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Progesterone Triggers Rapid Transmembrane Calcium Influx and/or Calcium Mobilization From Endoplasmic Reticulum, via a Pertussis-Insensitive G-Protein in Granulosa Cells in Relation to Luteinization Process

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Abstract We investigated the early effects (5–60 s) of progesterone (1 pM–0.1 μM) on cytosolic free calcium concentration ($[Ca^{2+}]_i$) and inositol 1,4,5-trisphosphate ($InsP_3$) formation in nonluteinized and in vitro luteinized porcine granulosa cells (pGCs). Progesterone increased $[Ca^{2+}]_i$ and $InsP_3$ formation within 5 s in both cell types. Progesterone induced calcium mobilization from the endoplasmic reticulum via the activation of a phospholipase C linked to a pertussis-insensitive G-protein. This process was controlled by protein kinases C and A. In contrast, only nonluteinized pGCs showed a Ca^{2+} influx via dihydropyridine-insensitive calcium channel. In both cell types, the nuclear progesterone receptor antagonist RU-38486 did not inhibit the progesterone-induced increase in $[Ca^{2+}]_i$; progesterone immobilized on bovine serum albumin, which did not enter the cell, increased $[Ca^{2+}]_i$ within 5 s and was a full agonist, but less potent than the free progesterone; pertussis toxin did not inhibit progesterone effect on $InsP_3$. In conclusion, progesterone may interact with membrane unconventional receptors that belong to the class of membrane receptors coupled to a phospholipase C via a pertussis toxin-insensitive G-protein. The source of the Ca^{2+} for the progesterone-induced increase in $[Ca^{2+}]_i$ also depends on the stage of cell luteinization.

Key words: phospholipase C, inositol-1,4,5-trisphosphate, protein kinase C, protein kinase A, progesterone

Although it is well established that progesterone acts as a local regulator of the ovarian functions in several species [Goodman and Hodgen, 1982; Rao and Maseh, 1986], the mechanisms by which progesterone affects target cells are not fully understood. Progesterone may act as an autocrine factor and it has been suggested that progesterone locally regulates the development and function of the follicle [Goodman and Hodgen, 1982] and the corpus luteum [Rothchild, 1981; Duffy et al., 1994]. Progesterone can regulate cell cycling in a variety of cells [Chaffkin et al., 1992]. Progesterone also blocks differentiation and controls the number of luteal cells that ultimately develop within the corpus luteum by regulating granulosa cell differentiation and proliferation. Lastly, it has been postulated that progesterone may regulate luteinizing hormone receptors and its own receptor [Jones et al., 1992; Duffy and Stouffer, 1995].

The action of progesterone is believed to be mediated by the binding of the hormone to specific intracellular receptors that interact with hormone-responsive elements located in the promoter region of steroid hormone-regulated genes to induce transcription. These classical receptors have been identified in luteal cells of corpus luteum and in luteinizing granulosa cells [Press

Abbreviations: P-CMO, progesterone-3-(O-carboxymethyl)-oxime; P-CMO BSA, progesterone-3-(O-carboxymethyl)-oxime bovine serum albumin; FCS, fetal calf serum; U-73122, 1-(6-((17β-3-metoxyestra-1,3,5(10)-trien-17-yl)-amino)-hexyl)-1H-pyrrole-2.5- dione; U-73343, 1-(6-((17β-3-metoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrolidine-2,5-dione; SpcAMPS, Sp-adenosine 3′,5′-cyclic monophosphothioate triethanolamine salt; PTX, pertussis toxin; $[Ca^{2+}]_i$, intracellular calcium concentration; PLC, phospholipase C; PKA, protein kinase A; PKC, protein kinase C; DAG, diacylglycerol; $InsP_3$, inositol-1,4,5-trisphosphate; GnRH, gonadotropin-releasing hormone; FSH, follicle-stimulating hormone; LH, luteinizing hormone; hCG, human chorionic gonadotropin; pGC, porcine granulosa cell.

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and Greene, 1988; Hild-Petito et al., 1988]. In granulosa cells, the expression of mRNA progesterone receptor is positively regulated by LH/hCG and tighly coupled to the preovulatory gonadotropin surge [Park and Mayo, 1991; Chandrasekher et al., 1994]. The granulosa layer of nonovulatory follicles is consistently negative for progesterone receptors.

Progesterone may also act nongenomically by triggering an increase in cytosolic free calcium via a Ca^{2+} channel different from the voltage-dependent Ca^{2+} channel [Blackmore et al., 1991]. Progesterone induces maturation in amphibian oocyte by acting on the plasma membrane [Liu and Patino, 1993], and the progesterone-initiated human sperm acrosome reaction requires a rise in intracellular calcium through putative membrane progesterone receptors [Blackmore et al., 1991; Tesarik and Mendoza, 1993; Turner et al., 1994]. Specific membrane binding sites for progesterone were investigated in rat brain [Tischkau and Ramirez, 1993], in human sperm [Blackmore et al., 1994; Aanesen et al., 1995], and in ovine corpus luteum [Bramley and Menzies, 1994].

