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Membrane Signalling and Progesterone in Female and Male Osteoblasts. I. Involvement of Intracellular Ca^{2+}, Inositol Trisphosphate, and Diacylglycerol, but Not cAMP

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Abstract Bone is a target tissue of progestins, but the mechanisms by which they act are still unclear. We examined the early (5–60 s) effects of progesterone and progesterone covalently bound to BSA (P-CMO BSA), which does not enter the cell, on the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]i$) and the formation of inositol 1,4,5 trisphosphate (InsP3) and diacylglycerol (DAG) in confluent female and male rat osteoblasts. P-CMO BSA like free progesterone increased $[Ca^{2+}]i$ via Ca^{2+} influx through L-type Ca^{2+} channels and Ca^{2+} mobilization from the endoplasmic reticulum. Both progestins increased $InsP_3$ and DAG formation within 10 s, and the increase was blocked by phospholipase C inhibitors (neomycin and U-73122). Progesterone and P-CMO BSA mobilized calcium from the endoplasmic reticulum via the activation of a phospholipase C linked to a pertussis toxin-insensitive G-protein in both osteoblast types, and this process was controlled by protein kinase C. Neither progestin had any effect on cAMP formation in male and female osteoblasts. The membrane effects were not blocked by a progesterone nuclear antagonist. They were independent of the concentration of nuclear receptors and not linked to gender. Thus, progesterone appears to act in female and male rat osteoblasts via unconventional cell-surface receptors which belong to the class of membrane receptors coupled to phospholipase C via a pertussis toxin-insensitive G-protein. The bifurcating pathways leading to the formation of $InsP_3$ and DAG may provide a certain flexibility in controlling cell responses, both by their nature and by their rates of formation and degradation. J. Cell. Biochem. 79:334–345, 2000. © 2000 Wiley-Liss, Inc.© 2000 Wiley-Liss, Inc.

Key words: protein kinase C; G-protein; gender

Most of the known effects of steroid hormones are mediated by intracellular receptors; the hormone-receptor complex modulates gene transcription activity in responsive cells [reviewed by Evans, 1998]. The human (hPR) and the chicken (cPR) progesterone receptors exist as two isoforms, PR-A and PR-B, each with different biological activities depending upon the cell and promoter context [Gronemeyer et al., 1991; Sylvava et al., 1997]. However, there is now considerable evidence that progesterone also acts very rapidly (in one second to a few minutes) on the membrane of several cells of the reproductive and nervous systems. The resulting effects cannot be explained by the classical nuclear receptor machinery [reviewed by Wheling, 1997; Revelli et al., 1998]. The non-genomic effects of progesterone involve the activation of signal transduction pathways that vary according to the cell type [reviewed by Wheling, 1997; Revelli et al., 1998]. Progesterone binds with a high affinity to the membrane fractions or cell surfaces of a number of cells and tissues, as shown by photoaffinity labeling or reversible binding studies [reviewed by Revelli et al., 1998], and the partial sequence of a high-affinity progesterone-binding site on porcine liver membranes was recently published [Meyer et al., 1996]. Progesterone or its metabolites have both stimulatory and inhibitory effects on the receptors of such neurotransmitters as GABA [Majewska, 1992], adrenalin [Petitti and Etgen, 1992], acetylcholine [Valera et al., 1992], and, very recently, oxytocin [Grazzini et al.,1998].

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But no information is available on the membrane effects of progesterone in bone and their gender specificity. As progesterone activates different transducing pathways, which are species- and tissue-specific, we have studied and compared the action of progestins on various signalling pathways in female and male rat osteoblasts, the bone-forming cells. We have established that progesterone and its derivative covalently bound to BSA use only the intracellular calcium/inositol trisphosphate/diacylglycerol pathway in osteoblasts. The membrane action is gender-independent and not related to the concentration of intracellular progesterone receptors in female and male osteoblasts.

MATERIALS AND METHODS

Materials

The ECL kit, and Fura-2/AM were purchased from Amersham (Les Ulis, France). Polyclonal rabbit anti-progesterone receptor (A and B progesterone receptors) antibody was 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 (P), progesterone-3-(O-carboxymethyl) oxime (P-CMO), progesterone-3-(O-carboxymethyl) oxime-bovine serum albumin (P-CMO BSA), and all other chemicals were from Sigma (St Quentin Fallavier, France). RU 38486 was a gift from Roussel-Uclaf, France. 1-(6-((17β-3-metoxyestra-1,3,5(10)-triene-17-yl)-amino)hexyl)-1H-pyrrole-2,5-dione (U-73122) and 1-(6-((17β-3-metoxyestra-1,3,5(10)-triene-17-yl)-amino) tj;1hexyl)-1H-pyrrolidine-2,5-dione (U-73343) were from Biomol Research Laboratory (Tebu). α Minimal Essential Medium (αMEM) and fetal calf serum were supplied by Gibco BRL (Life Science, Les Ulis, France).

