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Membrane Signaling and Progesterone in Female and Male Osteoblasts. II. Direct Involvement of Gαq/11 Coupled to PLC-β1 and PLC-β3

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Abstract   We have shown that progesterone (10 pM–10 nM) and progesterone covalently bound to bovine serum albumin (P-CMO BSA; 100 pM–1 μM) rapidly increased (within 5 s) the cytosolic free Ca^{2+} concentration and inositol 1,4,5 trisphosphate ($InsP_3$) formation in confluent female and male rat osteoblasts via a pertussis toxin-insensitive G-protein. The activation of G-proteins coupled to effectors such as phospholipase C (PLC) is an early event in the signal transduction pathway leading to $InsP_3$ formation. We used antibodies against the various PLC isoforms to show that only PLC-β1 and PLC-β3 were involved in the Ca^{2+} mobilization and $InsP_3$ formation induced by both progestins in female and male osteoblasts, whereas PLC-β2, PLC-γ1, andPLC-γ2 were not. We also used antibodies against the subunits of heterotrimeric G-proteins to show that the activation of PLC-β1 and PLC-β3 by both progestins involved the Gαq/11 subunit, which was insensitive to pertussis toxin, whereas Gαi, Gαs, and Gβγ subunits were not. The membrane effects were independent of the concentration of nuclear progesterone receptor, because the concentration of nuclear progesterone receptors was lower in male than in female osteoblasts. These data suggest that progesterone and P-CMO BSA, which does not enter the cell, directly activate G-protein leading to the very rapid formation of second messengers without involving the nuclear receptor. J. Cell. Biochem. 79:173–181, 2000.   © 2000 Wiley-Liss, Inc.

Key words: progesterone; phospholipase C; G-protein; calcium; osteoblast

Mammals have several types of G-proteins that are classified according to the amino acid sequences of the α subunits into subfamilies (Gq, Gs, Gi/Go, and G12). They are heterotrimers made up of α, β, and γ subunits, each of which has several similar isoforms. The β and γ subunits are tightly associated in a complex and linked to the GDP-bound α-subunit in an inactive state. When a membrane receptor is activated by its agonist, GTP-bound α-subunit dissociates from the receptor and βγ to regulate specific downstream effectors (Neer, 1995; Clapham and Neer, 1997; Exton, 1997; Hildebrandt, 1997; Rhee and Bae, 1997). The α-subunits of Gs and Gi can regulate adenylyl cyclase activity, whereas the α-subunits of the Gq group activate phospholipase C-β (PLC-β)

isoforms. The βγ subunits were thought to be an inactive complex for a long time, but experimental evidence now suggests that they activate several effectors [Kleuss et al., 1992; Conklin and Bourne, 1993; Clapham and Neer, 1997]. G-proteins are also divided classically into two types based on their sensitivity to *Bordetella pertussis* toxin (PTX). The PTX-sensitive G-proteins are inactivated by adenosine diphosphate (ADP)-ribosylation of the α subunit, and include members of the Gi and Go subfamilies. The PTX-insensitive G-proteins are resistant to ADP-ribosylation, and include members of the Gq subfamily.

Progesterone may activate membrane receptors coupled to G-proteins [Majewska, 1992; Petitti and Etgen, 1992; Valera et al., 1992; Grazzini et al., 1998]. Progesterone mobilizes calcium from the endoplasmic reticulum and increases the formation of inositol trisphosphate and diacylglycerol via a G-protein insensitive to pertussis toxin in osteoblasts, the bone forming cells, as well as in ovarian granulosa

cells [Lieberherr et al., 1999]. However, no information is available on the type of heterotrimeric G-protein directly involved in the nongenomic effects of progesterone. This study describes the G-protein and its effector that are directly involved in the membrane effect of progesterone and of progesterone covalently bound to bovine serum albumin (P-CMO BSA) in female and male osteoblasts.

MATERIALS AND METHODS
Materials

The enhanced chemiluminescence kit, L-3-phosphatidyl[2^{-}3H]inositol 4,5-bisphosphate, and Fura-2/AM were purchased from Amersham (Les Ulis, France). Polyclonal rabbit anti-progesterone receptor (A and B progesterone receptors) antibody, polyclonal rabbit anti-PLC antibodies, polyclonal rabbit anti–G-protein antibodies, and their blocking peptides were obtained from Santa Cruz Biotechnology, Inc. (Tebu, Le Perray-en-Yvelines, France). Peroxidase-conjugated goat anti-rabbit IgG was obtained from BioRad (Ivry-sur-Seine, France). Progesterone, progesterone-3-(O-carboxymethyl) oxime-bovine serum albumin (P-CMO BSA), and all other chemicals were from Sigma (St Quentin Fallavier, France). α Minimal essential medium (αMEM) and fetal calf serum were supplied by Gibco BRL (Life Technologies, Les Ulis, France).