The role of calcium in the regulation of steroidogenesis and progesterone release by granulosa cells is now well established [Flores and Velhuis, 1993]. These data support the view that progesterone may regulate its own production through the generation of a calcium signal. However, no information is available about the early effects of progesterone on the intracellular calcium concentration in granulosa cells, except one report showing no effect [Morley et al., 1992].

This study was therefore performed to describe the direct effects of progesterone on the intracellular calcium concentration and inositol 1,4,5-trisphosphate formation in porcine granulosa cells. Nonluteinized granulosa cells (pGCs) were taken from 1–3 mm follicles of immature porcine ovaries and treated in vitro with gonadotropins to induce luteinization. These cells were used to investigate whether the nongenomic effects of progesterone were correlated with the stage of pGC luteinization.

EXPERIMENTAL PROCEDURES

Materials

Progesterone (P), progesterone-3-(O-carboxymethyl) oxime (P-CMO), progesterone-3-(O-carboxymethyl) oxime-bovine serum albumin (P-CMO BSA, 32 mol progesterone/mol BSA), and all chemicals were from Sigma. RU 38486 was from Roussel-Uclaf (France), a minimal essential medium (α-MEM) without phenol red, Dulbecco's medium (DMEM), DMEM/F12 (1:1) medium, fetal calf serum (FCS), and trypsin/EDTA were from Gibco BRL (Eragny, France), and Fura2/AM was from Amersham (Les Ulis, France). 1-(6-((17β-3-metoxyestra-1,3,5(10)-trien-17-yl)-amino)hexyl)-1H-pyrrole-2,5- dione (U-73122) and 1-(6-((17β-3-metoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrolidine- 2,5-dione (U-73343) were from Biomol Research Laboratory (Plymouth, MA) and Tebu (Le Perray en Yvelines, France); thapsigargin, staurosporine and Sp-cAMPS (Sp-Adenosine 3′,5′-cyclic monophosphothioate triethylamine salt) were from Research Biochemicals International (Natick, MA) and Bioblock Scientific (Illkirch, France).

Isolation and Cell Culture

Ovaries of immature Large White sows (5–6 months of age) were obtained from a local slaughterhouse and collected in a 1:1 DMEM, Dakin-Cooper at 4°C. The pGCs were aseptically removed from 1–3 mm diameter follicles with a syringe within 2 h, washed in DMEM, and centrifuged at 250*g* for 10 min at 10°C. The pGCs from 10–30 ovaries were pooled, seeded at a density of 10^7 cells per 75 cm^2 flasks in DMEM/F12 with 10% FCS and cultured at 37°C in an atmosphere of 95% air/5% CO_2 for 48 h. After cell attachment and proliferation to confluency, the medium was replaced by DMEM/F12 containing 2% FCS, with or without 10 ng/ml FSH and hCG. Gonadotropins induced luteinization of the pGCs within 72 h, while untreated cells did not spontaneously luteinize. Cells were then removed with trypsin-EDTA and grown on glass coverslips or in 100 cm^2 petri dishes in α-MEM medium without phenol red and supplemented with 5% FCS until confluent. Cells were transferred to serum-free medium 24 h before their use in experiments.

The effect of gonadotropins on pGC luteinization was assessed by measuring the progesterone concentration in the culture medium by radioimmunoassay (COATRIA, Biomerieux Laboratories, Marnes l'Etoile, France). The assay sensitivity was 0.65 ng/ml, intra- and inter-assay coefficients of variation were 6% and 14%, respectively. The progesterone concentrations in nonluteinized pGCs were <1 nM and 1–10 μM in pGCs treated with gonadotropins for 72

h. The amount of progesterone decreased in serum-free cultures and dropped to indetectable levels at the time of calcium and $InsP_3$ measurement.

Calcium Measurement

The cells were washed three times with Hanks' Hepes, pH 7.4 (137 mM NaCl, 0.441 mM KH_2PO_4, 0.442 mM Na_2HPO_4, 0.885 mM $MgSO_47H_2O$, 27.7 mM glucose, 1.25 mM $CaCl_2$, 20 mM Hepes) and loaded with 1 μM Fura2/AM for 20 min in the same buffer at room temperature. The glass coverslip carrying the cells was inserted into a cuvette containing 2.5 ml Hanks' Hepes, pH 7.4. The cuvette was placed in a thermostatted (37°C) Hitachi 2000 spectrofluorimeter. Drugs and reagents were added directly to the cuvette with continuous stirring. Progesterone was dissolved in ethanol; the final concentration of ethanol never exceeded 0.01%. This ethanol concentration was without effect on any of the parameters tested in this study.