Progestins

Progestins were 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, inositol trisphosphate, and diacylglycerol formation, or cAMP. P-CMO BSA was treated with charcoal to eliminate any free progesterone or P-CMO. Charcoal treatment had no effect on the ability of P-CMO BSA to increase $[Ca^{2+}]i$ (data not shown). The effects of P-CMO BSA on $[Ca^{2+}]i$ were due to covalently bound P, not to free P or P-CMO in the P-CMO BSA.

Cell Isolation and Culture

Two-day-old Wistar rats were from the INRA breeding facility at Jouy-en-Josas, France. Osteoblasts were isolated from the parietal bones by sequential enzymatic digestion [Wong and Cohn, 1974]. Cells were grown on rectangular glass coverslips or in Petri dishes (150 cm^2) for 6 days in phenol red-free α-MEM supplemented with 10% heat-inactivated fetal calf serum (H-FCS). They 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.

Protein Separation and Immunoblotting

Cells were 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 benzamidine, 0.1 mM leupeptin, 2 mM phenylmethylsulfonyl fluoride [PMSF], 0.125 mM aprotinin, and 1 mM dithiothreitol). They were sonicated on ice (2×20 s) at 40 KH, and the homogenate was centrifuged for 10 min at 600*g* to remove nuclei. The pellet was suspended in extraction buffer, and centrifuged for 10 min at 600*g*. The pellet corresponding to the nucleus fraction was stored at −80°C. Protein was determined by the method of Bradford [1970].

Proteins were separated by SDS-polyacrylamide gel electrophoresis (8% resolving gel) in 25 mM Tris-base pH 8.3, 192 mM glycine, 0.1% SD. They were then electrophoretically transferred to polyvinyldifluoride membranes (Immobilon P, Millipore, St Quentin-en-Yvelines, France) in the same buffer with 20% ethanol for 2 h at 100 V. Non-specific binding to membrane was prevented by incubating the membranes in 10 mM Tris-buffered saline (TBS) pH 7.5 containing 150 mM NaCl, 5% skim powder milk and 0.1% Tween-20 (TN-TBM) with 1% normal goat serum for 12 h at 4°C. The membranes were washed in TBS containing 0.1% Tween-20, and incubated overnight at 4°C with 0.1 μg/ml polyclonal rabbit (PR-A and PR-B) progesterone receptor antibody. Unbound antibody was removed by four washes in PBS; the antibody bound to membrane was detected with peroxidase-conjugated goat anti-rabbit IgG (1 μg/ml) diluted in TN-TBM plus 0.2%

normal goat serum and revealed by chemiluminescence.

Calcium Measurement and Experimental Protocol

The cells were loaded with 1 μM Fura-2/AM for 30 min in Hank's 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 $MgSO_4$.7H2O, 27.7 mM glucose, 1.25 mM $CaCl_2$, and 25 mM HEPES) at room temperature as described previously [Lieberherr et al., 1993]. Drugs and reagents were added directly to the cuvette with continuous stirring. The Fura-2 fluorescence response to the intracellular calcium concentration ([Ca^{2+}]i) was calibrated from the ratio of the 340/380 nm fluorescence values after subtraction of the background fluorescence of the cells at 340 and 380 nm. The dissociation constant for the Fura-2-Ca^{2+} complex was taken as 224 nM [Grynkiewicz et al., 1985].

We first studied the direct effects of 1 pM to 100 nM progesterone, P-CMO and P-CMO/BSA on [Ca^{2+}]i. We then investigated whether the effects of progestins on [Ca^{2+}]i were due to an influx of Ca^{2+} from the extracellular milieu and/or Ca^{2+} mobilization from intracellular stores. 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 s before adding 1 nM progesterone. The second involved the selective blockers of L-type Ca^{2+} channels, nifedipine, and verapamil. We investigated how much of the transient increase in [Ca^{2+}]i was due to Ca^{2+} released from the endoplasmic reticulum, using neomycin, which inhibits phospholipase C (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].

Cell Labeling and Lipid Chromatography

The action of progestins on the formation of diacylglycerol (DAG) was assessed in cells incubated with [^{14}C] arachidonic acid (0.25 μCi/ml) for 24 h in phenol red-free medium with 1% H-FCS. The labeled cells were incubated for 2 h in fresh serum-free medium; ethanol solvent (0.01%) or progestins were added for 5–90 s. The reaction was stopped by removing the medium and adding cold methanol. The lipids were extracted according to Bligh and Dyer [1978], with a final amount of 2 ml methanol, 2 ml chloroform, and 1.6 ml aqueous salt (0.74% KCl, 0.04% $CaCl_2$, and 0.034% $MgCl_2$). The chloroform phases were combined, dried, and dissolved in 200 μl chloroform and methanol (2:1, vol/vol), and aliquot was taken for thin layer chromatography (TLC). Neutral lipids were separated in one dimension using n-heptane, diethyl ether, and acetic acid (75:25:4, by volume). The lipids were visualized by iodine staining, and the developed TLC plates were analyzed with a TLC linear analyser (LB 2820; Berthold, Munich, Germany). The ^{14}C-labeled lipids of interest were scraped off and counted by liquid scintillation with Econofluor. The percentage of radioactivity incorporated into diayglcerol was 2–4%.