Osteoblast Isolation and Culture

Osteoblasts were isolated from parietal bones of two-day-old female and male Wistar rats (Charles River, St Aubin les Elbeufs, France) by sequential enzymatic digestion [Wong and Cohn, 1974]. Cells were grown on rectangular glass coverslips or in Petri dishes for four days in phenol red-free αMEM supplemented with 10% heat-inactivated fetal calf serum (H-FCS). Cells were then incubated for 72 h in phenol red-free medium containing 1% H-FCS, and transferred to serum-free medium 24 h before use.

Phosphatidylinositol 4,5-Diphosphate Hydrolysis Assay

Cells were washed with ice-cold phosphate-buffered saline (PBS), pH 7.4, and scraped off 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 benzamidin, 0.1 mM leupeptin, 2 mM phenylmethylsulfonyl fluoride, 0.125 mM aprotinin, and 1 mM dithiothreitol). They were sonicated on ice (2×20 s) at 40 kHz, and the homogenate was centrifuged for 10 min at $600g$ to remove nuclei. The supernatant was centrifuged at $100,000g$ for 60 min, and the supernatant was saved. The resulting membrane pellet was suspended in extraction buffer. All the fractions (homogenate, cytosol, membrane, and nuclei) were stored at $-80°C$. Protein was assayed by the method of Bradford [1970].

Phospholipid vesicles were prepared according to Hofmann and Majerus [1982], and assays were done essentially as described by Wu et al. [1992]. Aliquots of membranes (10 μl, 10 μg protein) in buffer (50 mM HEPES, pH 7.0, 0.5 mM EGTA, 2 mM EDTA, 0.6 mM pepstatin, 0.5 mM benzamidine, 0.1 mM leupeptin, 2 mM phenylmethylsulfonyl fluoride, 0.125 mM aprotinin, and 1 mM dithiothreitol), were added to 40 μl assay buffer (50 mM HEPES, pH 7.0, 100 mM NaCl, 5 mM $MgCl_2$, 0.6 mM $CaCl_2$ and 2 mM EGTA) plus 10 μl PIP_2 (10,000–12,000 cpm [^{3}H]PIP_2), and incubated on ice for 10 min. For the antibody inhibition assay, membranes were incubated for 2 h with 10 μl antibody at the concentrations indicated, prior to adding the reaction mixture. For competition assays with the peptide control, 10 μl of the antibody was mixed with 2 μl of serially diluted control peptide before adding the membranes, and incubated for 2 h. The reaction was started by adding GTPγS followed by incubation at 37°C for 15 min. The reaction was stopped by adding 0.5 ml chloroform/methanol/HCl (40:20:0.5), mixing, and chilling on ice. Soluble inositol phosphates (indicating PIP_2 hydrolysis) were extracted with 150 μl chloroform and 200 μl 0.1 M HCl. Phases were separated by centrifugation, and 200 μl of the upper aqueous phase was taken for liquid scintillation counting.

Calcium Measurement and Experimental Protocol

The Ca^{2+} measurements were performed in a Hitachi F-2000 spectrofluorometer at a constant temperature of 37°C as previously described [Lieberherr et al., 1993]. We determined whether different PLC isoenzymes and various subunits of different G-proteins were involved in the effects of progesterone and P-CMO BSA on cytosolic-free Ca^{2+} concentration ([Ca^{2+}]i) by incubating osteoblasts for 5 min

with 20 μg/ml saponin in Hanks' HEPES, pH 7.4 (137 mM NaCl, 5.6 mM KCl, 0.441 mM KH_2PO_4, 0.442 mM Na_2HPO_4, 0.885 mM $MgSO_4.7H_2O$, 27.7 mM glucose, 1.25 mM $CaCl_2$ and 25 mM HEPES), plus excess anti-PLC antibody or anti–G-protein antibody. They were used at 1 μg/ml for PLC-β1, 5 μg/ml for PLC-β2, 1 μg/ml for PLC-β3, PLCγ1, and PLC-γ2 [Le Mellay et al., 1997], 2 μg/ml for Gαq/11, 5 μg/ml for Gαs and Gαi1->3, 3 μg/ml for Gβ, and 2,5 μg/ml for Gγ1 antibodies or nonimmune rabbit serum [Le Mellay et al., 1999]. Cells were washed thoroughly with Hanks' HEPES to remove saponin, and incubated with anti-PLC or anti-G-protein antibody or nonimmune rabbit serum for 1 h at 37°C [Le Mellay et al., 1997]. Fura-2/AM was then added for 30 min. In some experiments, anti-PLC antibody or anti–G-protein antibody was set up in competition with the antigen against which it was produced, or with the antigens for the other anti-PLC or anti–G-protein antibodies at room temperature (antibody:peptide 1:100, according to the specification of the manufacturer) prior to use. Progesterone and P-CMO BSA were used at 1 nM and 100 nM, respectively.