Calibration of fura-2 fluorescence in terms of intracellular calcium concentration ($[Ca^{2+}]_i$) was calculated from the ratio of 340/380 nm fluorescence values after subtraction of the background fluorescence of the cells at 340 and 380 nm as described by Grynkiewicz et al. (1985). The dissociation constant for the fura-2.Ca^{2+} complex was taken as 224 nM (Grynkiewicz et al., 1985). The values for R_{max}, R_{min} were calculated from measurements using 25 μM digitonin and 4 mM EGTA and enough Tris-base to raise the pH to 8.3 or higher. Each measurement on fura-2-loaded cells was followed by a parallel experiment with non-fura-2-loaded cells under the same conditions.

Extraction and Determination of Inositol-1,4,5-Trisphosphate

The reaction was stopped by removing the medium and adding ice-cold trichloroacetic acid (TCA) (final concentration of 5%). TCA was removed with diethyl ether. The final extract was neutralized before used for quantitative determination of inositol 1,4,5 trisphosphate (radiocompetition; Kit TRK 1000, Amersham, Les Ulis, France).

Experimental Protocol

The direct effects of progestins (1 pM to 0.1 μM) on intracellular calcium concentration ($[Ca^{2+}]_i$) of confluent granulosa cells were examined, first. We then investigated whether the action of progestins on $[Ca^{2+}]_i$ was due to an influx of Ca^{2+} from the extracellular milieu, and/or to Ca^{2+} mobilization from intracellular stores. Two types of blocking experiments were performed. In the first, a small excess of EGTA (2 mM) was added to the cuvette medium [Albert and Tashjian, Jr, 1984]. Replenishment of Ca^{2+} to a 1.25-mM excess following EGTA treatment restored the basal level. The substances to be tested were added 30 s after adding EGTA, when a new steady-state level of $[Ca^{2+}]_i$ had been reached [Albert and Tashjian, Jr, 1984]. In the second, verapamil, a selective blocker of Ca^{2+} entry via voltage-dependent Ca^{2+} channels, was added to a final concentration of 1 μM. Progestins were added 1 min after. Lastly, we determined the part of the $[Ca^{2+}]_i$ transient that was due to release from intracellular stores. The naturally occuring sesquiterpene lactone, thapsigargin, was used to inhibit the endoplasmic reticulum ATP-dependent Ca^{2+} pump and to release Ca^{2+} from the associated store, with little or no effect upon plasma membrane and sarcoplasmic reticulum [Jackson et al., 1988; Thastrup et al., 1990]. Neomycin, an indirect inhibitor of the phospholipase C (PLC) [Prentki et al., 1986], and U-73122 [Bleasdale et al., 1989], a direct inhibitor of PLC, were also used.

For inositol-1,4,5-trisphosphate ($InsP_3$), cells were incubated for 24 h in serum-free medium. The cells were then washed and incubated for 2 h in fresh medium. Ethanol solvent (0.01%) or progesterone (100 pM) were then added for 5–30 s.

Progestins

Progesterone was dissolved in ethanol; the final concentration of ethanol never exceeded 0.01%. This ethanol concentration was without effect on intracellular calcium concentration. In inositol phosphate experiments, there were no significant differences between the ethanol control values at 5–30 s, although the ethanol control values were higher (3%, $P < 0.001$, $n = 5$) than those of untreated controls.

To eliminate the possibility that there was no free progesterone or progesterone-CMO in the progesterone-CMO BSA preparation, the P-CMO BSA preparation was treated with charcoal to remove noncovalently bound P or P-CMO (Blackmore et al., 1991). The results showed that charcoal treatment had no effect on the ability of P-CMO BSA to increase $[Ca^{2+}]_i$. The observed

effects of P-CMO BSA on $[Ca^{2+}]_i$ were due to covalently bound steroid and not to free P or P-CMO in the P-CMO BSA preparation.

Statistical Analysis

The data on the changes in $[Ca^{2+}]_i$ and inositol-1,4,5-concentration were analyzed by one-way analysis of variance (ANOVA). The individual contrasts between treatment pairs were made by Dunnett's method. Differences of $P < 0.05$ were considered to be significant. A value of n represents n different cultures for a specific experiment.

RESULTS

Direct Effects of Progestins on $[Ca^{2+}]_i$ in Luteinized or Nonluteinized Granulosa Cells

The basal level of intracellular calcium concentration in the two types of confluent granulosa cells was 110 ± 10 nM (mean ± SE, n = 15). Figure 1 shows the transient increase in $[Ca^{2+}]_i$ induced by 100 pM progesterone; $[Ca^{2+}]_i$ dropped rapidly after 30 s, but remained higher than the basal level (20 ± 1%, mean ± SE, n = 15, $P < 0.001$) in nonluteinized cells (Fig. 1A). The $[Ca^{2+}]_i$ surge in luteinized cells consisted of a sharp spike followed by a rapid return to the resting levels (Fig. 1B). The concentration-dependent effects were bell-shaped, with a maximal activity at 100 pM (Table I) whatever the cell type. P-CMO and P-CMO BSA (Table I) induced a smaller increase in $[Ca^{2+}]_i$ in both cell types; the time course of the P-CMO BSA effect was similar to that of progesterone or P-CMO (data not shown). Progesterone was more potent than either P-CMO and P-CMO BSA, although these latter products were equipotent (Table I).