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 diethylether. The final extract was neutralized and inositol 1,4,5-trisphosphate mesured using a radiocompetition assay (Kit TRK 1000, Amersham, Les Ulis, France).

cAMP Measurement

Cells were incubated for 10 min or 2 h in medium containing 1% bovine serum albumin, 0.2 mM isobutyl methylxanthine (an inhibitor of phosphodiesterase) and the molecule to be tested or its vehicle. Cells were rinsed with PBS, and cAMP was extracted by sonication in 90% n-propanol. cAMP was measured by the protein binding assay of Lust et al. [1976], and expressed as pmoles per mg protein.

Statistical Analysis

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

RESULTS

Direct Effects of Progestins on Intracellular Calcium Concentration

The basal [Ca^{2+}]i was 116 ± 6 nM (mean ± S.E., n = 6) in confluent female osteoblasts and 120 ± 5 nM (mean ± S.E., n = 6) in male osteoblasts. One nM progesterone caused tran-

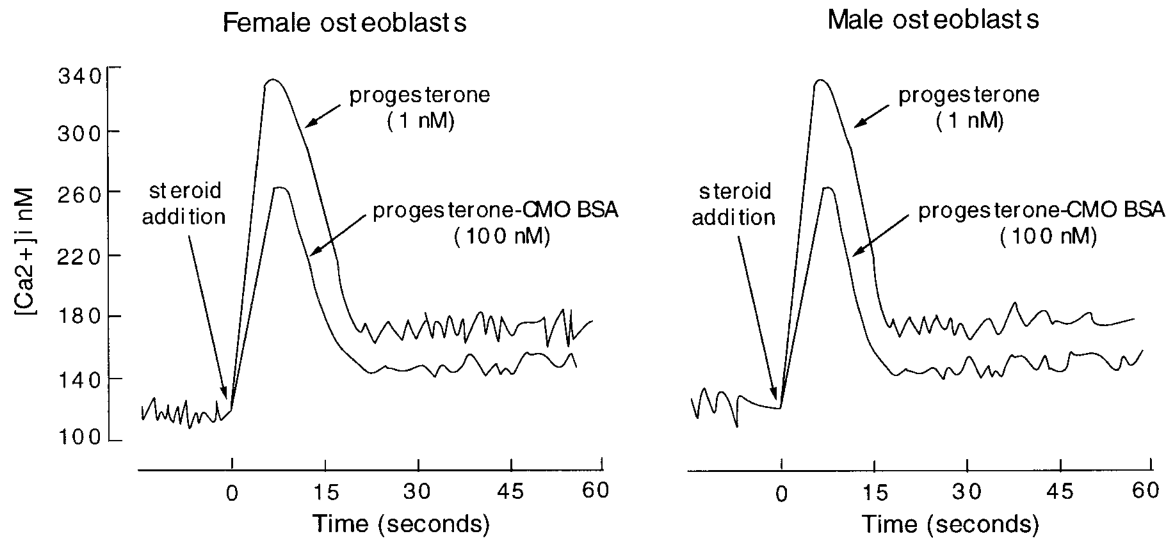

Fig. 1. Calcium responses of confluent female and male rat osteoblasts to 1 nM progesterone and 100 nM progesterone-CMO BSA. Arrow indicates the time at which the progestin was added. These results are representative of six cultures.

sient increases in $[Ca^{2+}]i$ of the same amplitude in both cell types (Fig. 1). The sharp peak fell rapidly after 15 s, but remained higher than the basal level (plateau phase) (25 ± 2%, mean ± S.E., n = 6, $P < 0.001$; Fig. 1). The concentration-dependent effects of progesterone were bell-shaped, with a maximum activity at 1 nM (Table I). P-CMO, which enters the cell, and P-CMO BSA, which does not, both produced smaller increases in $[Ca^{2+}]i$, but the time courses were similar to that of free progesterone (Fig. 1, data shown only for P-CMO BSA, and Table I). Free progesterone was more potent than either P-CMO or P-CMO BSA, while these two molecules were equipotent. There was a three-fold increase in $[Ca^{2+}]i$ by 1 nM free progesterone, the maximal active dose, while the maximal active concentration of P-CMO and P-CMO BSA (100 nM) induced a 2.2-fold increase (n = 6, $P < 0.001$). The smaller amplitude of the responses to P-CMO and P-CMO BSA was due to the carboxymethyl oxime radical necessary for the covalently bound; this modifies the structure of the molecule. 17α-hydroxyprogesterone (10 pM to 10 nM) was as active as progesterone, and medroxyprogesterone had no effect (Table I).

