterone production. The effects of A23187 were different in the large cells, in which it inhibited both basal and LH-stimulated progesterone production.

The effects of calcium ions on hormone-stimulated progesterone production in the small luteal cells were dependent on the availability of intracellular calcium pools, because increasing the concentrations of TMB-8, a putative inhibitor of intracellular calcium release and/or action, suppressed ($P < 0.05$) progesterone stimulated by LH, PGE_2 and $PGF_2\alpha$; whereas basal steroidogenesis was unaltered (Fig. 4). In contrast, both basal and LH and 8-Br-cAMP-stimulated progesterone production in large cells was inhibited ($P < 0.05$) by TMB-8 (Table 2). The action of this compound did not appear to result from its toxic effects, since it 'did not render the luteal cells permeable to trypan blue at the concentrations used

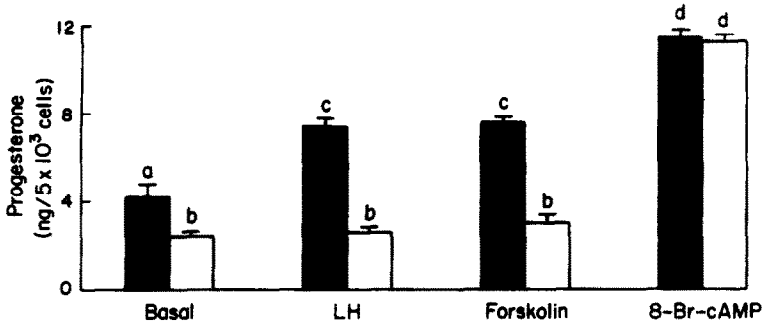

Fig. 2. Progesterone production by large luteal cells incubated in the presence of 2.0 mM (solid bars) or 5 μM (open bars) $CaCl_2$ in medium 199. The cells were incubated in 3 replicates per treatment per CL in the absence (basal) or presence of LH (1 μg/ml), forskolin (1 μM), and 8-Br-cAMP (0.5 mM). Each data point represents the mean $\pm$ SE for 4 CL. Bars with different superscripts are significantly different ($P < 0.01$).

($95 \pm 2\%$ vs $93 \pm 1.5\%$ in the absence and presence of 100 μM TMB-8 respectively, after 2 h incubation period).

The possible role of calmodulin/protein kinase C in mediating the actions of calcium in the luteal cells was investigated by assessing the responses of both small and large cells to a calmodulin/protein kinase C antagonist, W-7. This compound inhibited basal, LH and 8-Br-cAMP-stimulated progesterone production in both small and large luteal cells (Table 3). This compound had no effect cell viability at the concentrations used as determined by trypan blue dye exclusion.

The addition of verapamil, a compound that blocks voltage-sensitive calcium channels, had no influence on either basal or LH-stimulated progesterone in either small (10 ± 0.9, 9.1 ± 1.7, 57.4 ± 9 and 47.4 ± 6.1 ng/10^5 cells for control, 100 μM verapamil, 5 ng/ml LH and LH + verapamil, respectively, mean $\pm$ SE, $n = 3$ CL) or large luteal cells (5.0 ± 0.6, 6.0 ± 0.3, 8.0 ± 0.6, and 7.6 ± 0.3 ng/5 $\times$ 10^3 cells for control, 100 μM verapamil, 1 μg/ml LH, and LH + verapamil respectively, mean $\pm$ SE, $n = 3$ CL). Similarly, verapamil did not affect PGE_2, $PGF_2\alpha$ and 8-Br-cAMP-stimulated progesterone production in either cell type (data not shown).

DISCUSSION

The most significant result of this study was the demonstration that manipulation of the levels of extracellular and intracellular calcium ions exerted differential modulatory effects on steroidogenesis in the small and large bovine luteal cells. In the small luteal cells, only hormone-stimulated, but not basal, progesterone production was altered by removal of calcium from the medium. Similarly, the inhibition of the action and/or release of calcium with TMB-8 impaired the agonist-stimulated, but not basal, progesterone production in the small cells. Increasing intracellular calcium concentrations with the calcium ionophore A23187 also failed to alter basal progesterone production in the small cells. Collectively, these findings suggest that, in the small cells, only hormone-stimulated but not basal production of progesterone is dependent on calcium ions. In contrast, both basal and hormone-stimulated production of progesterone in the large luteal cells were depen-