Statistical Analysis

The data were analyzed by one-way analysis of variance. Treatment pairs were compared by Dunnett's method. A value of n represents n different cultures for a specific experiment.

RESULTS

We used 1 nM progesterone and 100 nM P-CMO BSA, which are the maximal active concentrations [Lieberherr et al., 1999]. Progesterone was dissolved in ethanol; the final concentration of ethanol in culture medium or buffer never exceeded 0.01%. This ethanol concentration was without effect on the intracellular calcium concentration or on inositol trisphosphate formation. P-CMO BSA was treated with charcoal to remove any free progesterone or progesterone-CMO [Lieberherr et al., 1993].

PLC Isoforms and G-Proteins Involved in the Effects of Progestins on Phosphatidylinositol 4,5-Diphosphate Hydrolysis

We used a cell-free membrane system to test the activity of the antibodies against PLC and G-proteins on the effect of progesterone on PIP_2 hydrolysis. Osteoblast membranes containing endogenous PLC and G-subunits were mixed with phospholipid vesicles containing radioactive substrate ($[^3H]PIP_2$).

The dose-response effect of GTPγS on PIP_2 hydrolysis showed that GTPγS itself had no effect below 1 μM (Fig. 1A). The effects of GTPγS on inositol 1,4,5 triphosphate ($InsP_3$) formation in the presence of 1 nM progesterone were dose-dependent (Fig. 1A, data shown for female osteoblasts). GDPβS (100 μM) inhibited the increase caused by 10 μM GTPγS (data not shown).

Osteoblast membranes were incubated with antibodies prior to stimulation with 100 μM GTPγS, the maximal active concentration on PIP_2 hydrolysis. Anti–PLC-β1, anti–PLC-β2, and anti–PLC-β3 antibodies inhibited the PIP_2 hydrolysis induced by 100 μM GTPγS, whereas anti–PLC-γ1 and anti–PLC-γ2 did not (Fig. 1B, data shown for female cells). The $[^3H]InsP_3$ concentration produced when membranes were incubated with both the anti–PLC-β antibody and its blocking peptide was the same as that obtained with preimmune serum (Fig. 1B). Anti-Gαq/11, anti-Gβ, and anti-Gβ plus anti-Gγ1 antibodies inhibited the PIP_2 hydrolysis induced by 100 μM GTPγS, whereas anti-Gγ1, anti-Gαs, and anti-Gαi antibodies did not (Fig. 1B, data shown for female osteoblasts). The $[^3H]InsP_3$ concentration produced when membranes were incubated with both the anti-Gαq/11 antibody and the blocking peptide or the anti-Gβ antibody and the blocking peptide was the same as that obtained with preimmune serum (Fig. 1B).

Figure 1C shows the inhibition of progesterone-induced $InsP_3$ formation by anti–PLC-β1 and anti–PLC-β3 antibody in the presence of 100 μM GTPγS. Anti–PLC-β2 antibody had no effect (Fig. 1C) although it prevented PIP_2 hydrolysis induced by 100 μM GTPγS (Fig. 3B). Anti-Gαq/11 antibody, but not anti-Gαi, anti-Gαs, anti-Gβ, anti-Gβ plus anti-Gγ1, and anti-Gγ1 blocked the $InsP_3$ response to progesterone in the presence of 100 μM GTPγS (Fig. 1C, data shown for female cells).

