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Phospholipase C-β and Ovarian Sex Steroids in Pig Granulosa Cells

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Abstract We compared the membrane effects of estradiol, progesterone, and androstenedione in a single experimental model, the ovarian granulosa cells collected from immature Large White sows. We measured changes in cytosolic free calcium concentration ($[Ca^{2+}]i$) in confluent Fura-2 loaded cells. We used pharmacological tools and polyclonal phospholipase C-β (PLC-β) antibodies. Each steroid (0.1 pM to 1 nM) transiently increased intracellular calcium concentration ($[Ca^{2+}]i$) within 5 sec. They mobilized Ca^{2+} from the endoplasmic reticulum as shown by using two phospholipase C inhibitors, neomycin and U-73122. Ca^{2+} mobilization involved PLC-β1 for progesterone, PLC-β2 for estradiol and PLC-β4 for androstenedione. A pertussis toxin-insensitive G protein was involved in the effects of progesterone on Ca^{2+} mobilization whereas estradiol and androstenedione effects were mediated via a pertussis toxin-sensitive G-protein. Ca^{2+} influx from the extracellular milieu was involved in the increase in $[Ca^{2+}]i$ induced by progesterone and estradiol, but not by androstenedione. Influx of Ca^{2+} was independent of Ca^{2+} mobilization from calcium stores, and it was suggested that L-type Ca^{2+} channels for estradiol and T-type Ca^{2+} channels for progesterone were involved. The three steroids had no effect on cAMP. Rapid effects of progesterone, estradiol, and androstenedione involved a direct action on cell membrane elements such as PLC-β, G-proteins, and calcium channels, and these mechanisms were hormone-specific. J. Cell. Biochem. 74:50–60, 1999.

Key words: ovarian steroids; granulosa; phospholipase C; intracellular calcium

Sex steroids including progestins, estrogens, and androgens are involved in various ways and play multiple roles in the physiological regulation of ovarian function in mammals. Locally produced steroid hormones have major autocrine and paracrine effects [Gougeon, 1996]. However, the mechanisms by which steroids affect target cells are not fully understood. Steroid activity is thought to involve the binding of the hormone to specific intracellular receptors leading to interactions of transcription factors with hormone-responsive elements in the promoter region of steroid hormone-regulated genes, inducing transcription. Classical receptors for progesterone, estradiol and androstenedione have been identified in granulosa cells [Hild-Petito et al., 1988]. Hence, there is considerable evidence for rapid effects of steroids on membranes in various cell types [reviewed in Wehling, 1997 and Revelli et al., 1998]. These rapid responses, which last only seconds or minutes are not consistent with the classical nuclear mechanism of steroid receptor action. They suggest instead activity triggered by a signal-generating receptor on the cell surface [reviewed in Revelli et al., 1997; Rae et al., 1998; Luconi et al., 1998]. These nongenomic effects involve the activation of various signal transduction pathways. They may involve second messengers as Ca^{2+} [Morley et al., 1992; Lieberherr et al., 1993; Tesarik and Mendoza, 1993; Lieberherr and Grosse, 1994; Gorczynska and Handelsman, 1995; Machelon et al., 1996, 1998; Moss et al., 1997], cyclic AMP [Maller et al., 1979], inositol trisphosphate [Lieberherr et al., 1993; Lieberherr and Grosse, 1994; Machelon et al., 1996], diacylglycerol [Lieberherr et al., 1993; Lieberherr and Grosse, 1994; Murase et al., 1996], or activation of enzymes such as phospholipase C [Le Mellay et al., 1997] or D [Murase et al., 1996], protein kinase C [O'Toole et al., 1996], or tyrosine kinase [Meizel and Turner, 1996; Tesarik et al., 1996]. All these

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mechanisms vary according to the cell type and seem to be tissue- and gender-specific. We used a single experimental model, the pig ovarian granulosa cells, to investigate the mechanisms underlying the nongenomic action of three ovarian sex steroids, progesterone, estradiol, and androstenedione. We focused on the characterization of the phosphoinositide-specific phospholipase C (PLC) involved in the mobilization of calcium from the endoplasmic reticulum induced by progesterone, estradiol, and androstenedione.

MATERIALS AND METHODS

Chemicals

Steroids and all chemical reagents were purchased from Sigma (St. Louis, MO). Dulbecco's Modified Eagle medium (DMEM), DMEM/F12 (1:1) medium without phenol red, fetal calf serum (FCS), and trypsin/EDTA were obtained from Gibco BRL (Eragny, France). The ECL kit and Fura-2/AM were supplied by Amersham (Les Ulis, France). Polyclonal antibodies against PLC-β1, PLC-β2, PLC-β3 and PLC-β4 were obtained from Santa Cruz Biotechnology, Inc. (Tebu, Le Perray-en-Yvelines, France). Peroxidase-conjugated goat anti-rabbit IgG was obtained from Bio-Rad (Ivry sur Seine, 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 supplied by Biomol Research Laboratory (Plymouth, MA) and Tebu (Le Perray-en-Yvelines, France).

Cell Isolation and Culture

Pig granulosa cells were collected from the ovaries of immature Large White sows (5–6 months of age) obtained from a local slaughter house. Cells were treated as previously described [Machelon et al., 1996]. Cells were removed from follicles, used to seed in DMEM/F12 containing 10% FCS and cultured at 37°C until confluent. They were transferred to trypsin-EDTA and cultured on glass coverslips or in Petri dishes in DMEM/F12 containing 2% FCS until confluent. The cells were transferred to serum-free medium 24 h before use.