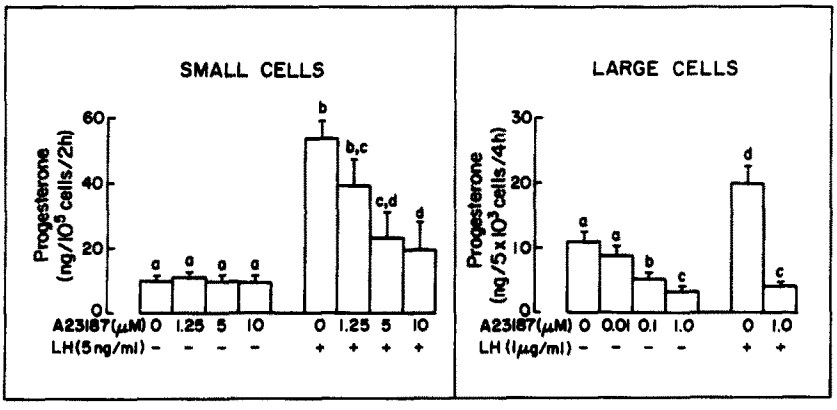

Fig. 3. A comparison of the effects of various concentrations of calcium ionophore, A23187, on progesterone production by small and large luteal cells. The cells were incubated with the ionophore (3 replicates per treatment for each CL) for 2 h in the absence or presence of LH. Values are mean $\pm$ SE for 4 CL. Bars with different superscripts are significantly different ($P < 0.05$).

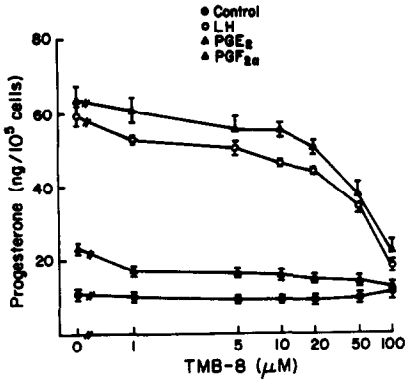

Fig. 4. Effects of increasing concentrations of the intracellular calcium blocker, TMB-8, on progesterone production by small luteal cells. The cells were incubated in 3 replicates per CL in the absence (control) or presence of LH (5 ng/ml), PGE_2 (1 μg/ml) and $PGF_2\alpha$ (1 μg/ml). Each data point represents mean ± SE for 3 CL.

dent on calcium ions. Removal of calcium ions from the medium reduced basal production as well as biosynthesis stimulated with LH and forskolin in the large luteal cells. TMB-8 also inhibited both basal and agonist-stimulated progesterone biosynthesis in the large luteal cells.

The results concerning the inhibitory effects of A23187 on the large luteal cells are similar to those reported for large ovine luteal cells [19] and rat luteal cells [11, 16]. The ionophore inhibited both LH and 8-Br-cAMP-stimulated progesterone production, indicating that its effects occur at a site distal to the generation of cAMP. Other effects of A23187 include its inhibitory action on protein synthesis [17], a result that suggests that protein synthesis is required for steroidogenesis in the large luteal cells. It is possible that the inhibitory effect of A23187 is due to its mobilization of calcium from intracellular organelles including mitochondria and endoplasmic reticulum [18], resulting in a reduced steroidogenesis. It is unlikely that the effects of A23187 were mediated via the activation of protein kinase C because the stimulation of this enzyme with phorbol esters increases progesterone production in small but not large luteal cells [2, 14]. It appears that intracellular calcium levels must be increased above some critical level for progesterone production to be reduced. Any level below this critical concentration of calcium would support stimulated progesterone biosynthesis. As shown in the present study, the concentration of calcium required to support or reduce progesterone

Table 2. The effects of TMB-8 on progesterone production (ng/5 × 10^3 cells) in large bovine luteal cells incubated for 4 h

	Alone	+LH (1 μg/ml)	+8-Br-cAMP (0.5 mM)
Controls	4.96 ± 0.55[b]	7.97 ± 0.64[c]	12.39 ± 0.12[e]
TMB-8			
30 μm	3.69 ± 0.12[a]	6.46 ± 0.94[c]	9.07 ± 0.28[f]
100 μM	3.03 ± 0.20[a]	3.95 ± 0.47[d]	7.48 ± 0.28[f]

Values are mean ± SE for 3 corpora lutea (3 replicates each). Numbers within columns with different superscripts are significantly different ($P < 0.05$).