Mechanisms of Progestin-Induced Changes in Intracellular Calcium

EGTA and nifedipine reduced the magnitude (40 ± 10%, mean ± S.E., n = 6, $P < 0.001$) of the transient peak induced by 1 nM progesterone, and the plateau phase was completely abolished in both cell types (Fig. 2A,C). Table II shows the blockade of progesterone and progesterone-CMO BSA-induced changes in intracellular calcium concentration by EGTA and verapamil in both cell types. Neomycin and U-73122 abolished the transient peak, but not the plateau phase in female and male osteoblasts (Fig. 2B,D and Table II). U-73343 (0.3–5 μM), an analogue similar to U-73122 but inactive [Bleasdale et al., 1989], had no effect (data not shown). Identical results were obtained for P-CMO BSA in both cell types (Table II).

Effects of Progestins on Inositol 1,4,5-Trisphosphate and Diacylglycerol Formation and cAMP Content

One nM progesterone increased the formation of inositol 1,4,5-trisphosphate (InsP3 ; Fig. 3A) and DAG (Fig. 3B) in female and male osteoblasts to the same degree. One hundred nM P-CMO BSA also increased InsP3 and DAG formation in the same period, but the amplitude was lower in both cell types (Fig. 3C, data shown for male osteoblasts and InsP3). Female and male rat osteoblasts were incubated for 1 min with U-73122, an inhibitor of phospholipase C, and 1 nM progesterone or 100 nM P-CMO BSA was then added. U-73122 inhibited the effect of progesterone or P-CMO BSA

TABLE I. Dose-Dependent Effects of Various Progestins on Intracellular Calcium Concentration in Confluent Female and Male Osteoblasts[a]

Progestins	Progesterone		Progesterone-CMO		Progesterone-CMO BSA		17α-hydroxy-progesterone		Medroxyprogesterone	
	$[Ca^{2+}]$i nM									
Concentration	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male
0	116 ± 6	120 ± 5	116 ± 6	120 ± 5	116 ± 6	120 ± 5	116 ± 6	120 ± 5	116 ± 6	120 ± 5
1 pM	115 ± 9	120 ± 4	117 ± 9	121 ± 4	123 ± 8	121 ± 7	113 ± 9	119 ± 3	123 ± 6	109 ± 11
10 pM	152 ± 5*	163 ± 9*	131 ± 10	129 ± 8	115 ± 8	123 ± 5	154 ± 3*	163 ± 9*	124 ± 7	106 ± 13
100 pM	249 ± 11*	256 ± 9*	123 ± 8	115 ± 9	126 ± 4	113 ± 11	267 ± 9*	249 ± 16*	109 ± 15	121 ± 9
1 nM	355 ± 11*	362 ± 9*	163 ± 3*	151 ± 13*	159 ± 7*	147 ± 14*	349 ± 13*	371 ± 14*	112 ± 9	122 ± 5
10 nM	232 ± 5*	241 ± 12*	189 ± 8*	187 ± 5*	191 ± 9*	185 ± 12*	223 ± 13*	234 ± 11*	108 ± 16	111 ± 10
100 nM	128 ± 9	135 ± 8*	263 ± 6*	259 ± 5*	271 ± 12*	285 ± 15*	112 ± 11	123 ± 6	119 ± 2	117 ± 6
1 μM	131 ± 12	123 ± 7*	201 ± 10*	198 ± 7*	188 ± 14*	191 ± 9*	118 ± 4	125 ± 8	115 ± 10	121 ± 5

[a]Intracellular Ca^{2+} concentrations were determined at t = 10 s after adding the progestin. Values are the mean ± S.E., n = 6 and are significantly different from the basal level of untreated cells.
*$P < 0.001$.

on InsP3 and DAG formation in both cell types whatever the incubation time with the progestin (Fig. 3C, data shown for P-CMO BSA and InsP3 in male osteoblasts).

The basal cAMP content of female osteoblasts was 8.3 ± 0.6 pmol/mg protein and in male osteoblasts it was 9.9 ± 0.9 pmol/mg protein, n = 5. The cAMP content of both cell types was not altered by 1 pM–1 μM progesterone, progesterone-CMO or progesterone-CMO BSA at any incubation time from 30 s to 60 min (data not shown). Forskolin (1 μM) increased the cAMP content in female (56.9 ± 1.5 pmol/mg protein, mean ± S.E., n = 5, $P < 0.001$) and male (61.3 ± 2.5 pmol/mg protein, mean ± S.E., n = 5, $P < 0.001$) osteoblasts within 5 min.