Blockade of Progesterone-Induced Changes in Intracellular Calcium Concentration

A small excess of EGTA (2 mM) was first used. 100 pM progesterone was added 30 s after EGTA. EGTA not only diminished the transient peak induced by progesterone (Fig. 2A), P-CMO, or P-CMO BSA (data not shown) but totally abolished the sustained plateau phase in nonluteinized cells. By contrast, EGTA did not block the progesterone effect on $[Ca^{2+}]_i$ in luteinized cells (Fig. 2B). The calcium entry blocker verapamil (1 μM) did not abolish the progesterone effect on $[Ca^{2+}]_i$ in nonluteinized and luteinized cells (data not shown).

Intracellular Organelle Responsible for Calcium Increase

Thapsigargin, which modifies calcium sequestration by the endoplasmic reticulum [Jackson et al., 1988; Thastrup et al., 1990], was used at 1 μM, at which concentration it had the greatest effect on $[Ca^{2+}]_i$. The rise in $[Ca^{2+}]_i$ induced by thapsigargin in nonluteinized and luteinized granulosa cells reached a peak within 90 s and then slowly decayed. 100 pM progesterone was added 10 min after thapsigargin. Pretreatment with thapsigargin totally abolished the spike phase induced by progesterone, but not the plateau phase in nonluteinized cells (Fig. 3A). Progesterone had no effect on $[Ca^{2+}]_i$ in thapsigargin-pretreated luteinized cells (Fig. 3B). The

TABLE I. Dose-Dependent Effects of Progestins on Intracellular Calcium Concentration in Nonluteinized and Luteinized Confluent Granulosa Cells†

	$[Ca^{2+}]_i$ nM					
	Progesterone		Progesterone–CMO		Progesterone–CMO BSA	
Concn	NL-GC	L-GC	NL-GC	L-GC	NL-GC	L-GC
0 pM	110 ± 2	115 ± 4	110 ± 2	115 ± 4	110 ± 2	115 ± 5
1 pM	204 ± 3*	216 ± 5*	120 ± 2	124 ± 3	121 ± 3	125 ± 1
10 pM	353 ± 5*	369 ± 6*	115 ± 5	121 ± 6	124 ± 4	123 ± 5
100 pM	395 ± 6*	395 ± 3*	332 ± 4*	315 ± 5*	315 ± 6*	319 ± 4*
1 nM	375 ± 6*	265 ± 4*	263 ± 6*	259 ± 7*	265 ± 2*	269 ± 7*
10 nM	235 ± 5*	243 ± 3*	234 ± 3*	229 ± 5*	231 ± 3*	230 ± 2*
100 nM	229 ± 4*	224 ± 5*	225 ± 5*	227 ± 4*	232 ± 5*	228 ± 4*

†Intracellular calcium concentrations were determined at 10 s. LN-GC and L-GC represent nonluteinized and luteinized granulosa cells, respectively. Values are the means ± SE (n = 10, three coverslips for each of the 10 cultures) and are significantly different from the control level.
*$P < 0.001$.

