BIOLOGY OF REPRODUCTION 41, 771–778 (1989)

Hormonal Regulation of Free Intracellular Calcium Concentrations in Small and Large Ovine Luteal Cells[1]

M. C. WILTBANK,[4] P. B. GUTHRIE,[5] M. P. MATTSON,[3,5]
S. B. KATER,[5] and G. D. NISWENDER[4]

Animal Reproduction Laboratory
Department of Physiology[4]
and
Department of Anatomy and Neurobiology[5]
Colorado State University
Fort Collins, Colorado 80523

ABSTRACT

The second messengers mediating hormonal regulation of the corpus luteum are incompletely defined, particularly for the primary luteolytic hormone prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). In this study, hormonally induced changes in free intracellular calcium concentrations were measured in individual small and large ovine luteal cells by using computer-assisted microscopic imaging of fura-2 fluorescence. This technique could readily detect transient increases in free calcium concentrations within both small and large luteal cells after treatment with 1 μM of the calcium ionophore, A23187. Treatment with $PGF_{2\alpha}$ (1 μM) caused a dramatic increase in free calcium concentrations in large (before = 73 ± 2 nM; 2 min after $PGF_{2\alpha}$ = 370 ± 21 nM; n = 33 cells) but not in small (before = 66 ± 4 nM; 2 min after $PGF_{2\alpha}$ = 69 ± 8 nM; n = 12 cells) luteal cells. The magnitude and timing of the calcium response was dose- and time-dependent. The $PGF_{2\alpha}$-induced increase in free intracellular calcium is probably due to influx of extracellular calcium, since inclusion of inorganic calcium channel blockers (100 μM manganese or cobalt) attenuated the response to $PGF_{2\alpha}$ and removal of extracellular calcium eliminated the response. In contrast to $PGF_{2\alpha}$, luteinizing hormone (LH) (100 ng/ml) caused no change in intracellular levels of free calcium in small or large luteal cells, even though this dose of LH stimulated ($p<0.01$) progesterone production by small luteal cells. Therefore, alterations in free calcium concentrations could be the intracellular second message mediating the luteolytic action of $PGF_{2\alpha}$ in the large ovine luteal cell.

INTRODUCTION

Secretion of progesterone by the corpus luteum is important for initiation and maintenance of pregnancy. The steroidogenic activity of the corpus luteum is regulated by a number of hormones via mechanisms that have not been completely defined. Luteinizing hormone (LH), an important luteotropic hormone in most species (Niswender and Nett, 1988), acts primarily through cyclic adenosine 3′,5′-monophosphate (cAMP)-dependent mechanisms (Hoyer and Niswender, 1985). However, phosphatidylinositol breakdown has also been reported following stimulation of luteal cells by LH (Davis et al., 1987a; Allen et al., 1988). Interestingly, the luteolytic actions of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) may also involve phosphatidylinositol metabolism (Leung et al., 1986; Davis et al., 1987b; Jacobs et al., 1987). An additional complexity is that the corpus luteum of many species contains two morphologically distinct steroidogenic cell types, termed large and small luteal cells (Ursely and Leymarie, 1979; Koos and Hansel, 1981; Fitz et al., 1982). The two cell types differ in hormonal responsiveness. Large cells contain essentially all the high-affinity receptor sites for $PGF_{2\alpha}$ (Fitz et al., 1982; Braden et al., 1988). In tissue obtained from normally cycling ewes, receptors for LH are found on both large and small luteal cells (Harrison et al., 1987), but LH dramatically stimulates progesterone production only in small luteal cells (Harrison et al., 1987). To more clearly define the second messengers involved in hor-

Accepted June 8, 1989.
Received February 10, 1989.
[1]This work is supported by the Colorado Experiment Station and NIH grant HD11590 and by NIH training grant HD07031 (M. Wiltbank).
[2]Reprint requests: G. D. Niswender, Department of Physiology, Colorado State University, Fort Collins, CO 80523.
[3]Present address: 2121 Sanders-Brown Bldg., University of Kentucky, Lexington, KY 40536-0230.

monal regulation of the two luteal cell types, we have investigated changes in free intracellular calcium concentrations in individual ovine luteal cells.