Calcium Determination and Experimental Protocols

The cells were washed with Hank's HEPES, pH 7.4 (137 mM NaCl, 5.6 mM KCl, 0.441 mM KH_2PO_4, 0.885 mM $MgSO_4$ $7H_2O$, 27.7 mM glucose, 1.25 mM $CaCl_2$, and 25 mM HEPES), and incubated with 1 µM Fura-2/AM for 30 min in the same buffer at room temperature. The glass coverslip supporting the cells was inserted into a cuvette containing 2.5 ml Hank's HEPES, pH 7.4 [Lieberherr et al., 1993]. The cuvette was placed in a thermostatically controlled (37°C) Hitachi F-2000 spectrofluometer. Drugs and reagents were added directly to the cuvette with continuous stirring. The Fura-2 fluorescence $[Ca^{2+}]i$ response was calibrated from the ratio of fluorescence at 340/380 nm after subtraction of the background fluorescence of the cells at 340 and 380 nm as described by Grynkiewicz et al. [1985]. A dissociation constant of 224 nM was used for the Fura-2-Ca^{2+} complex [Grynkiewicz et al., 1985]. Rmax and Rmin were calculated from determinations with 25 µM digitonin, 4 mM EGTA, and enough Tris base to bring the pH to at least 8.3. Each determination with Fura-2 loaded cells was followed by a parallel determination under the same conditions with nonloaded control cells.

We first studied the direct effects of 1 pM to 0.1 µM progesterone, estradiol, androstenedione, and testosterone. Steroids were first dissolved in ethanol; then the solutions were diluted to the required concentration with water. The final concentration of ethanol was less than 0.01%. This ethanol concentration had no effect on intracellular calcium concentration. We then investigated whether the effects of steroids on $[Ca^{2+}]i$ were due to an influx of Ca^{2+} from the extracellular medium. Two types of blocking experiments were performed. One involved adding a small excess of EGTA (2 mM), a chelator of extracellular calcium, to the medium in the cuvette and incubating for 30 sec before adding the steroid. The second type involved the addition of 1 µM nifedipine, a 1,4-dihydropyridine calcium channel antagonist which selectively blocks Ca^{2+} entry via voltage-dependent calcium channels, or 0.5 to 5 mM Ni^{2+} which blocks T-channels [Fox et al., 1987]. We investigated how much of the transient increase in $[Ca^{2+}]i$ was due to Ca^{2+} release from the endoplasmic reticulum [Berridge, 1993], using two drugs—neomycin, which inhibits PLC by binding to phosphoinositides [Prentki et al., 1986] and U-73122, a direct inhibitor of PLC involved in the hydrolysis of phosphatidylinositol 4,5 bisphosphate [Bleasdale et al., 1989]. We determined whether different PLC-β isozymes are

involved in the mobilization of Ca^{2+} induced by progesterone, estradiol, and androstenedione. Cells were incubated for 5 min with 10 μg/ml saponin in the presence of anti-PLC antibody or nonimmune rabbit serum, used at 10× the concentration used for western blotting. Cells were washed twice to remove saponin and were incubated with the anti-PLC antibody or nonimmune serum for 1 h at 37°C. 1 μM Fura-2/AM was added for the last 20 min of incubation. Progesterone, estradiol, and androstenedione were used at the concentration that gave the maximum increase in $[Ca^{2+}]i$ in confluent granulosa cells. We determined whether the G-protein involved in the activity of each steroid was sensitive to pertussis toxin (PTX) by incubating the cells with 100 ng/ml PTX for 16 h. Fura-2/AM loading and $[Ca^{2+}]i$ determinations were carried out with the toxin.

cAMP Determination

Cells were incubated for 10 min in medium containing 1% bovine serum albumin, 0.2 mM isobutyl methylxanthine (an inhibitor of phosphodiesterase) and the steroid to be tested or vehicle. Cells were rinsed twice with phosphate-buffered saline (PBS); and cAMP was extracted by sonication in the presence of 90% propanol. cAMP was determined by the protein binding assay of Lust et al. [1976] and expressed as pmoles per mg protein [Bradford, 1976].

Preparation of Homogenates

Cells were washed three times with ice-cold phosphate-buffered saline, pH 7.4. They were scraped into ice-cold extraction buffer (20 mM Tris-HCl, pH 7.5, 0.5 mM EGTA, 2 mM EDTA, 0.6 mM pepstatin, 0.5 mM benzamidine, 0.1 mM leupeptin, 2 mM phenylmethylsulphonyl fluoride, 0.125 mM aprotinin, and 1 mM dithiothreitol). Cells were sonicated on ice twice for 10 sec each at 40 KHz, and the homogenates were centrifuged for 10 min at 600*g* to remove nuclei. The resulting supernatant was collected and stored at −80°C. Protein was determined by the method of Bradford [1976].

Protein Separation and Immunoblotting

Proteins were separated by SDS-polyacrylamide gel electrophoresis (7.5% resolving gel) in 25 mM Tris-base, pH 8.3, 192 mM glycine, 0.1% SDS. They were electrophoretically transferred to nitrocellulose membranes (Immobilon P, Millipore, St. Quentin-en-Yvelines, France) in the same buffer with 20% ethanol for 2 h at 100 V [Le Mellay et al., 1997]. Nonspecific binding to nitrocellulose was prevented by incubating the membranes in 50 mM Tris-buffered saline (TBS), pH 7.5, containing 150 mM NaCl, 5% skimmed milk powder, and 0.05% Tween-20 for 12 h at 4°C. The membranes were washed in TBS containing 0.1% Tween-20 and incubated overnight at 4°C with polyclonal rabbit antibodies against specific isoenzymes of PLC (PLC-β1, PLC-β2, PLC-β3, and PLC-β4). The concentrations of PLC antibodies in TBS, 1.5% skimmed milk, 0.1% Tween-20 were as follows: 0.2 μg/ml for PLC-β1 and PLC-β2, 0.5 μg/ml for PLC-β3, and 1 μg/ml for PLC-β4. The antibodies bound to the proteins on the nitrocellulose were detected using peroxidase-conjugated goat anti-rabbit IgG (1 mg/ml) diluted ½, 000 in TBS, 1.5% skimmed milk, 0.1% Tween-20. The antigen was detected by enhanced chemiluminescence. We used molecular size standards to estimate the apparent molecular mass of the PLC: (from top to bottom of the gel), myosin, 205 kDa; β-galactosidase, 116 kDa; phosphorylase b, 97 kDa; fructose-6-phosphate-kinase, 84 kDa; albumin, 66 kDa; glutamic acid dehydrogenase 55 kDa. The specificity of the antibodies was checked before used by incubation at room temperature for 2 h with the corresponding peptide (antibody:peptide ratio, 1:100 for Santa Cruz Biotechnologies antibodies, as specified by the manufacturer).