Anti–PLC-β1 and anti–PLC-β3 antibodies and anti-Gαq/11 antibody blocked the PIP_2 hydrolys induced by 100 nM P-CMO BSA in the presence of 100 μM GTPγS in both types of osteoblasts and by 1 nM progesterone in male osteoblasts (data not shown).
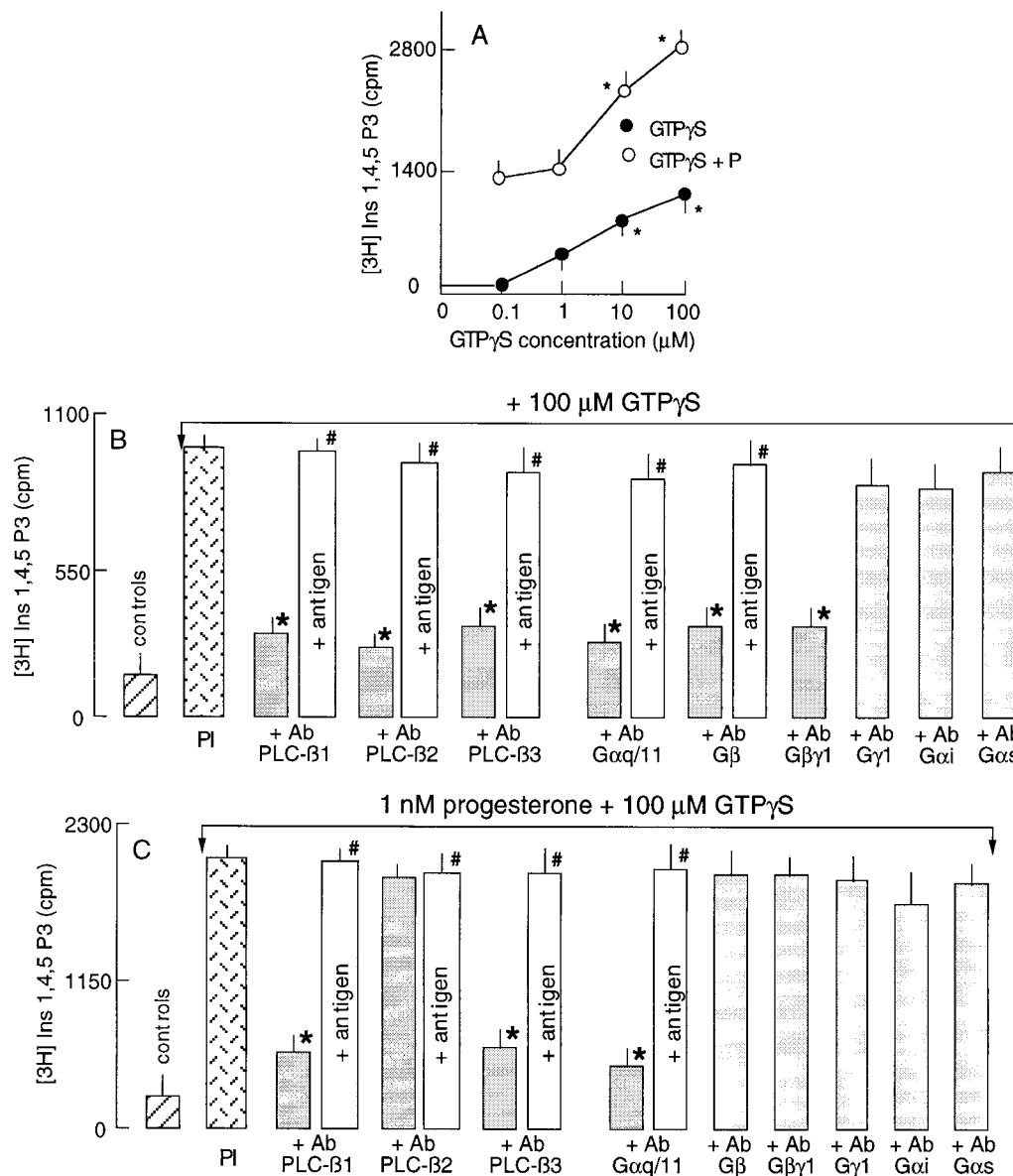
Fig. 1. Effects of anti-phospholipase C (PLC)-β antibodies and anti-G antibodies on PIP$_2$ hydrolysis induced by progesterone in membranes of female osteoblasts activated by GTPγS. **A:** Effects of GTPγS on the hydrolysis of PIP$_2$ in the presence or absence of 1 nM progesterone (P). **B:** Effects of anti-PLC antibodies and anti–G-protein antibodies (with or without their respective antigen) on GTPγS-induced PIP$_2$ hydrolysis. **C:** Effects of anti-PLC antibodies and anti–G-protein antibodies associated or not with their respective antigen on GTPγS-induced PIP$_2$ hydrolysis in the presence of 1 nM progesterone. Osteoblast membranes (5 μg) were incubated for 2 h with 1 μg/ml anti–PLC-β1 antibody, 5 μg/ml anti–PLC-β2 antibody, 1 μg/ml

anti–PLC-β3 antibody, 2 μg/ml anti-Gαq/11 antibody, 3 μg/ml anti-Gβ1->β4 antibody plus 2.5 μg/ml anti-Gγ1 antibody, 2.5 μg/ml anti-Gγ1 antibody, 5 μg/ml anti-Gαi1->3 antibody, 5 μg/ml anti-Gs antibody or preimmune serum (PI). PIP$_2$ hydrolysis was measured in the presence of 100 μM GTPγS. The PIP$_2$ hydrolysis in control incubations with GTPγS and without membranes was subtracted from all values. White columns show data obtained after incubating the antibody with its antigen prior to incubation with the membranes. Values are the mean ± SE, n = 4. significantly *$P < 0.001$ from the level in the presence of P or preimmune serum. #$P < 0.001$ from the level in the presence of antibody.