MATERIALS AND METHODS

Cells

Corpora lutea were collected from ewes on Day 10 of estrous cycle (Day 0 = estrus; n = 4–6 corpora lutea per experimental replicate), decapsulated, sliced, and dissociated into suspensions of single cells. Centrifugal elutriation was used to eliminate nonsteroidogenic cells and to separate small and large steroidogenic cells (Fitz et al., 1982). Large (2×10^4), small (1×10^5), or mixed (2×10^4 large plus 1×10^5 small) cell populations were placed in Medium 199 (GIBCO, Grand Island, NY; #400-1200; [Ca^{2+} = 1.3 mM]) containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 4.2 mM $NaHCO_3$, 100 IU/ml penicillin G, and 10 μg/ml streptomycin sulfate (this mixture was designed M199) with 5% ram serum and plated overnight onto polylysine-coated glass coverslips that had previously been adhered directly to an opening in the bottom of a 35-mm^2 culture dish. After plating, the cells were washed three times with M199 and loaded with the acetoxymethyl ester form of fura-2 (Molecular Probes, Eugene, OR; 2 mM in dimethylsulfoxide [DMSO] diluted to a final incubation concentration of 6 μM in M199) for 30 min at 34°C (Grynkiewicz et al., 1985). The cells were rinsed three times with M199 to remove extracellular dye and then incubated at 37°C for 1 h. De-esterification of fura-2 occurs within the cell, thereby trapping the fluorescent dye intracellularly (Malgaroli et al., 1987).

Calcium Measurements

The fura-2 fluorescence-imaging system has been described previously (Conner, 1986; Mattson et al., 1988). Individual cells were viewed with a Zeiss ICM inverted fluorescence microscope (20× and 40× Nikon UV-F objectives) attached to an RCA S.I.T. camera. The camera output was converted to a 640 × 480 digital image (307,200 pixels/screen) by the Quantex QX7-210 Image Processing System (Santa Clara, CA). The excitation wavelength was determined by a computer-controlled filter wheel that rotated between 350 ± 10 nm and 380 ± 10 nm interference filters. Neutral density filters (0.12 ND) were inserted in the excitation path to reduce bleaching of the fura-2 and prevent saturation of the camera by the fluorescent emission from the cells. The fluorescent emission was filtered with a 495-nm long-pass emission filter. Fluorescent images were obtained with each excitation filter. The ratio of fluorescence intensity (350 nm image/380 nm image) was converted to calcium concentration with the formula [free calcium] = K_d $[R-R_{min})/R_{max}-R)]$ (F_o/F_s) (Grynkiewicz et al., 1985; equation 5). For our system, R_{min} = 0.48; R_{max} = 11; (F_o/F_s) = 10; K_d = 224. The free intracellular calcium concentration for an individual luteal cell at a given time point represents the average of at least 25 pixels evaluated within the individual cell (normally 81 pixels were evaluated with each cell). In theory, calcium measurements using the ratio of two images should eliminate the effects of pathlength (thickness), excitation intensity, and dye concentration differences (Cohan et al., 1987). However, these measurements represent averaged cytoplasmic calcium concentrations and may not readily detect local changes in calcium concentration.

Experiments

Areas of the culture dish were chosen in which large and/or small luteal cells were clearly distinguishable,