Table 3. Effect of W-7, a calmodulin antagonist and protein kinase C inhibitor on progesterone production in small (ng/10^5 cells/2 h) and large (ng/5 × 10^3 cells/4 h) luteal cells incubated in the absence (control) or presence of LH (5 ng/ml for small cells and 1 μg/ml for large cells) or 8-Br-cAMP

	Small cells	Large cells
Control	10.12 ± 1.37[a]	10.29 ± 2.10[a]
W-7 (30 μM)	4.30 ± 0.57[b]	3.63 ± 0.15[b]
LH	68.33 ± 10.40[c]	21.06 ± 0.39[c]
LH + W-7	13.31 ± 2.65[a]	3.67 ± 0.10[b]
8-Br-cAMP (0.5 mM)	67.01 ± 19.03[c]	20.98 ± 0.46[c]
8-Br-cAMP + W-7	12.99 ± 04.71[a]	3.48 ± 0.44[b]

Values are mean ± SE for 3 corpora lutea. Numbers within columns with different superscripts are significantly different ($P < 0.05$).

production may differ between the small and large luteal cells.

The steroidogenic responses to LH, PGE_2, $PGF_2\alpha$ and 8-Br-cAMP in small luteal cells were attenuated by low extracellular calcium concentrations indicating that calcium is required for their maximal stimulatory actions. The action of calcium ion deprivation from the extracellular medium in modulating the hormone-stimulated progesterone production in the small luteal cells appears to occur at site(s) distal to the generation of cAMP. This conclusion was based on the following reasons. Firstly, LH and PGE_2-stimulated cAMP production in the small cells was not altered by the removal of calcium from the extracellular medium, indicating that adenylate cyclase activity could be sustained by intracellular calcium levels. This is in contrast to the results of Veldhuis and Klase[8] who showed that LH-stimulated cAMP production in pig granulosa cells was reduced in cells incubated in calcium-free medium. Other studies [11] with rat luteal cells demonstrated an enhancement of LH-stimulated cAMP accumulation after the removal of extracellular calcium. Secondly, the stimulatory action of 8-Br-cAMP on progesterone production was also impaired in cells incubated in calcium-deprived media, similar to other studies [8, 10]. Thirdly, the effect of $PGF_2\alpha$, which increased progesterone production without affecting cAMP, was also impaired in calcium-deficient medium. The actions of $PGF_2\alpha$ are mediated by increases in polyphosphoinositide hydrolysis and intracellular calcium concentrations, and subsequent activation of protein kinase C [2–5]. Thus, the inhibitory effect of calcium deprivation on agonist-stimulated steroidogenesis in the small luteal cells resides at a site distal to, or independent of the generation of cAMP.

The effect of removing calcium from the extracellular medium appears to be more complex in large than in small luteal cells. Both basal and agonist-stimulated progesterone production was reduced in the large cells in calcium-free medium. This suggests that calcium ions play a crucial role in the production of progesterone in the large luteal cells, in contrast to the small cells in which basal progesterone production was calcium-independent. LH-stimulated progesterone production in the large cells was also reduced

in calcium-free medium, as was production stimulated by forskolin. The action of forskolin is mediated by its stimulatory effects on adenylate cyclase [20] indicating that adenylate cylase enzyme in the large luteal cells is sensitive to calcium deprivation. Since, 8-Br-cAMP-stimulated progesterone production remained unaltered it seems likely that the inhibitory effects observed under calcium-deficient conditions were exerted prior to the generation of cAMP.

Steroidogenesis in both small and large luteal cells was probably dependent upon the availabaility of intracellular calcium, because TMB-8, the putative inhibitor of intracellular calcium action and/or release [21, 22] also suppressed LH and 8-Br-cAMP-stimulated progesterone production. However, it inhibited basal progesterone production in large but not small cells. The mechanism of action of TMB-8 on cAMP was not investigated; however, Veldhuis *et al.*[10] did not demonstrate an inhibitory effect on LH-stimulated cAMP in pig granulosa cells.