PLC Isoenzymes and G-Proteins Involved in the Effects of P-CMO BSA on Intracellular Calcium

The basal $[Ca^{2+}]$i was 116 ± 6 nM (mean $\pm$ SE, n = 6) in confluent female osteoblasts and 120 ± 5 nM (mean $\pm$ SE, n = 6) in male osteoblasts. Progesterone (1 nM) and P-CMO BSA (100 nM) caused a transient increase in $[Ca^{2+}]$i within 5 s (Fig. 2A, data shown for male osteoblasts). The sharp peak fell rapidly after 15 s, but remained higher than the basal level (plateau phase) ($25\% \pm 2\%$, mean $\pm$ SE, n = 6, $P < 0.001$; Fig. 1A). P-CMO BSA, which does not enter the cell, produced a smaller increase ($28\% \pm 5\%$, mean $\pm$ SE, n = 6, $P < 0.001$) in $[Ca^{2+}]$i, but the time course was similar to that of free progesterone.

Treatment of the cells with saponin for 5 min followed by incubation for 60 min with anti-PLC antibody or anti-G protein antibody in the absence of saponin did not affect the basal $[Ca^{2+}]$i. Nonimmune serum had no effect on the basal $[Ca^{2+}]$i or on the $[Ca^{2+}]$i response to free progesterone or P-CMO BSA.

The P-CMO BSA-induced increase in $[Ca^{2+}]$i was reduced by anti–PLC-β1 and anti–PLC-β3 antibodies, whereas antibody to PLC-β2 had no effect in either cell type (Fig. 2B, data shown only for male osteoblasts). The residual increase was due to Ca^{2+} influx, because it was totally blocked by incubating the cells for 30 s with 2 mM EGTA. Anti–PLC-γ1 and anti–PLC-γ2 antibodies had no effect (Fig. 2B). Anti–PLC-β4 antibody had no effect because rat osteoblasts lack this PLC-β isoform [Le Mellay et al., 1997]. Anti–PLC-β1 and anti–PLC-β3 antibodies were incubated for 2 h with their antigen or with the antigens used to produce the other anti-PLC antibodies (antibody: antigen 1:10 or 1:100) before use. The inhibition of the P-CMO BSA-induced increase in $[Ca^{2+}]$i due to anti–PLC-β1 and PLC-β3 antibodies totally disappeared only when the anti–PLC-β1 antibody or anti–PLC-β3 antibody was incubated with its antigen, but not with the antigens corresponding to the other anti-PLC antibodies (Fig. 2B, data shown only for the antigen corresponding to PLC-β1).

The P-CMO BSA-induced transient peak in $[Ca^{2+}]$i was blocked by anti-Gαq/11 antibody in both cell types (Fig. 3, data shown only for male osteoblasts). The residual increase was due to a Ca^{2+} influx, because this remaining increase was totally blocked by preincubating the cells for 30 s with 2 mM EGTA (Fig. 2). The anti-Gαi antibody (1 μg/ml), which cross-reacts with Gαi1, Gαi2, and Gαi3, and the anti-Gαs antibody had no effect (Fig. 3). The anti-Gβ antibody, which is broadly reactive with Gβ1, Gβ2, Gβ3, and Gβ4, and the anti-Gγ1 antibody, added simultaneously in the cell, had no effect (Fig. 3).

Similar results were observed when using 1 nM free progesterone in both cell types and 100 nM P-CMO BSA in female cells (data not shown).

DISCUSSION

We find that Gαq/11 coupled to phospholipases C-β1 and β3 is directly involved in the membrane signaling of free progesterone and its covalently bound derivative P-CMO BSA, which does not enter the cell, in female and male osteoblasts.