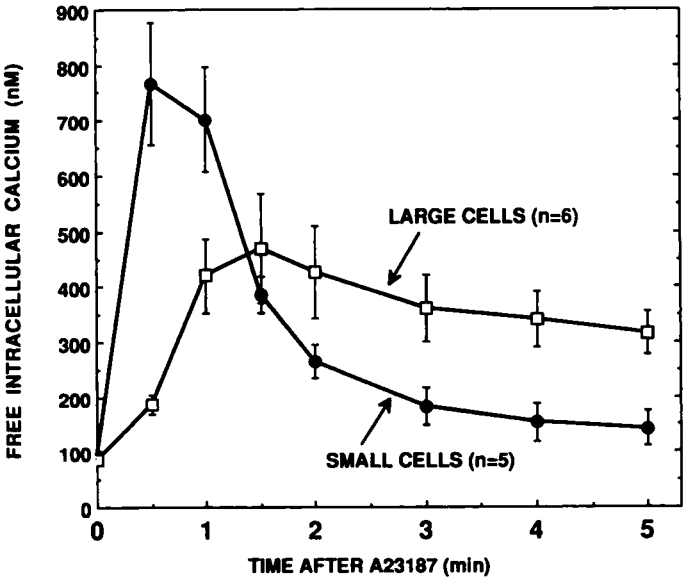

FIG. 1. Effect of 1 μM A23187 on free intracellular calcium concentration within small and large luteal cells (Mean ± SEM). For large cells, free intracellular calcium concentration is greater ($p<0.05$) than at Time 0 for all time points except 30 s after A23187. For small cells, free intracellular calcium concentration is greater ($p<0.05$) than Time 0 at 30, 60, and 90 s, but is not different at 2, 3, 4, or 5 min after A23187. Similar results were obtained on two other occasions when measurements were made every 20 s after A23187 or every minute after A23187 (n = total of 26 large cells and 16 small cells evaluated). Treatment with DMSO (vehicle) caused no significant change in free intracellular calcium concentrations in either cell type.

and intracellular free calcium measurements were made on these cells before any treatment. $PGF_{2\alpha}$ (UpJohn, Kalamazoo, MI; $PGF_{2\alpha}$-tromethamine salt; #U-14583E), LH (NIADDK-oLH-25), forskolin (Calbiochem-Behring Diagnostics, La Jolla CA; #D0627; diluted in ethanol), A23187 (Sigma Chemical Co., St. Louis, MO, C7523; diluted in DMSO) or appropriate control medium was added directly to culture dishes and mixed rapidly (<10 s). Free calcium concentrations were measured in individual luteal cells at times ranging from 10 s to 30 min after treatment. In certain experiments, progesterone was measured in the media by radioimmunoassay (Niswender, 1973).

Statistics

Data were evaluated by repeated measures analysis of variance followed by Dunnett's *t*-test (Dunnett, 1964) using the Statview 512+ statistics program. For graphical representations, standard error of the mean (standard deviation divided by the number of cells) was used because the free calcium concentration for an individual luteal cell represents the average of multiple free calcium determinations within an individual cell (normally 81 pixels evaluated per cell).

RESULTS

A preliminary experiment was performed to test for possible effects of photo damage in large and small ovine luteal cells by taking consecutive calcium concentration measurements (one measurement every 30 s). There was no significant change in free calcium concentrations after 12 measurements (56.3 ± 5.4 vs. 65.3 ± 6.9 nM), therefore, subsequent experiments made 12 or fewer calcium concentration measurements in an individual luteal cell.