Statistical Analysis

Data were analyzed by one-way analysis of variance. Treatment pairs were compared by Dunnett's method. n is the number of cultures used for a specific experiment.

RESULTS

Direct Effects of Steroids on Intracellular Calcium Concentration

A transient increase in $[Ca^{2+}]i$ was induced by 0.1 pM to 100 pM progesterone, estradiol, or androstenedione. There was a sharp peak in $[Ca^{2+}]i$ which fell rapidly after 15 sec, but remained higher than the basal level (plateau phase) with estradiol and progesterone whereas $[Ca^{2+}]i$ fell to resting levels for androstenedione (Fig. 1). Adding 1 pM to 10 nM testosterone had no effect (Fig. 1). The increase in $[Ca^{2+}]i$ was dose-dependent with maximum activity at 1 nM androstenedione, 100 pM 17-β estradiol, and 100 pM progesterone (Table I).

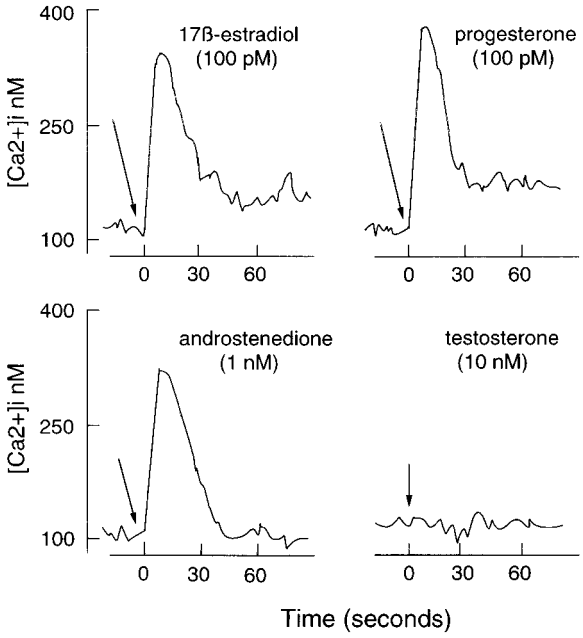

Fig. 1. Profile of calcium responses to estradiol, progesterone, androstenedione, and testosterone in confluent pig granulosa cells. Arrows indicate the time at which the steroid was added. These results are representative of at least six cultures.

TABLE I. Dose-Dependent Effects of the Steroids on Intracellular Calcium Concentration in Confluent Granulosa Cells[a]

	$[Ca^{2+}]$i nM		
Concentration	17β-estradiol	Progesterone	Androstenedione
0 pM	115 ± 10	118 ± 9	120 ± 11
1 pM	190 ± 9*	203 ± 8*	130 ± 5
10 pM	247 ± 11*	345 ± 10*	165 ± 6*
100 pM	327 ± 12*	395 ± 10*	209 ± 4*
1 nM	253 ± 9*	339 ± 11*	323 ± 17*
10 nM	214 ± 7*	241 ± 7*	205 ± 6*

[a]Intracellular Ca^{2+} concentrations were determined at 10 sec. Values are the means ± S.E.M., n = 6 for each steroid, and are significantly different from the basal level.
*$P < 0.001$.

Mechanisms Involved in the Steroid-Induced Changes in Intracellular Calcium Concentration

The steroid was added 30 sec after 2 mM EGTA. EGTA reduced the magnitude of the transient peak induced by 100 pM estradiol or progesterone, and the plateau phase was completely inhibited (Fig. 2A,B). In contrast, treatment with 2 mM EGTA did not inhibit the response to 1 nM androstenedione (Fig. 2C). Cells were then incubated for 1 min with 1 µM nifedipine, a Ca^{2+} entry inhibitor, or for 5 min with 0.5 to 5 mM Ni^{2+}, which mainly blocks T-type Ca^{2+} channels, before adding the steroid. Treatment with nifedipine partly inhibited the $[Ca^{2+}]$i response to 100 pM estradiol (Fig. 2A), but had no effect on the response to 100 pM progesterone or 1 nM androstenedione (data not shown). The $[Ca^{2+}]$i response to 100 pM progesterone was lower in cells treated with Ni^{2+}, this response being dose-dependent (Fig. 2B, data shown only for 5 mM Ni^{2+}). Ni^{2+} at any concentration did not block the response to androstenedione (Fig. 2C, data shown only for 5 mM Ni^{2+}).

Cells were incubated for 3 min with 2 mM neomycin, 2 µM U-73122, or 2 µM U-73343, an inactive analogue of U-73122, before adding the maximal effective concentration of steroid. Treatment with neomycin or U-73122 totally abolished the transient peak induced by 1 nM androstenedione, 100 pM estradiol, and progesterone, but the plateau phases induced by 100 pM estradiol and progesterone were unaffected (Table II). Two µM U-73343 had no effect (data not shown).