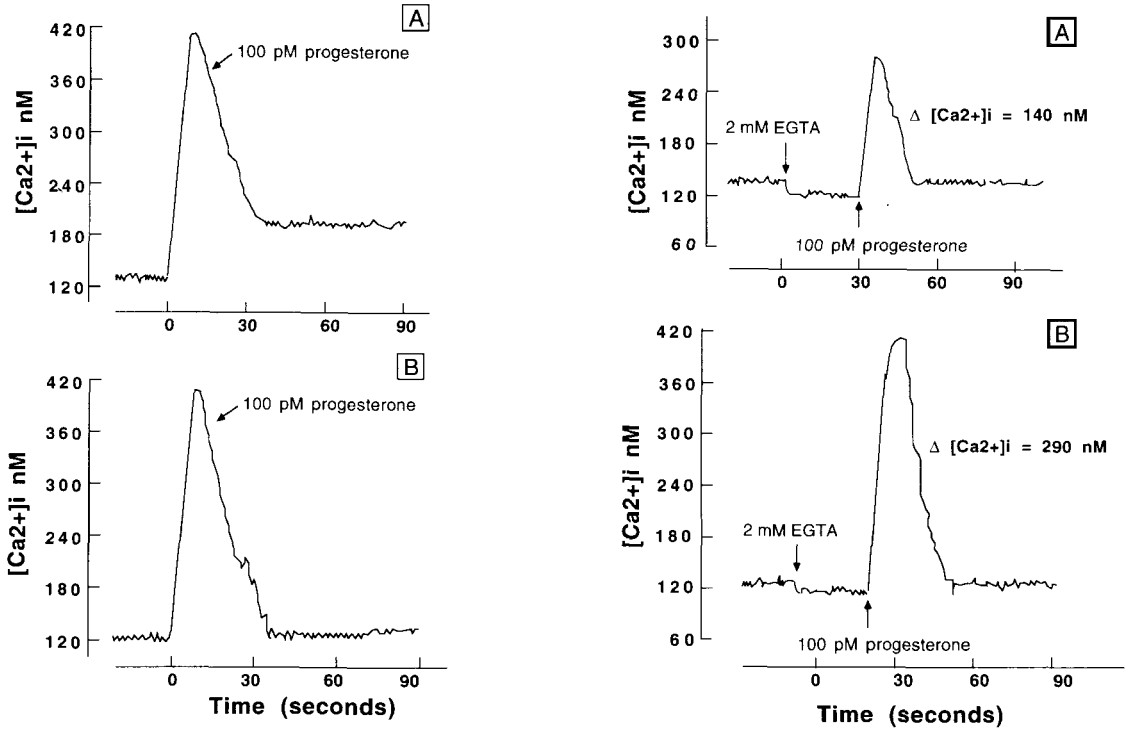

Fig. 1. Effects of 100 pM progesterone on intracellular calcium in nonluteinized (**A**) and luteinized (**B**) confluent granulosa cells. This experiment is representative of at least 15 cultures (five coverslips for each of the 15 cultures).

Fig. 2. Effects of EGTA on the $[Ca^{2+}]_i$ response to progesterone in nonluteinized (**A**) and luteinized (**B**) granulosa cells. Cells were incubated with 2 mM EGTA for 30 s before adding 100 pM progesterone. These experiments are representative of at least 10 cultures (three coverslips for each of the 10 cultures).

responses to P-CMO or P-CMO BSA were similar to those to progesterone in both cell types (data not shown).

Cells were pretreated for 5 min with 1 mM neomycin (which inhibits phospholipase C via binding to phosphoinositides) [Prentki et al., 1986] with 0.5–5 μM U-73122 or with 0.5–5 μM U-73343 (an inactive analogue of U-73122) [Bleasdale et al., 1989; Smith et al., 1990] before 100 pM progesterone was added. Neomycin totally blocked the spike phase in untreated and gonadotropin-treated cells, while the plateau phase remained unchanged in nonluteinized granulosa cells (Table II). U-73122 (1–5 μM) itself triggered transient dose-dependent (1–5 μM) increases in $[Ca^{2+}]_i$ (data not shown). Pretreatment with 5 μM U-73122 did not alter the plateau phase in nonluteinized cells, but abolished the transient peak in nonluteinized and luteinized cells (Table II). U-73343 (0.5–5 μM) had no effect on the response of either cell type to progesterone (data not shown).

Effects of Protein Kinases C and A on the Intracellular Calcium Response to Progesterone

Cells were pretreated for 1 min with 1 nM staurosporine, a potent inhibitor of protein kinases with some selectivity for protein kinase C, before adding 100 pM progesterone. The protein kinase C blockade by staurosporine enhanced the $[Ca^{2+}]_i$ response to progesterone in both nonluteinized and luteinized cells (Table II).

Cells were pretreated for 1 min with 0.1 mM Sp-cAMPS, a potent membrane activator of cAMP-dependent protein kinase I and II, before 100 pM progesterone was added. The protein kinase A stimulation by Sp-cAMPS diminished the $[Ca^{2+}]_i$ response to progesterone in nonluteinized and luteinized cells (Table II).

Effects of Pertussis Toxin on the Intracellular Calcium Response to Progesterone

Nonluteinized and luteinized granulosa cells were incubated for 24 h with 100 ng/ml pertus-

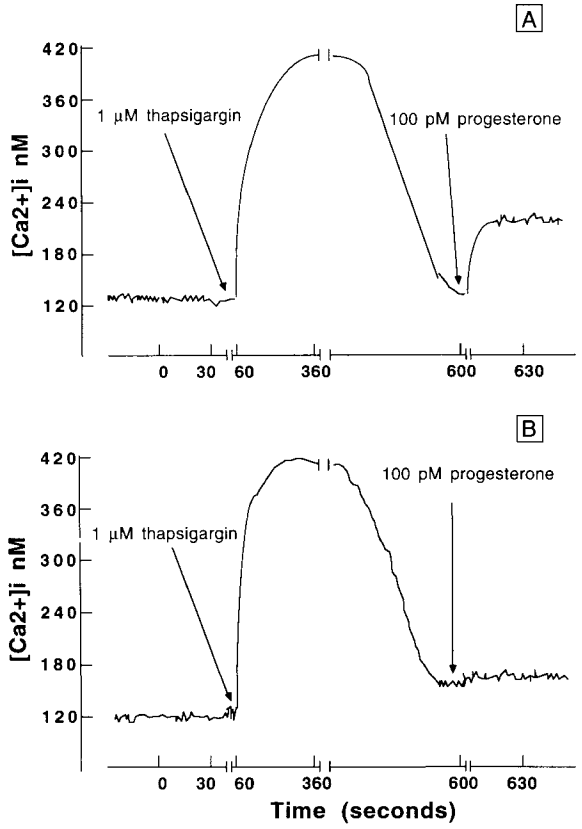

Fig. 3. Effects of thapsigargin on the $[Ca^{2+}]_i$ response to progesterone in nonluteinized (A) and luteinized (B) granulosa cells. Cells were incubated with 1 μM thapsigargin for 10 min before adding 100 pM progesterone. These experiments are representative of at least 10 cultures (three coverslips for each of the 10 cultures).