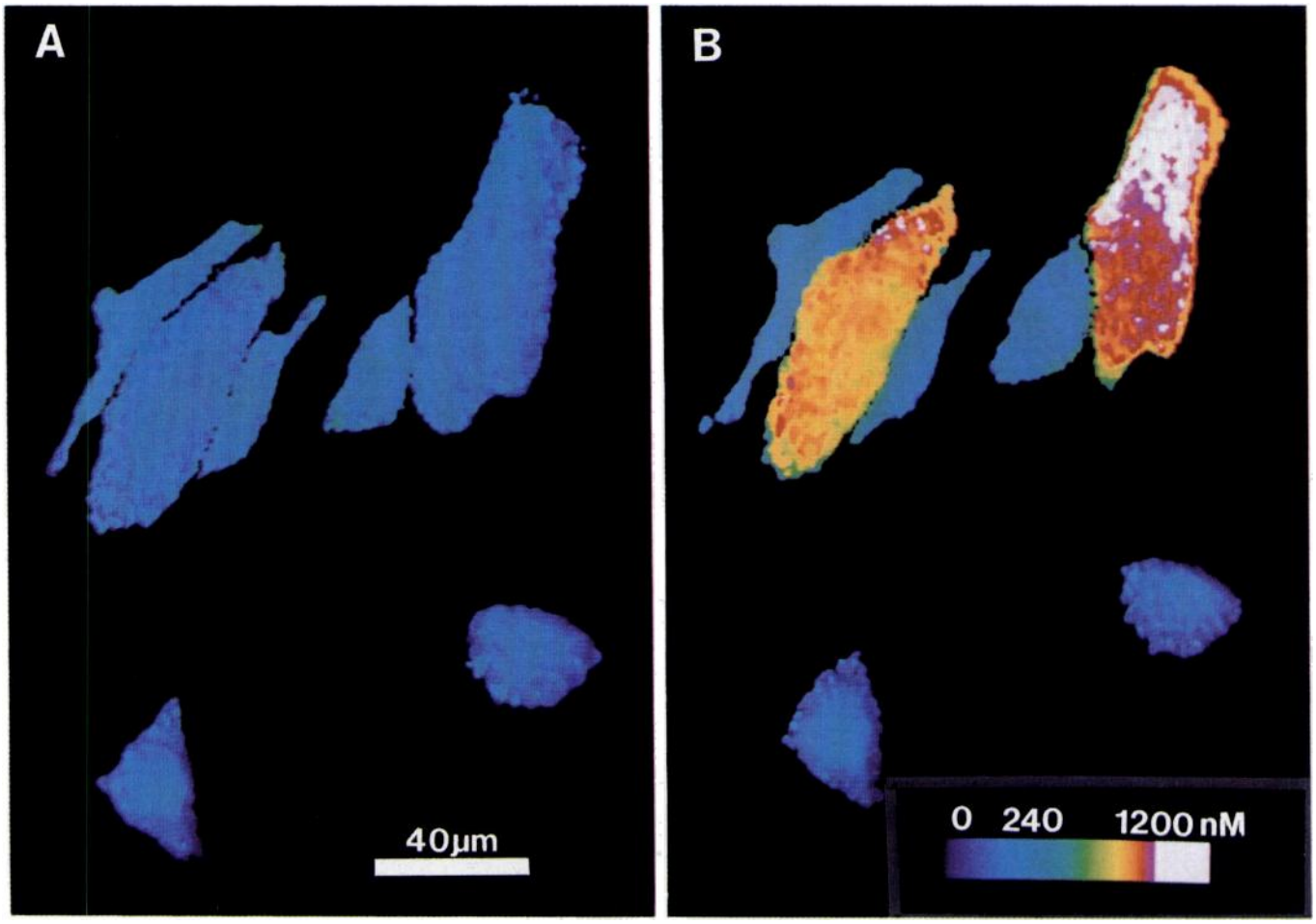

FIG. 2. Computer-generated color maps of free intracellular calcium concentrations in adjacent small and large luteal cells, before *(left)* and 40 s after *(right)* 1 μM $PGF_{2\alpha}$. Cell color is indicative of free calcium concentrations as shown in the color scale on the *right*. Cell size is indicated by the *bar* on the *left*.

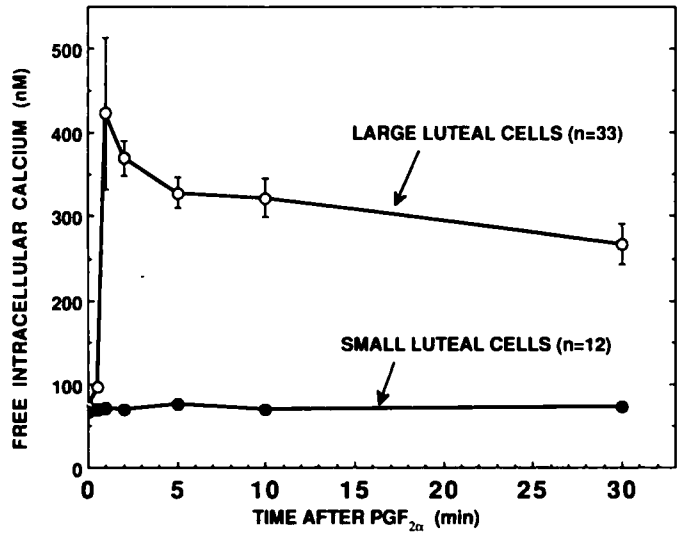

FIG. 3. Effect of 1 μM $PGF_{2\alpha}$ on free intracellular calcium concentrations in small and large luteal cells. Calcium concentrations were measured as described in *text* by using computer imaging of fura-2 fluorescence. Note that time on *Y axis* is in minutes. Data are from 4 replicates of this experiment with 5–8 large cells and 2–4 small cells evaluated per replicate. Points represent mean ± SEM (if SEM larger than dot).