Effects of Progesterone Nuclear Antagonist on the Membrane Response to Progestins

Cells were incubated for 45 min to 240 min with 10 nM or 1 μM RU-486, a nuclear antagonist of progesterone. The basal $[Ca^{2+}]$i was not altered by the antagonist, and incubation of female and male cells with RU-486 did not alter the $[Ca^{2+}]$i response to 100 pM progesterone (Table III) or 100 nM P-CMO BSA (data not shown) whatever the incubation time. RU-486 did not alter the basal level of inositol 1,4,5-trisphosphate, or the effects of progesterone on this parameter in both cell types (data not shown).

Effects of Staurosporine and Pertussis Toxin on the Intracellular Calcium Response to Progestins

Cells were incubated for 1 min with 1 nM staurosporine, a potent inhibitor of protein kinases with some selectivity for protein kinase C (PKC), before adding 100 pM progesterone. The PKC blockade by the drug enhanced the $[Ca^{2+}]$i response to progesterone in both female and male osteoblasts (Table III).

Cells were incubated for 18 h with 100 ng/ml pertussis toxin (PTX). Fura-2/AM loading and measurement were carried out with 100 ng/ml PTX. PTX did not alter the response to 100 pM progesterone (Table III) and P-CMO BSA (data not shown) of either osteoblast type.

Progesterone Nuclear Receptors and Intracellular Calcium Response to Progesterone

Female osteoblasts were incubated for 12 h or 24 h with 1 nM 17β-estradiol or ethanol