sis toxin (PTX). Fura-2/AM loading and $[Ca^{2+}]_i$ measurements were carried out with 100 ng/ml PTX. Preincubation with PTX did not alter the $[Ca^{2+}]_i$ response of either cell type to progesterone (Table III).

Effects of RU 38486 on the Intracellular Calcium Response to Progesterone

Nonluteinized and luteinized granulosa cells were incubated for 4 h with 1 μM RU 38486; 100 pM progesterone was then added. Preincubation with RU 38486 did not alter the basal $[Ca^{2+}]_i$, and did not inhibit the effects of progesterone on intracellular calcium of either cell type (Table III).

Effects of Progesterone on Inositol-1,4,5-Trisphosphate Formation

The basal concentration of inositol-1,4,5-trisphosphate, measured by radiocompetition in ethanol controls, was 4.6 ± 0.4 pmol/mg protein (n = 5) [Bradford, 1976]. Preincubation of the cells with pertussis toxin did not modify the basal level of $InsP_3$ (data not shown).

Figure 4 shows the response profile of $InsP_3$ to 100 pM progesterone in luteinized cells. Progesterone increased $InsP_3$ formation within 5 s. Identical results were observed in nonluteinized cells (data not shown). Preincubation of the cells with 100 ng/ml pertussis toxin did not abolish the increasing effect of progesterone on $InsP_3$ (data not shown).

DISCUSSION

This is, to our knowledge, the first report that progesterone increases the formation of inositol-1,4,5-trisphosphate and the intracellular calcium concentration via calcium mobilization from the endoplasmic reticulum in granulosa cells. These effects occur within 5 s and for progesterone concentrations as low as 1 pM. Moreover, the increase in $[Ca^{2+}]_i$ associates two mechanisms. One involves the mobilization of calcium from the endoplamic reticulum, and is common to both luteinized and nonluteinized cells. The second involves the influx of calcium from the extracellular milieu, and is specific to nonluteinized cells.

We have also obtained evidence that the initial progesterone-induced increase in the concentration of cytosolic free calcium is due to the release of Ca^{2+} from the endoplasmic reticulum. This is mediated by inositol-1,4,5-trisphosphate, the putative Ca^{2+}-mobilizing signal generated by the hydrolysis of phosphatidylinositol-4,5-bisphosphate [Michell, 1975; Joseph and Williamson, 1989; Berridge and Irvine, 1989]. There are three lines of evidence for this. First, when the extracellular Ca^{2+} was reduced to very low levels (below 100 μM) using the Ca^{2+} chelator EGTA, the ability of progesterone to increase $[Ca^{2+}]_i$ was not affected, or only partially blocked, depending on the cell type. Second, thapsigargin (which alters calcium sequestration by the endoplasmic reticulum) and phospholipase C inhibitors (indirect, like neomycin, or direct, like U-73122) partially or totally blocked the transient peak in both cell types without any effect on the sustained plateau phase in nonluteinized cells. Third, progesterone is able to increase the formation of $InsP_3$, which is responsible for calcium release from the endoplasmic reticulum [Berridge and Irvine, 1989].

TABLE II. Effects of Different Substances on Intracellular Calcium Response to Progesterone†

	$[Ca^{2+}]_i$ nM			
	Basal level (P = 0 pM)		Stimulated level (P = 100 pM)	
	Nonluteinized cells	Luteinized cells	Nonluteinized cells	Luteinized cells
Neomycin				
0 mM	107 ± 7	111 ± 5	389 ± 7*	405 ± 6*
1 mM	99 ± 6	101 ± 7	148 ± 3*,**	104 ± 5**
U-73122				
0 mM	112 ± 4	110 ± 3	395 ± 6*	400 ± 4*
5 μM	139 ± 3*	143 ± 2	179 ± 3*,**	142 ± 3**
Staurosporine				
0 mM	112 ± 3	107 ± 8	397 ± 3*	423 ± 9*
1 nM	128 ± 9	111 ± 6	542 ± 3*,**	545 ± 5*,**
Sp-cAMPS				
0 mM	113 ± 2	114 ± 5	399 ± 6*	418 ± 5*
0.1 mM	118 ± 4	109 ± 7	236 ± 4*,**	229 ± 4*,**

†Granulosa cells were incubated with either neomycin for 5 min, U-73122 for 5 min, staurosporine or Sp-CAMPS for 1 min before the addition of 100 pM progesterone (P). Data are the intracellular calcium concentrations 10 s after the steroid addition. Values are the means ± SE (n = 6; three coverslips in six different cultures) and significantly different from their respective controls (*$P < 0.001$) and from progesterone-treated cells (**$P < 0.001$).