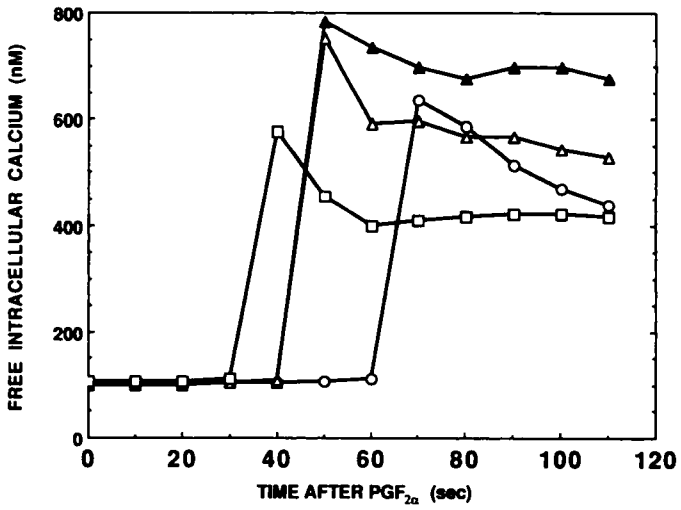

FIG. 4. Time course of effects of 1 μM $PGF_{2\alpha}$ on free intracellular calcium concentrations within individual large luteal cells in one microscopic field. Calcium measurements were made every 10 s by using computer imaging of fura-2 fluorescence.

Treatment with 1 μM of calcium ionophore, A23187, caused a significant increase in free intracellular calcium concentrations in both large and small luteal cells; however, the magnitude and timing of the responses clearly differed between the two cell types (Fig. 1). Small luteal cells showed an abrupt and marked increase in free calcium that was greatest by 30 s and subsequently returned to baseline by 3 min. Large luteal cells required 90 s before maximal free intracellular calcium concentrations were attained, and free calcium in these cells had not returned to basal levels by 30 min.

Treatment with $PGF_{2\alpha}$ (1 μM) caused a dramatic increase in free intracellular calcium concentration in large but not small luteal cells (Figs. 2 and 3). The difference in responsiveness was graphically demonstrated in mixed-cell preparations, with free calcium concentrations increasing in large luteal cells without a detectable change in adjacent small luteal cells (Fig. 2). There was a significant increase in free intracellular calcium concentrations in large cells ($p<0.01$; compared to 0 time) by 60 s after $PGF_{2\alpha}$ and at all subsequent times, while in small cells there was no significant change at any time measured (Fig. 3). Examination of individual large luteal cells in one microscopic field at 10-s intervals (Fig. 4) indicated that individual large luteal cells responded to $PGF_{2\alpha}$, with a rapid and consistent rise in free intracellular calcium. However, there appeared to be a difference in the lag time to responsiveness between individual cells with, for example, one cell responding after 30 s and an adjacent cell not responding until after 60 s (Fig. 4). Cells treated with medium alone showed no change in free calcium concentration within large or small luteal cells at any of the times examined (data not shown). To determine if the calcium response was terminated when $PGF_{2\alpha}$ was removed from the medium, large luteal cells were treated for 2 min with 1 μM $PGF_{2\alpha}$, and then washed 3 times with either M199 or with M199 containing $PGF_{2\alpha}$. Washing out the $PGF_{2\alpha}$ caused a significant decrease in free intracellular calcium concentration as compared to cells washed with medium containing $PGF_{2\alpha}$, but the free calcium still had not returned to basal levels by 20 min (Fig. 5).

The timing and degree of intracellular calcium elevation were dependent upon the dose of $PGF_{2\alpha}$ (Table 1). Large luteal cells did not respond to treatment with 0.1 μM of $PGF_{2\alpha}$ by 1.5 min, and less than 50% responded by 5 min. Responding cells were distinguished by a 2-fold or greater increase in free intracellular calcium concentrations. Nonresponding cells had free intracellular calcium concentrations similar to those measured before treatment. Nearly all large luteal cells responded to the 0.5 μM dose; however, the lag time to response was longer than in cells treated with 1 μM (Table 1). Responding cells showed less of a calcium increase in