Isoenzymes of the PLC-β family are thought to be involved in the hydrolysis of phosphatidylinositol 4,5-diphosphate upon agonist activation [Rhee et al., 1991]. We identified the phospholipase C-β isoforms involved in Ca^{2+} mobilization and $InsP_3$ formation by progesterone and P-CMO BSA in female and male osteoblasts. The PLC-β family consists of four isoforms, β1, β2, β3, and β4, and we have previously shown that rat osteoblasts possess PLC-β1, β2, and β3, but not β4 [Le Mellay et al., 1997]. PLC-β1 and PLC-β3 are involved in Ca^{2+} mobilization and $InsP_3$ formation, as anti–PLC-β1 and anti–PLC-β3 antibodies inhibit the progestin-induced increase in $InsP_3$ formation and in $[Ca^{2+}]$i. Anti-PLC antibodies only block the increase in $[Ca^{2+}]$i that is due to Ca^{2+} mobilization from the endoplasmic reticulum. The inhibition of the enzyme activity by anti–PLC-β1 and anti–PLC-β3 antibodies is abolished in competition experiments, in which polyclonal PLC-β1 or PLC-β3 antibodies are incubated with their own antigens, but not when incubated with the antigen corresponding to the other PLC-β. This type of enzyme inhibition by selective antibodies suggests that the antibody directed against amino acids at the carboxy terminus of the PLC-β binds to a site on the enzyme that is critical for the correct geometry of the active site. PLC-β2 is not involved in the signal transduction of progesterone, although its antibody prevents PIP_2 hydrolysis in the presence of GTPγS. Similarly, PLC-γ1 and -γ2 take no part in progestin ef-
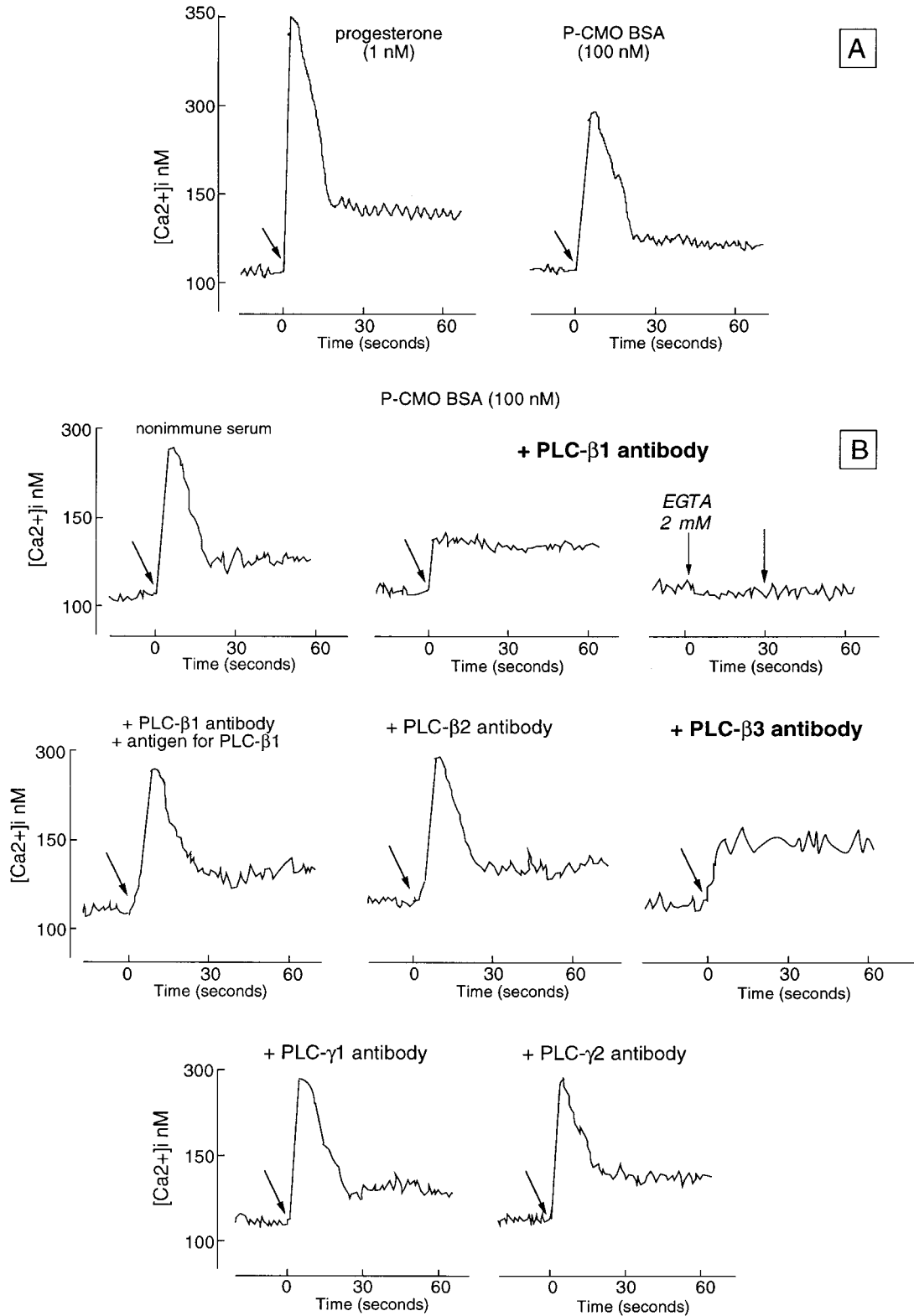
Fig. 2. Changes in the intracellular calcium in male osteoblasts treated with anti-phospholipase C (PLC) isoform antibodies in responses to progesterone covalently bound to bovine serum albumin (P-CMO BSA). Cells were cultured, loaded with Fura2/AM, and incubated with the anti-PLC antibodies. Arrows indicate the time at which 1 nM progesterone (**A**) and 100 nM P-CMO BSA (**A and B**) were added. In some experiments, P-CMO BSA was added 30 s after 2 mM EGTA, and cells were incubated with both the anti–PLC-β1 antibody and its own antigen before the experiment. These results are representative of six cultures for each experimental case.
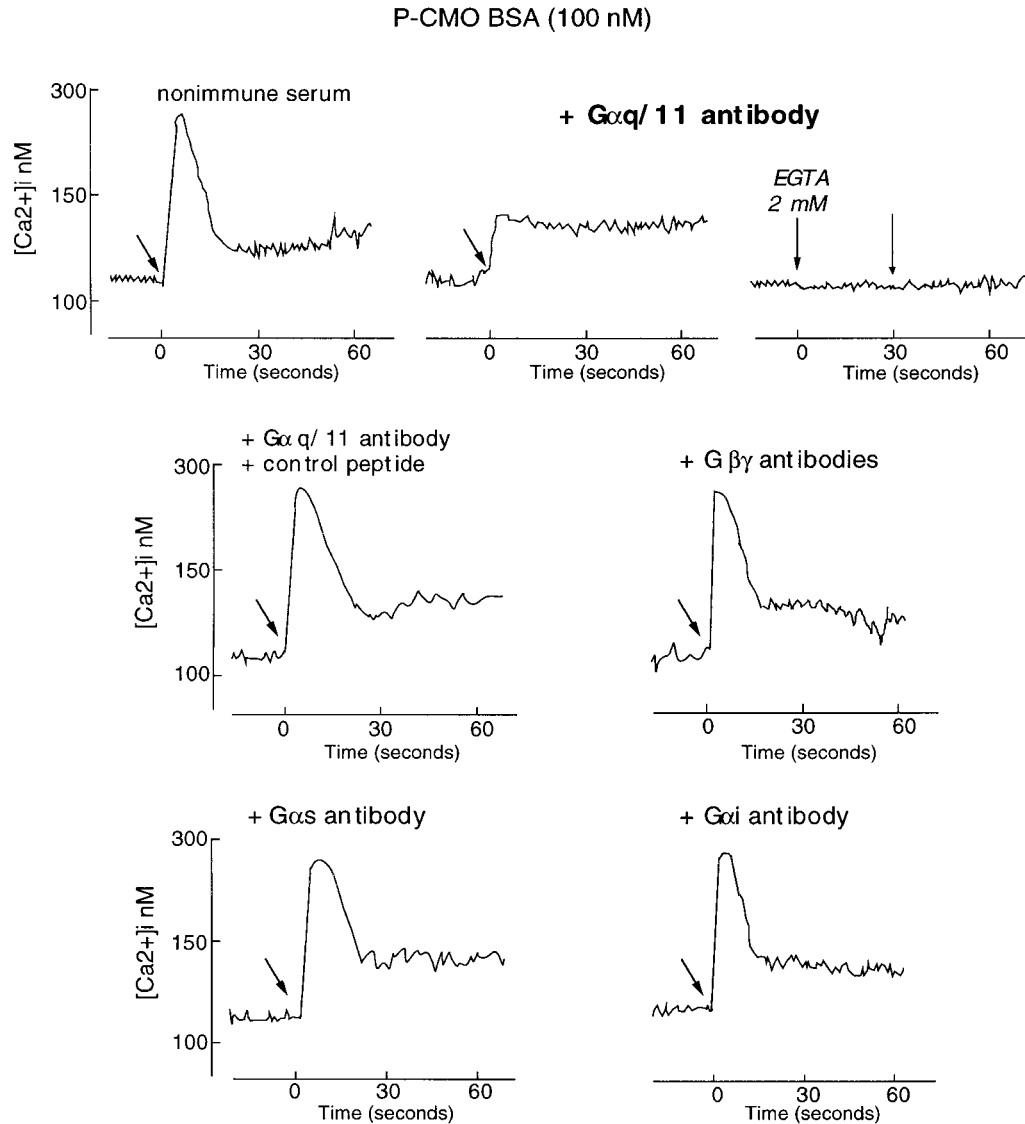
P-CMO BSA (100 nM)