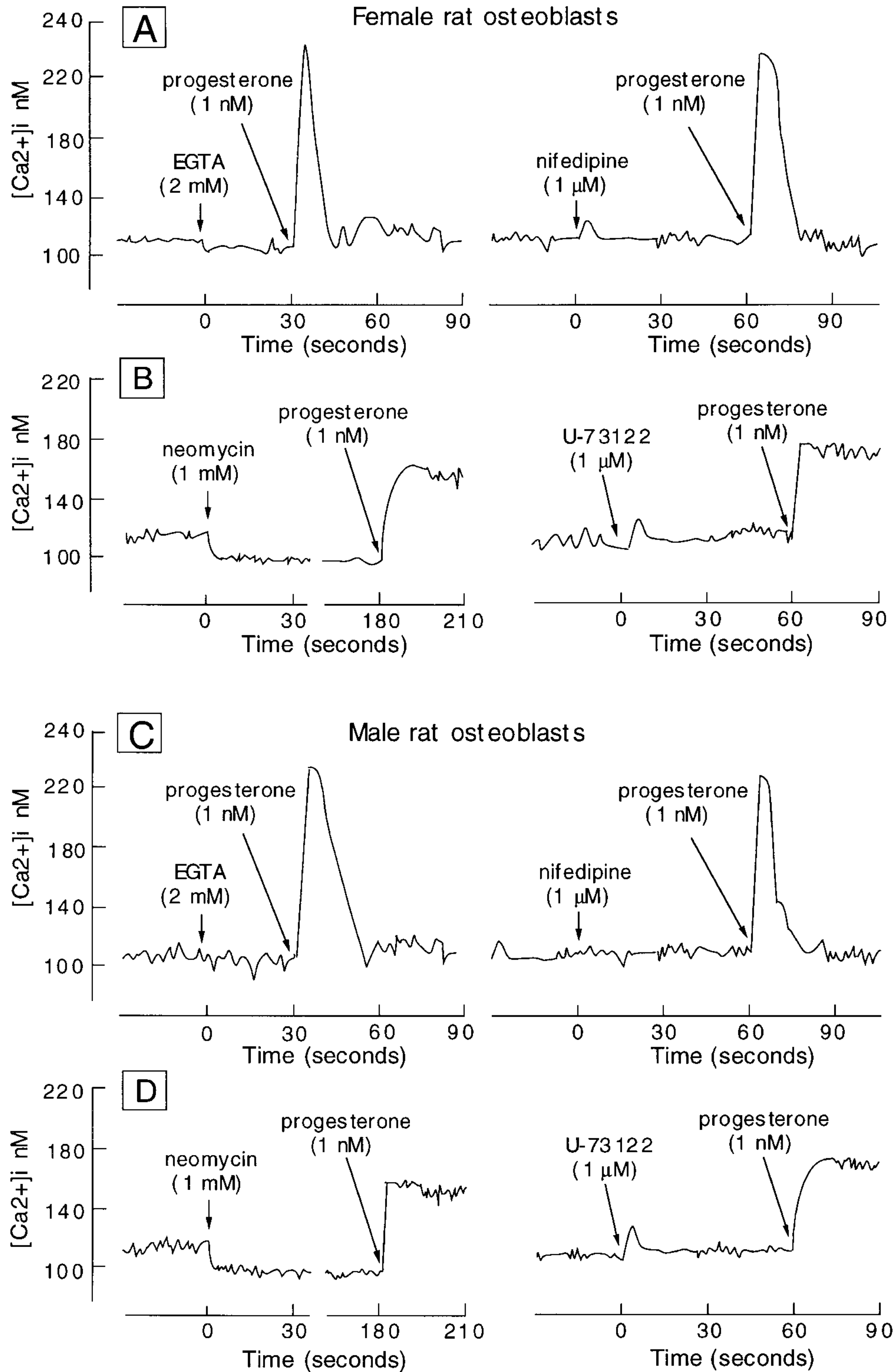

Fig. 2. Mechanisms involved in the intracellular calcium increase induced by progesterone. Female osteoblasts were incubated for 30 s with 2 mM EGTA and for 60 s with 1 μM nifedipine (**A**), or for 3 min with 1 mM neomycin and for 60 s with 1 μM U-73122 (**B**) before adding 1 nM progesterone. Male osteoblasts were incubated for 30 s with 2 mM EGTA and for 60 s with 1 μM nifedipine (**C**), or for 3 min with 1 mM neomycin and for 60 s with 1 μM U-73122 (**D**) before adding 1 nM progesterone. Arrows indicate the time at which drugs were added. These results are representative of six cultures.

TABLE II. Blockade of Progesterone and P-CMO BSA-Induced Changes in Intracellular Calcium Concentration in Confluent Female and Male Rat Osteoblasts[a]

	Female rat osteoblasts $[Ca^{2+}]$i nM			Male rat osteoblasts $[Ca^{2+}]$i nM		
		Stimulated level			Stimulated level	
Treatment	Basal level	Progesterone	P-CMO BSA	Basal level	Progesterone	P-CMO BSA
	116 ± 6	349 ± 8*	258 ± 11*	120 ± 5	369 ± 10*	249 ± 8*
EGTA (2 mM)	116 ± 5	268 ± 10*,**	192 ± 9*,**	109 ± 14	272 ± 8*,**	189 ± 15*,**
Verapamil (1 μM)	121 ± 7	278 ± 12*,**	162 ± 5*,**	112 ± 3	264 ± 11*,**	171 ± 9*,**
Neomycin (1 mM)	109 ± 8	158 ± 9*,**	145 ± 9*,**	110 ± 5	161 ± 5*,**	148 ± 6*,**
U-73122 (1 μM)	139 ± 5	168 ± 6*,**	160 ± 3*,**	141 ± 8	173 ± 9*,**	169 ± 3*,**

[a]Osteoblasts were incubated for 30 s with 2 mM EGTA, for 60 s with 1 μM verapamil, for 3 min with 1 mM neomycin, or for 60 s with 1 μM U-73122 before adding 1 nM progesterone or 100 nM progesterone-CMO BSA (P-CMO BSA). Intracellular calcium concentrations were determined at t = 10 s after the addition of the progestin. Values are the mean ± S.E., n = 6.
*$P < 0.001$ for the difference between the basal level in the presence or absence of blocker and the level of cells treated with the progestin in the presence or absence of blocker.
**$P < 0.001$ for the difference between cells treated with the progestin alone and cells treated with progestin plus blocker.

vehicle, and male osteoblasts for 24 h with either 1 nM 5α-dihydrotestosterone (DHT), 1 nM testosterone or ethanol vehicle before using them for Western immunoblotting and calcium measurement. Incubation with estradiol increased the synthesis of progesterone nuclear receptor in female osteoblasts (Fig. 4A). Testosterone increased the synthesis of progesterone receptors in male osteoblasts, whereas DHT had no effect (Fig. 4B). Increasing the concentration of progesterone receptor in both osteoblast types did not modify the $[Ca^{2+}]$i response to progesterone (Table IV).

DISCUSSION

Progesterone-CMO BSA and free progesterone use the Ca^{2+}/inositol trisphosphate/ diacylglycerol pathway instead of cAMP/ adenylyl cyclase pathway in female and male rat osteoblasts.

These effects occur at physiological concentrations of progesterone (10 pM to 1 nM). The response curve is bell-shaped with a maximum at 1 nM. The bell-shaped dose-dependent action on $[Ca^{2+}]$i and $InsP_3$ is a general phenomenon that occurs with several steroids [Lieberherr et al., 1993, 1999 ; Lieberherr and Grosse, 1994; Sömjen et al., 1997]. The non-genomic effects generally fade at concentrations which induce the genomic effects, suggesting a chronology in the events of the steroid action.