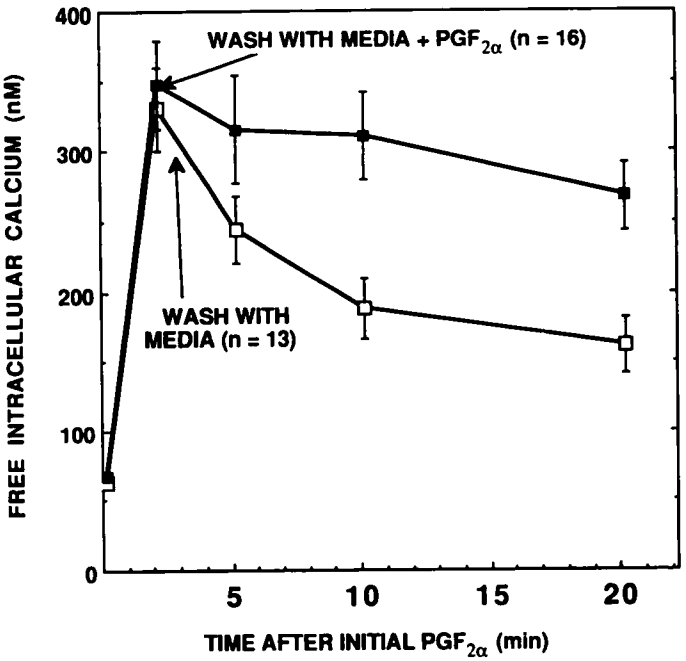

FIG. 5. Changes in free intracellular calcium concentrations after washing $PGF_{2\alpha}$-treated large luteal cells. Cells were treated with 1 μM $PGF_{2\alpha}$ for 2 min and then washed three times with M199 or M199 containing 1 μM $PGF_{2\alpha}$ (Mean ± SEM). For cells washed with medium + $PGF_{2\alpha}$, measurements at 2, 5, 10, and 20 min after treatment are different from Time 0 ($p<0.05$) but not different from each other. For cells washed with medium, measurements at 2, 5, 10, and 20 min after treatment are different from Time 0, and 5, 10, and 20 min are different from 2-min time point ($p<0.05$). Data are from one replicate of this experiment with statistical differences determined by repeated measures analysis of variance.

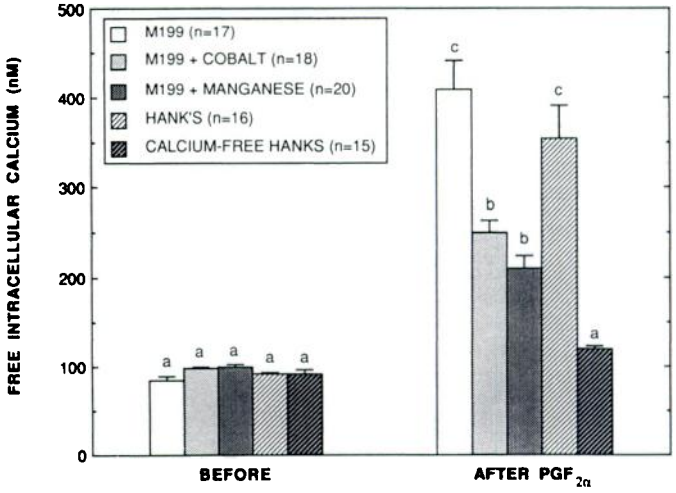

FIG. 6. Effect of addition of inorganic calcium channel blockers or removal of extracellular calcium on $PGF_{2\alpha}$-induced increase in free intracellular calcium concentrations. Large luteal cells were incubated in M199 (M199) until 1 min before $PGF_{2\alpha}$ treatment. Cells were then washed 5 times with Hanks', calcium-free Hanks', M199, M199 + 100 μM cobalt, or M199 + 100 μM manganese. Free intracellular calcium concentrations from 3 replicates of this experiment. *Bars* with different *letters* are different ($p<0.05$).

cells treated with 0.1 or 0.5 μM than in cells treated with 1 μM (Table 1; $p<0.01$). Small cells (n = 16) showed no response to any of the doses of $PGF_{2\alpha}$.

To evaluate whether the increase in free intracellular calcium was due to an influx of extracellular calcium, cells were examined in either normal Hanks', in calcium-free Hanks', in M199, or in M199 containing 100 μM cobalt or manganese (inorganic calcium channel blockers). Cells incubated in calcium-free Hanks' showed no significant response to treatment with 1 μM $PGF_{2\alpha}$, whereas cells in normal Hanks' showed the characteristic increase in free intracellular calcium concentrations (Fig. 6). Cells incubated with 100 μM cobalt or manganese showed an attenuated response to $PGF_{2\alpha}$ (Fig. 6).