Fig. 3. Changes in the intracellular calcium in male osteoblasts treated with anti-G protein antibodies in responses to progesterone covalently bound to bovine serum albumin (P-CMO BSA). Cells were cultured, loaded with Fura2/AM, and incubated with anti–G-protein antibodies. Arrows indicate the time at which 100 nM P-CMO BSA was added. These results are representative of six cultures for each experimental case.

fects, because PLC-γ are substrates for growth factor receptor protein-tyrosine kinases [Rhee et al., 1991]. Finally, the progestin effect is specific to this steroid, because calcitriol uses a PLC-β1 linked to a PTX-insensitive G-protein in the same cells, and estradiol uses a PLC-β2 coupled to a PTX-sensitive G-protein in female osteoblasts [Le Mellay et al., 1999]. The activation of PLC-β by progestins seems to be tissue-specific, because progesterone activates only PLC-β1 in ovarian granulosa cells [Lieberherr et al., 1999].

PLC-β enzymes are regulated via heterotrimeric G-proteins in response to agonist binding to receptors [Neer, 1995; Exton, 1997]. Receptor activation of PLC via G-proteins occurs through pertussis toxin-sensitive and toxin-insensitive signaling pathways [Neer, 1995; Exton, 1997]. The α subunits of the Gq/11 family are presumed to mediate the toxin-insensitive pathway, but the nature of the G-proteins mediating the toxin-sensitive pathway is less well understood [Rhee et al., 1991; Neer, 1995; Exton, 1997]. The PLC-β1 and
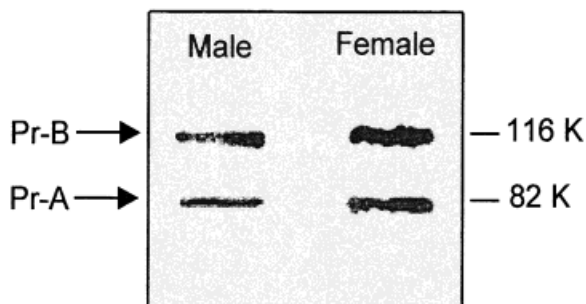
Fig. 4. Immunoblots of progesterone receptors in female and male rat osteoblasts. Cell nuclei were prepared as described. Fifty-microgram aliquots were separated by electrophoresis on sodium dodecyl sulfate-polyacrylamide (8%) gel, transferred to Immobilon P membrane, and probed with 0.1 µg/ml polyclonal rabbit (Pr-A and Pr-B) progesterone receptor antibody (see arrows). The antibody bound to membrane was detected with peroxidase-conjugated goat anti-rabbit IgG (1 µg/ml) and revealed by chemiluminescence. These results are representative of three cultures.

PLC-β3 involved in the action of progestins are linked to a pertussis toxin-insensitive G-protein, which is a member of the Gq family. These results are consistent with the fact that the G-proteins Gq/11 are the most prominent G-protein activators in the receptor-mediated regulation of the PLC-β1 and PLC-β3 [Wu et al., 1992; Neer, 1995]. Treatment with anti-Gαi and anti-Gαs antibodies, which are involved in the adenylyl cyclase pathway [Exton, 1997], does not block the $[Ca^{2+}]i$ response to progestins or the stimulation of PIP_2 hydrolysis caused by GTPγS. Gβγ subunits like PLC-β2, which is the preferential target effector for Gβγ subunits [Zhu and Birnbaumer, 1996], are not involved in Ca^{2+} mobilization or $InsP_3$ formation induced by progesterone and P-CMO BSA. Although osteoblasts possess these classes of the G-subunits [Le Mellay et al., 1999], this indicates that the effects of progestins on $[Ca^{2+}]i$ and $InsP_3$ formation in both osteoblasts are not mediated via the subunits of the G-protein linked to the cAMP pathway.

The membrane signaling of progestins seems to be independent of gender in osteoblasts, which is not the case for estradiol [Lieberherr et al., 1993] and testosterone [Lieberherr and Grosse, 1994]. This is in agreement with data obtained with progesterone in spermatozoa [Blackmore et al., 1996]. Although male osteoblasts have a lower basal level of classical receptors than female osteoblasts (see Fig. 4), the calcium and $InsP_3$ responses to progestins have the same amplitude in both cell types. These data corroborate the finding that progesterone may act on the membranes of cells lacking PR, and suggest that the membrane signaling of progesterone is not linked to the presence of nuclear PR.

Finally, the data define a new type of non-genomic mechanism of progesterone action occurring at physiologic concentration (1 nM), and provide the first demonstration of a direct interaction between progesterone and a G-protein. This does not involve G-protein–coupled receptors, but may be important for the understanding of the sperm-cell membrane progesterone receptor [Blackmore et al, 1996]. The cloning of the "membrane" receptor should help in the evaluation of its physiologic role in normal and pathological conditions.

ACKNOWLEDGMENTS

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