TABLE III. Effects of RU-38486 and Pertussis Toxin on Intracellular Calcium Response to Progesterone†

	$[Ca^{2+}]_i$ nM			
	Basal level (P = 0 mM)		Stimulated level (P = 100 pM)	
	Nonluteinized cells	Luteinized cells	Nonluteinized cells	Luteinized cells
RU-38486				
0 μM	103 ± 7	101 ± 5	389 ± 5*	402 ± 4*
1 μM	109 ± 6	111 ± 7	388 ± 3*	404 ± 5*
Pertussis toxin				
0 ng/ml	106 ± 4	105 ± 5	403 ± 6*	399 ± 5*
100 ng/ml	109 ± 6	111 ± 7	388 ± 7*	410 ± 9*

†Granulosa cells were incubated with either RU-38486 for 4 h or pertussis toxin for 24 h before the addition of 100 pM progesterone (P). Data are the intracellular calcium concentrations 10 s after the steroid addition. Values are the means ± SE (n = 6; three coverslips in six different cultures) and significantly different from their respective controls (*$P < 0.001$).

Since it is now clear that a G-protein is essential for phospholipase C activation leading to the formation of $InsP_3$, and diacylglycerol (DAG) [Lo and Hughes, 1987], the next step was to identify the putative G-protein involved. Several types of mammalian G-proteins have been identified and have been divided traditionally into two types based on their sensitivity to the toxin derived from *Bordella* pertussis [Simon et al., 1991]. The PTX-sensitive G-proteins are inactivated by ADP-ribosylation on the α-subunit and include members of G_i and G_o family. The PTX-insensitive G-proteins are resistant to ADP-ribosylation. The G_q class of G-proteins is PTX-insensitive and generally activates PLC-β isoforms [Bristol and Rhee, 1994]. In our experimental system, preincubation of the pGC with PTX does not abolish the progesterone effect on $[Ca^{2+}]_i$ and $InsP_3$ formation. This result suggests that the G-protein involved in the rapid effects of progesterone belongs to the Gq family and activates a PLC-β isoenzyme.

Progesterone immobilized by covalent linkage to bovine serum albumin also increases $[Ca^{2+}]_i$ via a calcium influx and/or calcium mobilization, depending on the cell type. However, the presence of a carboxymethyl oxime group on the carbon 3 of progesterone reduces its capacity to increase $[Ca^{2+}]_i$. Since P-CMO and P-CMO BSA are equipotent, the presence of BSA esterified to

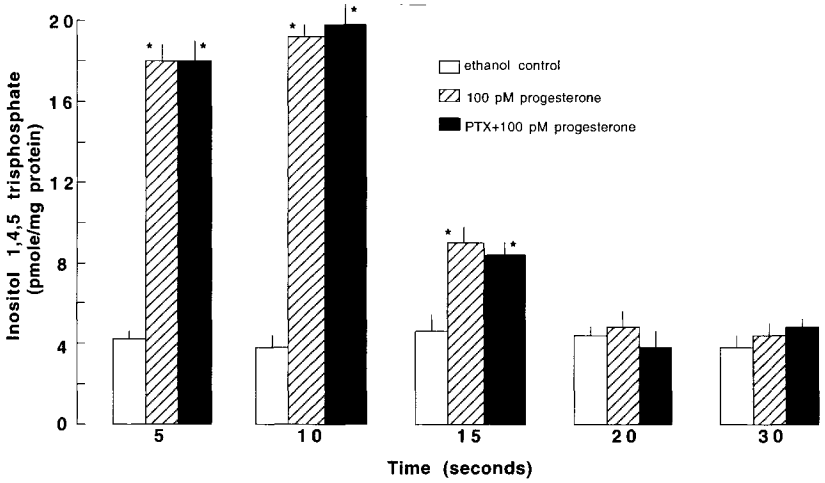

Fig. 4. Time course of changes in inositol-1,4,5-trisphosphate formation induced by 100 pM progesterone in luteinized cells pretreated or not with pertussis toxin. Cells were incubated for 24 h with 100 ng/ml pertussis toxin. Data are the mean ± SE (n = 5). Values significantly different from ethanol controls are indicated as follows: *, $P < 0.001$.

the CMO linker has no influence on the activity of P-CMO. Both P-CMO and P-CMO BSA have the same action time courses as the steroid alone, but lower potencies, showing that only the CMO group is responsible for the reduced potency. The increase in $[Ca^{2+}]_i$ is not due to steroid or steroid-CMO liberated during the incubation, since P-CMO BSA, P-CMO and progesterone have identical time courses of action. These results suggest that the progesterone receptor responsible for mediating Ca^{2+} mobilization from the endoplasmic reticulum resides on the outer surface of the granulosa cells. Further evidence in favor of a specific membrane progesterone receptor is that neomycin and U-73122, two inhibitors of phospholipase C, block the rapid responses induced by progesterone, and RU-38486, which competes with progesterone in the nucleus in these cells, does not inhibit the rapid responses to progesterone. This suggests that the membrane receptor may be different from the classic nuclear receptor. Further research is needed to assess the membrane association of progesterone and the role of the pertussis toxin-insensitive GTP-binding protein involved.