Treatment with 100 ng/ml of LH had no detectable effect on free calcium concentrations in either luteal cell type (data not shown). There was no detectable alteration in free intracellular calcium when measurements were made every 10 s for 100 s (n = 2 experiments with 10 individual small luteal cells) nor when measurements were made at 0.5, 1, 2, 3, 5, 7, and 10 min after LH treatment (n = 4 experiments with mixed or small luteal cells and a total of 26 individual luteal cells; 5 large, 21 small). Incubation for 30 min with 100 ng/ml of this LH preparation stimulated progesterone production in small luteal cells (0.56 ± 0.03 vs. 2.14 ± 0.05 fg/cell^{-1} × min^{-1}; $p<0.01$).

DISCUSSION

The objective of this study was to determine if LH or $PGF_{2\alpha}$ influences free intracellular calcium concentrations in large or small luteal cells, thereby implicating calcium as a second messenger for these hormones. Treatment with the calcium ionophore A23187 caused a differential increase in free calcium concentration within the two types of luteal cells and the increase could be readily quantified. Our most significant finding was that PGF_{α} markedly increased free intracellular calcium concentrations in large but not small luteal cells. This action had a clear dose-, time-, and extracellular calcium-dependence, as well as cellular specificity. In contrast, LH did not influence free calcium concentrations in either cell type. These results suggest that the calcium- regulatory mechanisms differ within the two types of steroidogenic luteal cells.

In the small steroidogenic luteal cell, LH stimulates production of progesterone by increasing intracellular

TABLE 1. The effect of different doses of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) on free intracellular calcium concentrations in large luteal cells.

Dose of $PGF_{2\alpha}$	Free calcium after $PGF_{2\alpha}$ in responding cells (nM)*			% of cells responding** after given time (min)				
	0 min	5 min	10 min	0.5	1.0	1.5	5.0	10.0
0.1 μM (n = 13)	73 ± 3^{aA}	177 ± 15^{bA}	167 ± 10^{bA}	0	0	0	46	46
0.5 μM (n = 13)	79 ± 3^{aA}	240 ± 25^{bB}	216 ± 24^{bA}	0	23	77	92	92
1 μM (n = 16)	75 ± 2^{aA}	315 ± 29^{bC}	308 ± 29^{bB}	0	100	100	100	100

*Means (± SEM) in a row with different small letter superscripts or in a column with different capital letter superscripts are statistically different ($p < 0.05$). Cells were from 3 replicates of this experiment.

**Responding cells were those cells showing 2-fold or greater increases in free intracellular calcium concentrations.

cAMP concentrations (Hoyer and Niswender, 1985). It has also been reported that LH either increases (Davis et al., 1987a; Allen et al., 1988) or has no effect (Jacobs et al., 1987) on the concentrations of phosphoinositides, including IP_3, within mixed luteal cell preparations. IP_3 can increase free calcium concentrations either by releasing sequestered intracellular calcium (Berridge, 1987) or by opening plasma membrane calcium channels (Kuno and Gardner, 1987). In our experiments, it is not known if IP_3 concentrations were altered by LH, but there was no detectable change in free calcium concentrations. In this respect, our data do not support the results of Davis et al. (1987a), who reported that LH increased free intracellular calcium concentrations in mixed bovine luteal cells as measured by spectrophotometric changes in quin-2 fluorescence. The reason(s) for this discrepancy is not apparent. Our LH preparation was biologically active since it significantly increased progesterone production by small cells, and the 100 ng/ml dose has been found to be maximally stimulatory (Jordan et al., 1978). It could be argued that our fura-2 system could not detect any increase in free calcium because the changes were too rapid or they occurred in a very localized region. However, the finding that free calcium concentrations in small luteal cells were elevated for minutes after treatment with A23187 suggests that measurement of calcium every 10 s should be more than adequate to detect a hormonally induced change. This result also indicates that we could reliably detect alterations in free intracellular calcium concentrations within this cell type. Therefore, our data indicate that neither LH nor $PGF_{2\alpha}$ treatment influenced free calcium concentrations within small luteal cells.