Western Immunoblotting of the PLCs

All Western blots were carried out with cell homogenates (35 µg of proteins). A 160-kDa band was detected by western blotting with the PLC-β1 antibody (Fig. 3). A 157-kDa band was detected on immunoblots probed with the PLC-β2 antibody (Fig. 3). A 154-kDa band was detected with the PLC-β3 antibody and a 163-kDa band with the PLC-β4 antibody (Fig. 3). A competitive Western blot with polyclonal PLC-β1, PLC-β2, PLC-β3, and PLC-β4 antibodies and the antigens against which they were raised showed that immunoreactivity was completely abolished when the antigen was used at 100× the concentration of the corresponding antibody (data not shown). This also shows that each antibody was reacting with its intended target isoform.

Characterization of the PLC-β Isoform Involved in the Intracellular Calcium Response to Steroids

Treatment of the cells with saponin for 5 min followed by incubation for 60 min with the anti-PLC antibody in the absence of saponin did not affect basal $[Ca^{2+}]$i. Nonimmune serum had no effect on basal $[Ca^{2+}]$i or on the $[Ca^{2+}]$i response to the steroids. The estradiol-induced increase in $[Ca^{2+}]$i was reduced by anti-PLC-β2

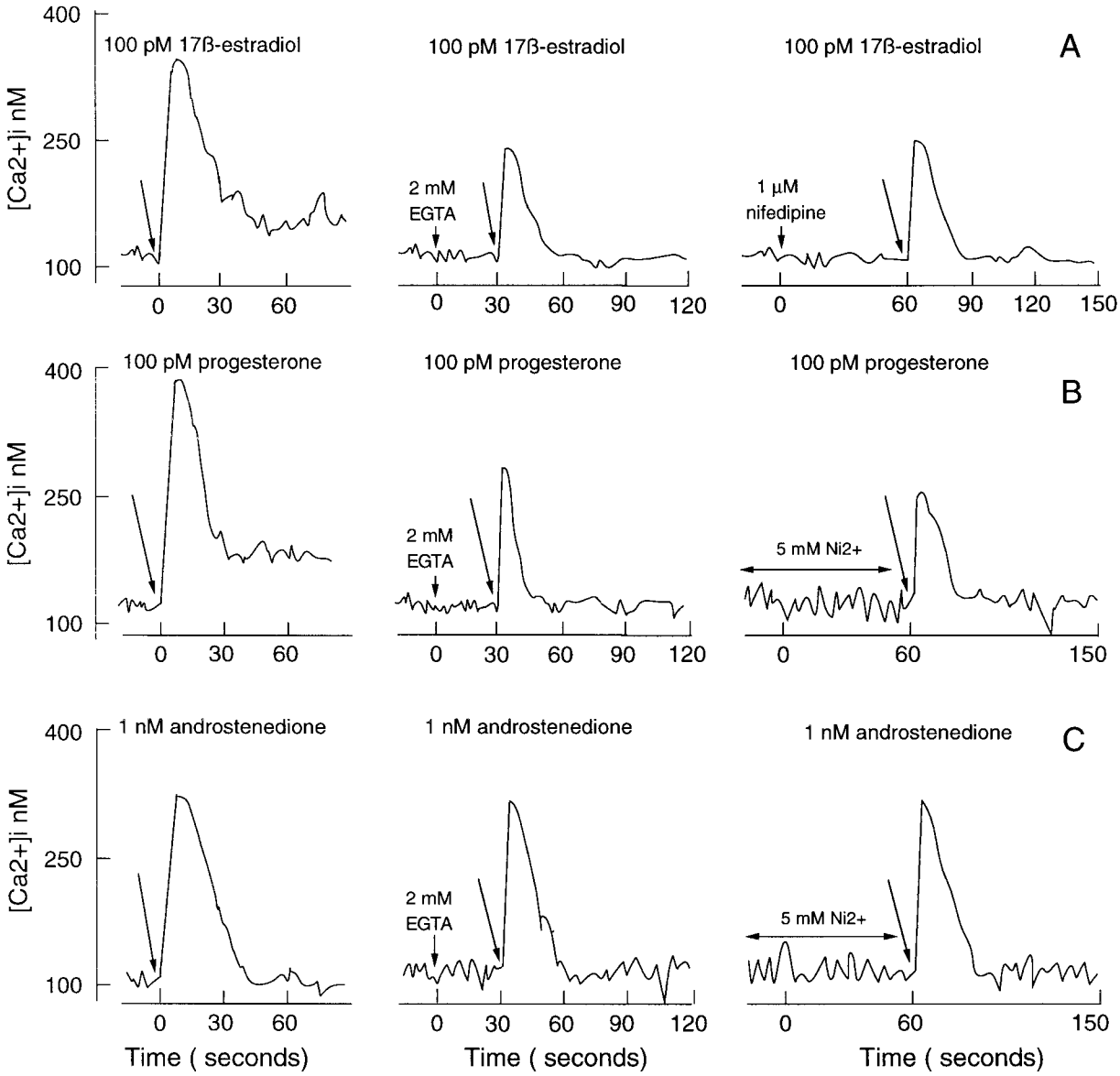

Fig. 2. Mechanisms involved in the intracellular calcium increase induced by steroids. Cells were incubated for 30 sec with 2 mM EGTA, for 1 min with 1 µM nifedipine, or for 5 min with 5 mM Ni^{2+} before adding 100 pM 17β-estradiol (**A**), 100 pM progesterone (**B**), or 1 nM andostenedione (**C**). Arrows indicate the time at which the steroid was added. These results are representative of at least six cultures.

antibody whereas the antibodies to PLC-β1, PLC-β3 and PLC-β4 had no effect (Fig. 4A and Table III). The residual increase was due to Ca^{2+} influx because it was blocked by incubating the cells for 30 sec with 2 mM EGTA before addition of the steroid (Fig. 4A). The progesterone-induced increase in $[Ca^{2+}]i$ was totally abolished by the anti-PLC-β1 antibody, but not by the anti-PLC-β2, anti-PLC-β3, and anti-PLC-β4 antibodies (Fig. 4B and Table III). Only anti-PLC-β4 antibody blocked the $[Ca^{2+}]i$ response to androstenedione (Fig. 4C and Table III).