W-7, a naphthalenesulfonamide, which is both a calmodulin antagonist and a protein kinase C inhibitor [23, 24] suppressed basal and agonist-stimulated progesterone production in both small and large luteal cells. This finding is consistent with our previous results [7, 14], showing that activation of protein kinase C in the small luteal cells with phorbol esters or synthetic diacylglycerols stimulates progesterone production. However, phorbol esters or synthetic diacylglycerols, which activate protein kinase C [25, 26] do not alter progesterone production in the large cells [14] indicating that protein kinase C in these cells is probably not associated with progesterone production or that the enzyme activity is already high and cannot be stimulated further with phorbol esters or diacylglycerols. If protein kinase C is not associated with progesterone production in the large luteal cells, then the effects of W-7 are due to its inhibition of calmodulin action. Hence, calmodulin may be the intracellular regulator of basal progesterone production in the large cells, although further studies are needed to confirm this possibility. Veldhuis *et al.*[10] and Lahav *et al.*[27], also demonstrated suppressive effects of W-7 on progesterone biosynthesis by pig granulosa and rat luteal cells, respectively. Other effects of W-7, including its inhibition of protein kinase A could contribute to the observed effects, although W-7 inhibits this enzyme at higher concentrations than those used in the present study [23].

Verapamil had no influence on progesterone production by either cell type, suggesting that the bovine luteal cells, similar to rat luteal cells [11, 27], lack voltage-dependent calcium channels. Ovine luteal cells [28] and porcine granulosa cells [8]) probably contain voltage-dependent calcium channels, since depolarization with high potassium and the addition of verapamil influence steroidogenesis in these cells.

In summary, these findings demonstrate the requirement of calcium ions in the actions of hormones that stimulate progesterone production in bovine luteal cells. They are consistent with the previous results that showed the hormone's ability to mobilize calcium in these cells [2–4]. In the small cells, the locus of steroidogenesis that requires calcium ions appears to reside distal to the generation of cAMP. However, the basal progesterone production was independent of calcium. In the large cells, the primary effects of calcium ions were exerted prior to the generation of cAMP, probably on adenylate cyclase. Furthermore, our observations suggest that other intracellular calcium-dependent processes including calmodulin and protein kinase C-dependent mechanisms are involved in the actions of hormones that regulate steroidogenesis in the bovine corpus luteum.

Acknowledgements—We thank Raymond Saatman, Laura Weakland and Douglas Bianchi for technical assistance and Virginia Slator for assistance in preparation of this manuscript. We also thank Bill Harris (Baker Institute of Animal Health) for his skilful help with the cell sorter. This work was supported by USDA grant No. 85-CRCR-1-1830, NIH grant HD-22248 and the Veterans Administration.

REFERENCES

1. Davis J. S., Weakland L. L., Farese R. V. and West L. A.: Luteinizing hormone increases inositol triphosphate and cytosolic free Ca^{2+} in isolated bovine luteal cells. *J. Biol. Chem.* **262** (1987a) 8515–8521.
2. Alila H. W., Corradino R. A. and Hansel W.: Differential effects of luteinizing hormone on intracellular free calcium in small and large bovine luteal cells. *Endocrinology* **124** (1989) 2314–2320.
3. Alila H. W., Corradino R. A. and Hansel W.: Arachidonic acid and its metabolites increase cytosolic free calcium in bovine luteal cells. *Prostaglandins* **39** (1990) 481–496.
4. Davis J. S., Weakland L. L., Weiland D. A., Farese R. V. and West L. A.: Prostaglandin $F_2\alpha$ stimulates phosphatidylinositol 4,5-bisphosphate hydrolysis and mobilizes intracellular calcium in bovine luteal cells. *Proc. Natn. Acad. Sci. U.S.A.* **84** (1987b) 3728–3732.
5. Davis J. S., Alila H. W., West L. A., Corradino R. A. and Hansel W.: Acute effects of prostaglandin $F_2\alpha$ on inositol phospholipid hydrolysis in the large and small cells of the bovine corpus luteum. *Molec. Cell. Endocr.* **58** (1988) 43–50.
6. Hansel W. and Dowd J. P.: New concepts of the control of corpus luteum function. *J. Reprod. Fert.* **78** (1986) 755–768.
7. Hansel W., Alila H. W., Dowd J. D. and Yang X.: Control of steroidogenesis in small and large bovine luteal cells. *Aust. J. Biol. Sci.* **40** (1987) 331–347.
8. Veldhuis J. D. and Klase P. A.: Mechanisms by which calcium ions regulate the steroidogenic actions of luteinizing hormone in isolated ovarian cells *in vitro*. *Endocrinology* **111** (1982a) 1–6.
9. Veldhuis J. D. and Klase P. A.: Calcium ions modulate hormonally stimulated progesterone production is isolated ovarian cells. *Biochem. J.* **202** (1982b) 381–386.
10. Veldhuis J. D., Klase P. A., Demers L. M. and Chafouleas J. G. Mechanisms subserving calciums' modulation of luteinizing hormone action in isolated swine granulosa cells. *Endocrinology* **114** (1984) 441–449.
11. Dorflinger L. J., Albert P. J., Williams A. T. and Behrman H. R. Calcium is an inhibitor of luteinizing hormone-sensitive adenylate cyclase in the luteal cell. *Endocrinology* **114** (1984) 1208–1215.