In the large luteal cell, there are receptors for LH, but LH treatment produces no increase in cAMP or progesterone production by these cells (Hoyer and Niswender, 1986; Harrison et al., 1987). In addition, our data indicate that LH receptors are not coupled to calcium entry in the large cell. Other hormones, particularly the prostaglandins, are probably important in regulating this cell type, since $PGF_{2\alpha}$ receptors are present exclusively on the large cells of the ovine corpus luteum (Fitz et al., 1982). Our data indicate that binding of $PGF_{2\alpha}$ to these receptors is coupled to an increase in free intracellular calcium concentrations. This is primarily due to influx of calcium from extracellular sources, since inclusion of inorganic calcium channel blockers attenuated the response to $PGF_{2\alpha}$ and removal of extracellular calcium eliminated the response. The reason that manganese and cobalt only partially blocked the calcium response is probably because these inhibitors were present at a much lower concentration (100 μM) than was calcium (1.3 mM) in the M199. The abruptness of the calcium influx in individual luteal cells, after a 30- to 60-s lag time, suggests that an intracellular signal must build up to a threshold level, at which point numerous calcium channels respond in a given cell. The response to A23187 in the large luteal cell was more gradual, which further suggests that $PGF_{2\alpha}$ must cause the sudden opening of many calcium channels. Possible mechanisms for the $PGF_{2\alpha}$-induced calcium influx include 1) the occupied $PGF_{2\alpha}$ receptor activates phospholipase C, causing increases in IP_3 and subsequent opening of calcium channels (Leung et al., 1986; Davis et al., 1987b; Jacobs et al., 1987); 2) the $PGF_{2\alpha}$ receptor is an ion channel, as is the case for certain neurotransmitter receptors (Barnard and Seeburg, 1988); 3) the $PGF_{2\alpha}$ receptor regulates membrane potential and the calcium influx is mediated through voltage-dependent calcium channels; 4) a G-protein couples the occupied $PGF_{2\alpha}$ receptor to ion channels (Brown and Birnbaumer, 1988; Exton, 1988). Our present results cannot distinguish among these possibilities. However, depolarization of the cell with high levels of potassium caused no change in free intracellular calcium concentration (unpublished re-

sults), indicating that voltage-dependent changes are unlikely.

Once calcium enters the cell there are probably a multitude of intracellular responses. Calcium sequestering/extrusion mechanisms may be activated such as the calcium-dependent, high-calcium-affinity, nucleoside triphosphatase that has been found in luteal tissue from rats (Minami and Penniston, 1987). From our experiments using A23187, it is clear that the large luteal cell has less efficient mechanisms for eliminating free intracellular calcium than the small luteal cell.

The high calcium concentrations in large luteal cells after treatment with $PGF_{2\alpha}$ may mediate this hormone's action by activating multiple calcium-dependent pathways including protein kinase C, calmodulin, and calcium-stimulated phospholipases and proteases (Carafoli, 1987). We have recently found that protein kinase C is present and inhibitory to steroidogenesis in large ovine luteal cells (Wiltbank et al., 1989). Therefore, the antisteroidogenic effects of $PGF_{2\alpha}$ (Pate and Nephew, 1988) may be mediated through calcium influx and activation of protein kinase C. Other possible effects of the high, free intracellular calcium concentrations include decreased adenylate cyclase activity (Behrman et al., 1986), activation of cAMP phosphodiesterase (Mattson and Spaziani, 1986), decreased metabolic enzyme activities (Rao et al., 1984), and increased autolytic/cytotoxic activities (McClellan et al., 1977; Rao et al., 1984; Braden et al., 1988). During $PGF_{2\alpha}$-induced luteolysis, large and small steroidogenic luteal cells undergo cell death, as measured by decreases in luteal cell numbers (Braden et al., 1988). In other cell types, sustained high levels of free intracellular calcium are considered the mediator of cell death (Choi, 1985; Mayer and Westbrook, 1987; Mattson et al., 1988). Therefore, it is reasonable to consider that the cytotoxic effects of $PGF_{2\alpha}$ on the large luteal cell (Fitz et al., 1984; Braden et al., 1988) could be due to the induction of high concentrations of free intracellular calcium by $PGF_{2\alpha}$ within this cell type. The in vivo cytotoxic effect of $PGF_{2\alpha}$ treatment on the small luteal cell (Braden et al., 1988) is probably through indirect mechanisms such as intercellular communication from large to small cell or decreases in tissue blood flow (Niswender et al., 1975).

ACKNOWLEDGMENTS

We thank Dr. M. H. Mayan for elutriation of luteal tissue and Ms. Kathy Miller for manuscript preparation. Surgical assistance was kindly provided by C. Belfiore, N. Capitan, G. DiGregorio, Dr. M. Diskin, Dr. J. Flores, M. Gallegos, D. Gregg, Dr. J. Kile, Dr. J. Knickerbocker, and G. Wiepz.

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