Polyclonal anti-PLC-β1 and anti-PLC-β2 antibodies were incubated for 2 h with their corresponding antigens (antibody:antigen ratio, 1:100) before use. There was no inhibition of the estradiol-induced increase in $[Ca^{2+}]i$ by anti-PLC-β2 antibody if the antibody was first incubated with PLC-β2 antigen (data not shown). Alternatively, the progesterone-induced increase in $[Ca^{2+}]i$ was restored when the anti-PLC-β1 antibody was co-incubated with PLC-β1 antigen (data not shown). The $[Ca^{2+}]i$ response to androstenedione was unaffected if cells were incubated with

TABLE II. Effects of Different Substances on Intracellular Calcium Responses to Sex Steroids[a]

	$[Ca^{2+}]$i nM			
		Stimulated level		
Substances	Basal level	100 pM E2	100 pM P	1 nM A
Neomycin				
0 mM	117 ± 9	335 ± 10*	383 ± 12*	319 ± 7*
2 mM	105 ± 6	155 ± 6*,**	158 ± 9**,*	117 ± 9**
U-73-122				
0 mM	115 ± 10	341 ± 10*	391 ± 9*	331 ± 10*
2 μM	130 ± 4	165 ± 2**,*	176 ± 12[a]	120 ± 11**
Pertussis toxin				
0 ng/ml	116 ± 7	335 ± 10*	391 ± 12*	329 ± 12*
100 ng/ml	108 ± 5	155 ± 6**,*	378 ± 9*	117 ± 9**

[a]Granulosa cells were incubated with either neomycin and U-73122 for 5 min, or pertussis toxin for 16 h before adding 100 pM estradiol (E2), 100 pM progesterone (P), or 1 nM androstenedione (A). Intracellular calcium concentrations were determined at 10 sec after the addition of each steroid. Values are the means ± S.E.M., n = 6 for each steroid, and are significantly different from their respective controls (*$P < 0.001$) and from steroid-treated cells (**$P < 0.001$).

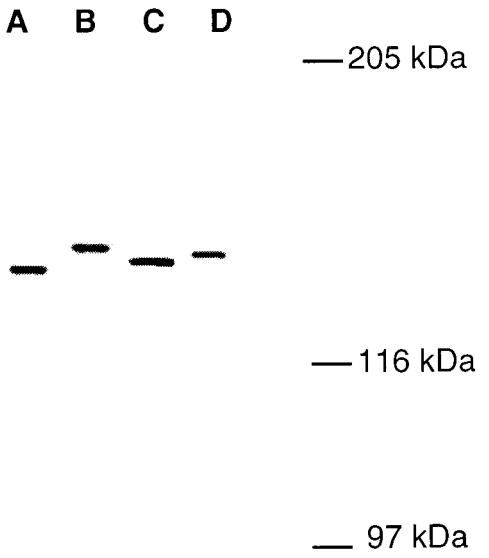

Fig. 3. Immunoblots of the PLC-β isoforms of pig granulosa cells. Thirty-five μg aliquots of homogenate were subjected to electrophoresis on SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and probed with antibodies against the various PLC-β isoforms. A 154 kDa band was detected for PLC-β3 (**A**), a 163 kDa band for PLC-β4 (**B**), a 157 kDa band for PLC-β2 (**C**), and a 160 kDa band for PLC-β1 (**D**). Lines indicate the standards: myosin, 205 kDa; β-galactosidase, 116 kDa; and phosphorylase b, 97 kDa. These results are representative of at least six cultures.

the anti-PLC-β4 antibody after it had been incubated with the corresponding antigen (Fig. 4C).

Effects of Pertussis Toxin on the Intracellular Calcium Response to Steroids

Granulosa cells were incubated for 16 h with 100 ng/ml PTX. Fura-2/AM loading and $[Ca^{2+}]$i determinations were carried out with 100 ng/ml PTX. PTX did not affect the basal $[Ca^{2+}]$i; PTX blocked the part of the $[Ca^{2+}]$i corresponding to mobilization of Ca^{2+} by estradiol and androstenedione (Table II), but had no effect on the plateau phase induced by estradiol (data not shown). PTX had no effect on both mechanisms involved in the increase of $[Ca^{2+}]$i induced by progesterone (Table II).

Effects of Steroids on cAMP Content

The cAMP content of granulosa cells was not affected by 1 pM–1 μM estradiol, progesterone, or androstenedione, whereas 1 μM forskolin increased cAMP content (Table IV).

Effects of Nuclear Antagonists on Intracellular Calcium Responses to Steroids

Granulosa cells were incubated for 4 h with 1 μM tamoxifen, a nuclear antagonist of estradiol, 1 μM flutamide, a nuclear antagonist of androstenedione, or 1 μM RU-486, a nuclear antagonist of progesterone. The basal level of $[Ca^{2+}]$i was not affected by any of these antagonists. Incubation of the cells with tamoxifen, flutamide, or RU-486 did not affect the $[Ca^{2+}]$i response to 17β-estradiol, androstenedione, or progesterone (Table V).

DISCUSSION

Using a single experimental model, the pig ovarian granulosa cells in culture, we have shown that the three ovarian sex steroids, progesterone, estradiol, and androstenedione, used specific transmembrane signaling pathways and different isoforms of PLC-β to mobilize calcium from the endoplasmic reticulum.