The rapid (within 5 s) Ca^{2+} response of female and male osteoblasts to progesterone involves both the influx of extracellular Ca^{2+} and Ca^{2+} release from the endoplasmic reticulum, although progesterone uses only one or the other mechanism in many cell systems. The progesterone-induced Ca^{2+} influx involves voltage-gated Ca^{2+} channels, whereas depleting extracellular Ca^{2+} in ovarian pig granulosa cells [Machelon et al., 1996] and spermatozoa from several species [Blackmore et al., 1991; Turner et al., 1994] involves non-voltage-gated Ca^{2+} channels. This confirms that the type of response to progesterone may be tissue- or species-dependent. On the other hand, progesterone mobilizes Ca^{2+} from intracellular calcium stores in both osteoblast types, as it does in ovarian granulosa cells [Machelon et al., 1996]. The progesterone-induced Ca^{2+} mobilization follows an increase in inositol 1,4,5-trisphosphate ($InsP_3$), which is responsible for Ca^{2+} mobilization from the endoplasmic reticulum [Berridge, 1993]. Increasing $[Ca^{2+}]$i by releasing Ca^{2+} from the endoplamic reticulum seems to be a general mechanism used by progesterone and other steroids in osteoblasts [Lieberherr et al., 1993; Grosse et al.,1993; Lieberherr and Grosse, 1994] and other cell types [Kostellow et al., 1993; Machelon et al., 1996].

Progesterone causes an increase in $InsP_3$ and DAG in female and male osteoblasts. The response to progesterone seems to follow the pattern described for agonist-stimulated phosphoinositide turnover in general [Berridge, 1993]. Progesterone triggers the formation of $InsP_3$ in reproductive cells [Machelon et al., 1996; Murase and Roldan, 1996; Kostellow et al., 1993]. Progesterone may also increase DAG