The action of progesterone on intracellular calcium is modulated by protein kinase C (PKC). Pretreatment of the cells with staurosporine, a PKC inhibitor, induced a greater $[Ca^{2+}]_i$ response to progesterone. As well as generating inositol-1,4,5-trisphosphate, the hydrolysis of phosphatidylinositol-4,5-bisphosphate increases intracellular DAG, which may activate PKC. A characteristic feature of the inositol lipid receptor mechanism is that the DAG/PKC pathway feeds back onto the transduction process to inhibit further hydrolysis of phosphatidylinositol-4,5-bisphosphate [Nishizuka, 1984; Swann et al., 1989]. Our results suggest that PKC exerts a negative feedback on the $[Ca^{2+}]_i$ response to progesterone.

Protein kinase A also modulates the $[Ca^{2+}]_i$ response to progesterone. Inositol-1,4,5-trisphosphate releases calcium from intracellular stores by binding to specific receptors that are coupled to calcium channels. The 260-kDa protein, known to be the inositol trisphosphate receptor, is the major substrate for cAMP-dependent kinase [Mignery et al., 1990]. Phosphorylation of the inositol trisphosphate receptor has no effect on ligand binding but prevents the ligand-induced opening of calcium channels intrinsic to the receptor [Supattapone et al., 1988]. Stimulating protein kinase A activity with Sp-cAMPS results in a decreased $[Ca^{2+}]_i$ response to progesterone, probably through the phosphorylation of the inositol trisphosphate receptor.

Progesterone stimulates the Ca^{2+} influx into nonluteinized cells in a way comparable to that found in human sperm (Blackmore, 1993). Although lowering the extracellular calcium by EGTA partially blocks the $[Ca^{2+}]_i$ response to progesterone, antagonist of voltage-dependent Ca^{2+} channels, verapamil, has no effect. This is much like the pattern of Ca^{2+} response to gonadotropin-releasing hormone (GnRH) in granulosa cells [Davis et al., 1986] in which GnRH

induces a rapid increase in $[Ca^{2+}]_i$ that is not altered by dihydropyridines, in contrast to the responses of gonadotrophs and GnRH neurons [Marchetti et al., 1990; Chang et al., 1989]. Treatment with high K^+ buffer (data not shown) also has no effect, corroborating that voltage-sensitive calcium channels (VSCC) are not expressed by the granulosa cells [Wang et al., 1992]. This result suggests that the Ca^{2+} channel involved is not one of the voltage-dependent types but is more likely to be similar to the poorly characterized receptor-operated calcium channel [Blackmore, 1993; Foresta et al., 1993].

Finally, the sources of Ca^{2+} for progesterone-induced increase in $[Ca^{2+}]_i$ depends on the stage of differentiation. Progesterone acts on the granulosa cell surface at two distinct levels in nonluteinized cells: opening of plasma membrane Ca^{2+} channel and activation of a complex membrane associated with a G-protein. In luteinized cells, only the second process is involved. As a consequence, the ability of progesterone to trigger the release of Ca^{2+} from internal stores of both nonluteinized and luteinized granulosa cells via inositol-1,4,5-trisphosphate formation is a new feature of progesterone action, which implies that the current model of progesterone, linked to luteinization and acquisition of nuclear receptors by these cells, may require some revisions. It is also pertinent to note that the action of progesterone on calcium signaling depends on the stage of differentiation of the granulosa cells as do the action of gonadotropic hormones. Calcium signaling induced by FSH involves both calcium influx and calcium mobilization from intracellular stores in nonluteinized cells [Flores et al., 1990], while calcium mobilization from intracellular stores is the only route involved in the response of luteinized cells to LH/hCG [Carnegie and Tsang, 1984; Veldhuis et al., 1983].

In conclusion, the membrane effects of progesterone initiate a series of steps leading to a cascade of intracellular responses. The first step is a G-protein-mediated activation of PLC, leading to the formation of $InsP_3$ and DAG. These initial changes determine the second step; $InsP_3$ increases $[Ca^{2+}]_i$, and Ca^{2+} and DAG activate PKC. Subsequently, PKC serves as an interacting signal in the control of Ca^{2+} signaling as does PKA. Such feedback mechanisms provide additional degree of complexity in signaling that is important in the amplification, maintenance and termination of specific aspects of the activation pathways.

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