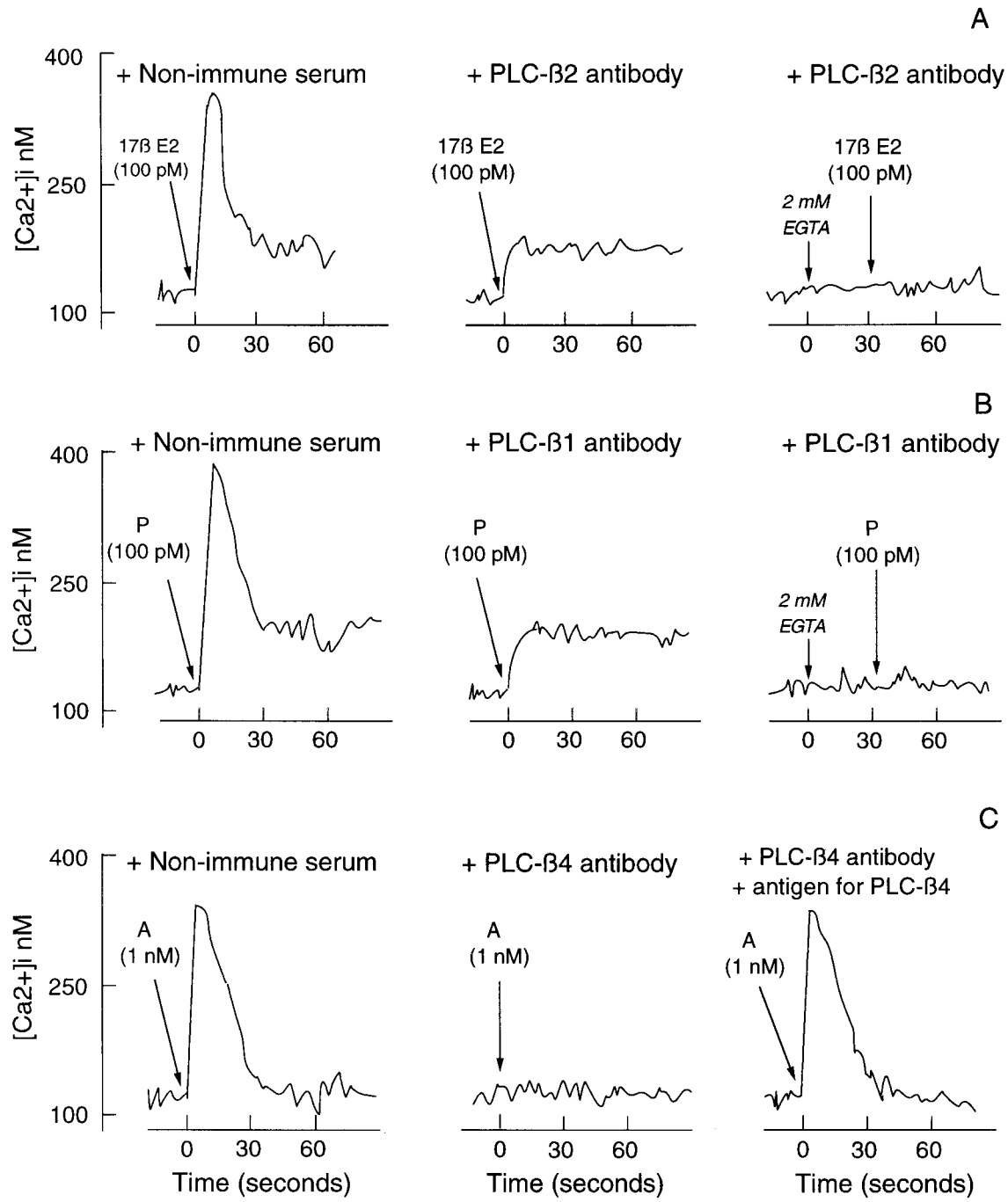

Fig. 4. Intracellular calcium responses after treatment of the granulosa cells with antibodies against the PLC-β isoforms. Cells were permeabilized with 10 µg/ml saponin to allow the penetration of the antibody or nonimmune serum, well washed to remove saponin, then incubated for 1 h in medium without saponin plus the antibody or nonimmune serum. Arrows indicate the time at which estradiol (**A**), progesterone (**B**), and androstenedione (**C**) were added. In some experiments, EGTA was added 30 sec before the steroid, and cells were incubated simultaneously with the anti-PLC-β4 antibody and the corresponding antigen before the experiment. These results are representative of at least six cultures.

We used cytosolic free calcium concentration as a biological parameter to reflect and compare the rapid responses to the three steroids. Each steroid induced an immediate (within 5 sec) and transient increase in $[Ca^{2+}]i$ in Fura-2 loaded cells. However, the profile of the response depended on the steroid tested. Estradiol and progesterone triggered a transient peak in $[Ca^{2+}]i$ followed by a sustained plateau phase whereas the increase induced by androstedione

TABLE III. Effects of Anti-PLCβ Antibodies on the Intracellular Calcium Responses to Estradiol, Progesterone, and Androstenedione[a]

	$[Ca^{2+}]i$ nM		
Substances	17β-estradiol (100 pM)	Progesterone (100 pM)	Androstenedione (1 nM)
Nonimmune serum	371 ± 11	393 ± 13	324 ± 8
anti-PLC β1 Ab (2 μg/ml)	365 ± 7	157 ± 6*	309 ± 9
anti-PLC β2 Ab (2 μg/ml)	145 ± 3*	378 ± 11	315 ± 11
anti-PLC β3 Ab (5 μg/ml)	355 ± 10	388 ± 12	321 ± 13
anti-PLC β4 Ab (10 μg/ml)	363 ± 12	369 ± 14	115 ± 9*

[a]Cells were permeabilized for 5 min with 10 μg/ml saponin to allow the penetration of the antibody (Ab) or nonimmune serum, well washed to remove saponin, then incubated for 1 h with the antibody or nonimmune serum. Intracellular calcium concentrations were determined 10 s after the addition of the steroid. Values are the means ± S.E.M., n = 4 for each steroid, and significantly different from the values obtained in the presence of nonimmune serum.
*$P < 0.001$. See also Figure 4.