12. Ursely J. and Leymaire P.: Varying response to luteinizing hormone of two luteal cell types isolated from bovine corpus luteum. *J. Endocr.* **83** (1979) 303–310.
13. Koos R. D. and Hansel W.: The large and small cells of the bovine corpus luteum: ultrastructural and functional differences. In *Dynamics of Ovarian Function* (Edited by N. B. Schwartz and M. Hunzicker-Dunn). Raven Press, New York (1981) p. 197.
14. Alila H. W., Dowd J., Corradino R. A., Harris W. V. and Hansel W.: Control of progesterone production in small and large bovine luteal cells separated by flow cytometry. *J. Reprod. Fert.* **82** (1988) 645–655.
15. Alila H. W., Corradino R. A. and Hansel W.: A comparison of the effects of cycloxygenase prostanoids on progesterone production by small and large bovine luteal cells. *Prostaglandins* **36** (1988) 259–270.
16. Baum M. S. and Roseberg S.: A phorbol ester, phorbol 12-myristate 13-acetate, and calcium ionophore, A23187, can mimic the luteolytic effect of prostaglandin $F_2\alpha$ in isolated rat luteal cells. *Endocrinology* **120** (1987) 1019–1026.
17. Veldhuis J. D. and Klase P. A.: Role of calcium ions in the stimulatory actions of luteinizing hormone in isolated ovarian cells: studies with divalent-action ionophores. *Biochem. Biophys. Res. Commun.* **104** (1982c) 603–610.
18. Asem E. K., Molnar M. and Hertelendy F.: Luteinizing hormone-induced intracellular calcium mobilization in granulosa cells: comparison with forskolin and 8-bromo-adenosine 3′,5′-monophosphate. *Endocrinology* **120** (1987) 853–859.
19. Hoyer P. B. and Marion S. L.: Influence of agents that affect intracellular calcium regulation on progesterone secretion in small and large luteal cells of the sheep. *J. Reprod. Fert.* **86** (1989) 445–455.
20. Seamon K. and Daly J. W.: Activation of adenylate cyclase by the diterpene forskolin does not require the guanine nucleotide regulatory protein. *J. Biol. Chem.* **256** (1981) 979–981.
21. Chiou C. Y. and Malagodi M. H.: Studies on the mechanism of action of a new Ca^{2+} antagonist, 8-(*N*,*N*-diethylamino)-octyl3,4,5-trimethoxybenzoate hydrochloride in smooth and skeletal muscle. *Br. J. Pharmac.* **53** (1975) 279–285.
22. Charo I. F., Feinman R. D. and Detwiler T. C.: Inhibition of platelet secretion by an antagonist of intracellular calcium. *Biochem. Biophys. Res. Commun.* **72** (1976) 1462–1467.
23. Tanaka T., Ohmura T. and Hidaka H.: Hydrophobic interaction of the Ca^{2+}-calmodulin complex with calmodulin antagonists. *Molec. Pharmac.* **22** (1982) 403–407.
24. Schatzman R. J., Raynor R. L. and Kuo J. F.: *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), a calmodulin antagonist, also inhibits phospholipid-sensitive calcium-dependent protein kinase. *Biochem. Biophys. Acta* **755** (1983) 144–147.
25. Nishizuka Y.: Turnover of inositol phospholipids and signal transduction. *Science* **225** (1984) 1365–1367.
26. Nishizuka Y.: Studies and perspectives of protein kinase C. *Science* **233** (1986) 305–312.
27. Lahav M., Rennert H., Sabag K. and Barzilai D.: Calmodulin inhibitors and 8-(*N*,*N*-diethylamino)-octyl-3,4,5-trimethoxybenzoate do not prevent the inhibitory effect of prostaglandin $F_2\alpha$ on cyclic AMP production in isolated rat corpora lutea. *J. Endocr.* **113** (1987) 205–212.
28. Higuchi T., Kaneko A., Abel J. H. and Niswender G. D.: Relationship between membrane potential and progesterone release in ovine corpora lutea. *Endocrinology* **99** (1976) 1023–1032.