TABLE IV. Effects of the Three Steroids and Forskoline on cAMP Content of Pig Granulosa Cells[a]

Treatment	cAMP (pmole/mg protein)
Ethanol (control)	27.9 ± 2.6
Forskoline (1 μM)	119.1 ± 11*
17β-oestradiol (100 pM)	25.9 ± 1.6
Progesterone (100 pM)	26.2 ± 2.1
Androstenedione (1 nM)	22.9 ± 0.5

[a]Values are the means ± S.E.M., n = 4 for each steroid, and significantly different from the control cells.
*$P < 0.001$.

involved a sharp peak followed by a rapid return to resting levels. These effects occurred at physiological concentrations and started at concentrations lower than those needed for the genomic effects [Lieberherr et al., 1993; Lieberherr and Grosse, 1994]. The response curve was bell-shaped with a maximum at 100 pM for estradiol and progesterone, and at 1 nM for androstenedione. The bell-shaped dose-dependent action on $[Ca^{2+}]i$ and $InsP_3$ formation is a general phenomenon observed for various steroids [Lieberherr et al., 1993; Lieberherr and Grosse, 1994; Machelon et al., 1996, Sömjen et al., 1997]. The nongenomic effects faint at concentrations which induce genomic effects, suggesting a chronology in the events of the steroid action. There was no response to testosterone (1 pM to 1 μM) confirming the gender-specificity of the calcium response to testosterone [Lieberherr and Grosse, 1994; Sömjen et al., 1997].

All three steroids caused the release of calcium from the endoplasmic reticulum via phosphoinositide breakdown, shown using inhibitors of PLC. PLC inhibitors (neomycin and U-73122) abolished the peak in $[Ca^{2+}]i$ induced by estradiol, progesterone, and androstenedione in pig granulosa cells. The mobilization of Ca^{2+} from the endoplasmic reticulum follows an increase in the formation of $InsP_3$ via the hydrolysis of phosphatidylinositol 4,5 bisphosphate [Berridge, 1993]. This reaction is catalysed by a phosphoinositide-specific PLC generally coupled to a G-protein. The PLC involved in this mechanism is thought to belong to the PLC-β family, which consists of four isoforms [Lee and Rhee, 1995]. We identified the PLC-β isoforms present in granulosa cells. These cells contain the PLC-β1, -β2, -β3, and -β4 isoforms as shown by Western immunoblotting. We further characterized the PLC-β isoform activated by each steroid, by incubating the cells with specific anti-PLC antibodies. Only PLC-β1, PLC-β2, and PLC-β4 were involved in the effects of progesterone (β1), estradiol (β2), and androstenedione (β4), on the mobilization of Ca^{2+} from intracellular Ca^{2+} stores. Anti-PLC-β1, anti-PLC-β2, and anti-PLC-β4 antibodies inhibited the steroid-induced increase in $[Ca^{2+}]i$ in a similar way to direct or indirect inhibitors of PLC (see Table II and Fig. 4). Anti-PLC antibodies, like PLC inhibitors, blocked only the part of the increase in $[Ca^{2+}]i$ that is due to Ca^{2+} mobilization from the endoplasmic reticulum. The inhibition of enzyme activity is totally abolished in competition experiments in which polyclonal PLC-β1, PLC-β2, and PLC-β4 antibodies were incubated with the antigens against which they were raised [Le Mellay et al., 1997].

PLC-β enzymes are generally regulated via heterotrimeric G-proteins in response to an agonist binding to a receptor [Lee and Rhee, 1995].

TABLE V. Effects of Nuclear Antagonists on Intracellular Calcium Response to 17β-Estradiol, Progesterone, and Androstenedione[a]

	$[Ca^{2+}]i$ nM			
		Stimulated level		
Antagonists	Basal level	17β-estradiol (100 pM)	Progesterone (100 pM)	Androstenedione (1 nM)
Tamoxifen				
0 mM	105 ± 10	341 ± 11*	—	—
1 μM	120 ± 4	365 ± 2*	—	—
Flutamide				
0 mM	109 ± 7	—	—	321 ± 11*
1 μM	121 ± 9	—	—	317 ± 9*
RU-486				
0 mM	108 ± 9	—	388 ± 7*	—
1 μM	119 ± 7	—	401 ± 15*	—

[a]Granulosa cells were incubated for 4 h with either tamoxifen, flutamide, or RU-486 before adding 100 pM estradiol, 100 pM progesterone, or 1 nM androstenedione. Intracellular calcium concentrations were determined at 10 sec after the addition of each steroid. Values are the means ± S.E.M., n = 6 for each steroid, and are significantly different from their respective controls.
*$P < 0.001$.

Heterotrimeric G-proteins are a large family of homologous proteins classified on the basis of the amino acid sequences of the α subunits [reviewed in Exton, 1997]. G-proteins have also been classified into two types based on their sensitivity to PTX. The PTX-sensitive G-proteins are inactivated by ADP-ribosylation of the α-subunit and include Gi and Go subfamily. PTX-insensitive G-proteins are resistant to ADP-ribosylation and include members of the Gq subfamily of which α subunits activate specific PLC isoenzymes [Smarcka et al., 1991]. Progesterone-mediated Ca^{2+} mobilization in pig granulosa cells involved PLC-β1 and was not affected by PTX as previously shown [Machelon et al., 1996]. This suggests that the mechanisms mediating transmembrane progesterone signaling involve a PTX-insensitive G-protein, probably Gq, coupled to phospholipase C-β1. Although estradiol and androstenedione activate different PLC-β isoforms, PLC-β2, and PLCβ-4 respectively, their effect on the mobilization of Ca^{2+} involved a PTX-sensitive G-protein. PTX-sensitive G-proteins, such as Gi/Go-proteins, may activate PLC-β isozymes via the Gβγ dimer only [Camps et al., 1992; Exton, 1997]. However, this cannot rule out the involvement of the G βγdimer arising from Gq [Lee and Rhee, 1995]. Thus, estradiol might activate PLC-β2 via a G-protein βγcomplex. The PLC-β4 isoenzyme is thought to be unaffected by the βγ complex, but is activated by Gα subunits of the Gq class [Lee et al., 1994]. Since no information is available on the sensitivity to PTX of the G-protein involved in PLC-β4 activation, further investigations are required to identify the G-subunits involved in androstenedione-induced PLC-β4 activation in pig granulosa cells.

Estradiol and progesterone triggered a transient peak in $[Ca^{2+}]i$ followed by a sustained plateau phase. The plateau phase, which corresponds to influx of Ca^{2+}, disappeared in calcium-free medium whereas the transient peak persisted with a smaller amplitude. PLC inhibitors and anti-PLC-β antibodies had no effect on the plateau phase induced by both steroids whereas they abolished the transient peak in $[Ca^{2+}]i$. As for progesterone, PTX had no effect on estradiol-induced calcium influx although the toxin inhibited estradiol-induced mobilization of Ca^{2+} from intracellular organelles. These data suggest that the influx of calcium induced by both steroids is independent of the activation of G-proteins. In other respects, the increase in $[Ca^{2+}]i$ induced by estradiol was inhibited by nifedipine, an L-type calcium channel blocker, suggesting that the $[Ca^{2+}]i$ increase is partly due to an influx of Ca^{2+} from the extracellular milieu via activation of voltage-gated channels in pig granulosa cells like in other cell types [Lieberherr et al., 1993; Sömjen et al., 1997]. The $[Ca^{2+}]i$ response to progesterone was not affected by nifedipine, but was reduced by Ni^{2+}, which blocks mostly T-type Ca^{2+} channels [Fox et al., 1987], but also Ca^{2+}-release-activated channels (CRAC) [Dolmetsh and Lewis, 1994]. These channels are

activated by depletion of intracellular stores in many cell types including granulosa cells [Squires et al., 1997], and are responsible for capacitative influx [Putney and Bird, 1993]. However, the response to progesterone was not altered by KCl [Machelon et al., 1996], although KCl induces cell depolarization and CRAC currents blocking [Dolmetsh and Lewis, 1994]. This suggests that the influx of Ca^{2+} induced by progesterone is not due to capacitative Ca^{2+} entry. T-type currents have been identified in pig granulosa cells [Kusaka et al, 1993], and L-type channels might be involved in the action of LH [Veldhuis and Klase, 1982]. Thus, progesterone probably activates a T-type Ca^{2+} channel whereas estradiol presumably activates a dihydropyridine-sensitive L-type Ca^{2+} channel. EGTA, nifedipine, and Ni^{2+} had no effect on the $[Ca^{2+}]i$ response to androstenedione, showing that the increase in $[Ca^{2+}]i$ is not due to an influx of Ca^{2+}. The nongenomic effects of the three steroids in pig granulosa cells involve Ca^{2+} ions as second messengers, but not cAMP. This rules out a possible activation of calcium channels via their phosphorylation by cAMP [Mooren and Kinne, 1998].

In conclusion, our data provide further evidence for specific membrane responses for ovarian steroid hormones. In this single experimental model, the nongenomic effects of progesterone, estradiol, and androstenedione involve different PLC-β isoenzymes, probably coupled to different G-proteins, and variant types of Ca^{2+} channels. Androstenedione only mobilizes Ca^{2+} from the endoplasmic reticulum, whereas the transient Ca^{2+} increase induced by estradiol and progesterone is due to both the release of Ca^{2+} from intracellular stores and the influx of Ca^{2+} from the extracellular milieu probably via the activation of different Ca^{2+} channels. The $[Ca^{2+}]i$ response to androstenedione involves the activation of voltage-gated calcium channels in human granulosa cells [Machelon et al., 1998], but not in pig granulosa cells, and the estradiol-induced increase in $[Ca^{2+}]i$ in chick granulosa is only due to the mobilization of Ca^{2+} from intracellular stores [Morley et al., 1992]. This suggests that the response to androstenedione or estradiol is species-specific. However, estradiol may use the same transmembrane signaling pathway in different cell types [Morley et al., 1992; Lieberherr et al., 1993]. Progesterone increases cAMP content in oocytes [Maller et al., 1979], but not in pig granulosa cells, suggesting that the response to progesterone is tissue-specific.

Finally, Ca^{2+} is the second messenger involved in most of the nongenomic activities of steroids [reviewed in Revelli et al., 1997], and may regulate several bifurcating pathways such as the protein kinase C, protein kinase A, and tyrosine kinase pathways. In other respects, all steroid nuclear receptors studied to date are phosphorylated on multiple sites (Zhang et al., 1997], and the tyrosine kinase pathway is involved in phosphorylation of the nuclear estrogen receptor [Arnold et al., 1997]. Thus, an individual steroid ligand might activate a membrane-bound receptor and, simultaneously or consecutively, modulate the classic nuclear receptor. The resultant overall effect on the cell may be the surimposition of the two receptor-mediated events, one with a rapid onset and the other with long term persistence. One of these events may affect or regulate the other, which one may condition or modulate the other. A better understanding of the nature and physiological role of the membrane receptors is therefore required to determine the mechanisms of steroid activity in normal and pathological conditions.

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