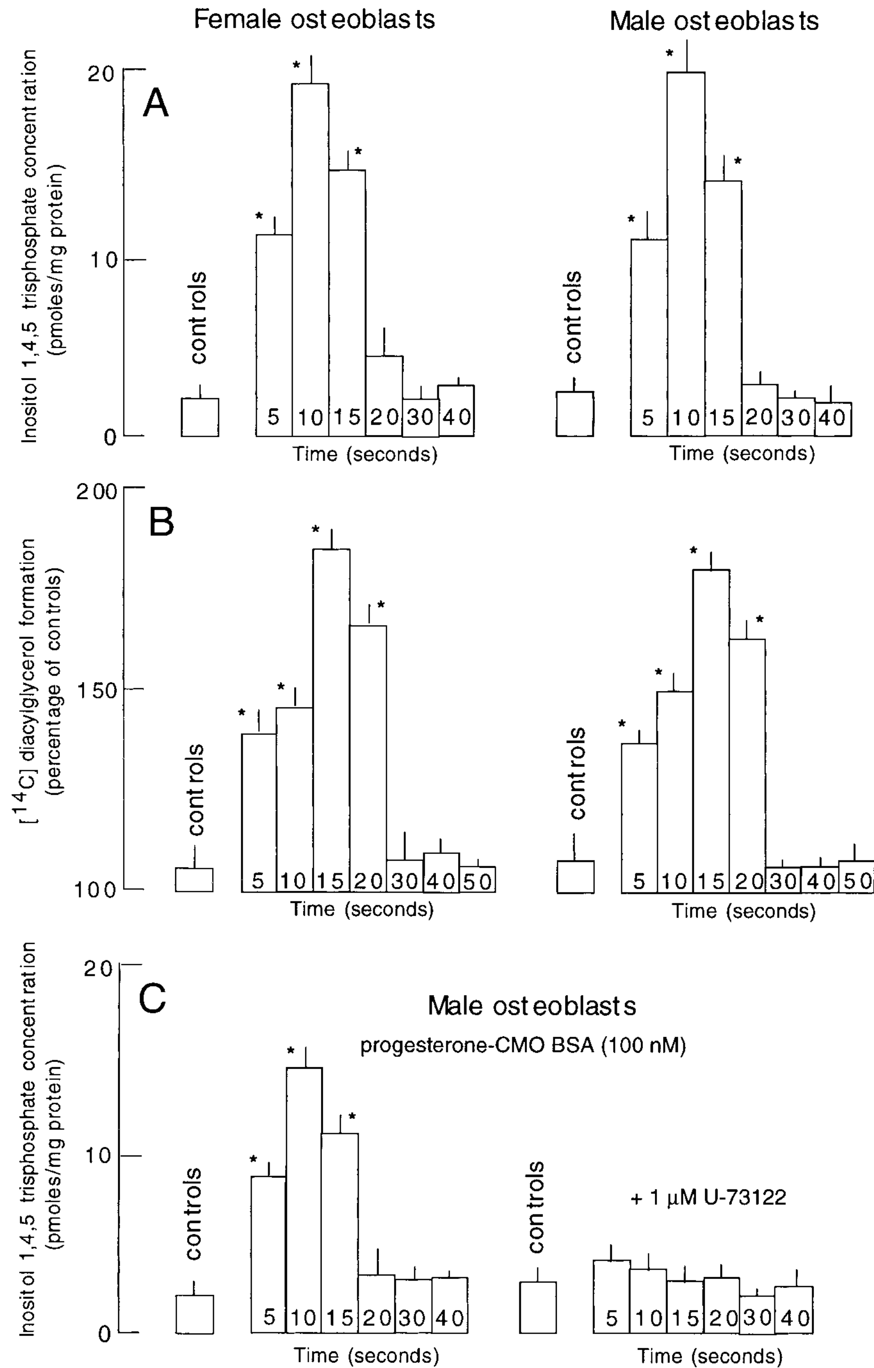

Fig. 3. Effects of progestins on inositol 1,4,5-trisphosphate and diacylglycerol formation in female and male osteoblasts. Female and male osteoblasts were incubated for various times with 1 nM progesterone, and the inositol trisphosphate concentration was measured (**A**). Female and male osteoblasts were incubated for various times with 1 nM progesterone, and diacylglycerol formation was measured (**B**). Male osteoblasts were incubated for various times with 100 nM progesterone-CMO BSA, and diacylglycerol formation was measured (**C**). Male osteoblasts were incubated for 1 min with 1 µM U-73122 then for various times with 100 nM progesterone-CMO BSA, and diacylglycerol was measured (**C**). The results are the mean ± S.E., n = 5 cultures, and significantly different from the respective controls (*$P < 0.001$).

TABLE III. Effects of RU-38486, Staurosporine, and Pertussis Toxin on Calcium Response to Progesterone[a]

	$[Ca^{2+}]i$ nM			
	Basal level (P = 0 pM)		Stimulated level (P = 100 pM)	
Treatment	Female osteoblasts	Male osteoblasts	Female osteoblasts	Male osteoblasts
RU-38486				
0 μM	121 ± 8	114 ± 5	249 ± 5*	246 ± 9*
10 nM	116 ± 3	120 ± 6	251 ± 11*	247 ± 10*
1 μM	112 ± 8	122 ± 9	243 ± 13*	256 ± 8*
Staurosporine				
0 nM	115 ± 5	111 ± 9	252 ± 9*	249 ± 11*
1 nM	113 ± 7	119 ± 7	399 ± 10**	412 ± 14**
Pertussis toxin				
0 ng/ml	116 ± 5	110 ± 6	245 ± 6*	253 ± 10*
100 ng/ml	120 ± 4	112 ± ″	252 ± 9*	261 ± 9*

[a]Cells were incubated with RU-38346 for 4 h, pertussis toxin for 18 h or staurosporine for 1 min before the addition of 100 pM progesterone (P). Data are the intracellular calcium concentration 10 s after the steroid addition. Values are the mean ± S.E., n = 5, and significantly different from their respective controls (*$P < 0.001$) or stimulated levels (**$P < 0.001$).

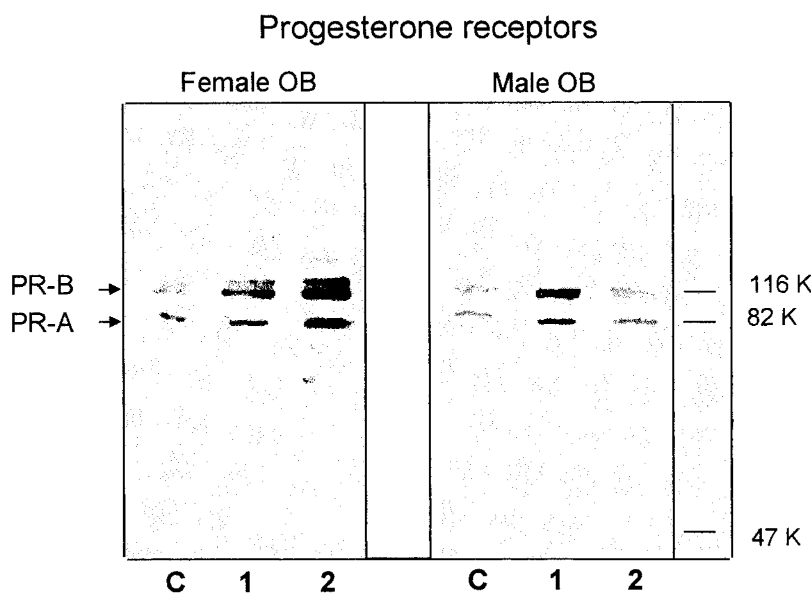

Fig. 4. Immunoblots of progesterone receptors in female and male osteoblasts. 35 μg aliquots of the nucleus fraction were separated by electrophoresis on SDS-polyacrylamide gel, transferred to nitrocellulose membranes, and probed with antibody against progesterone receptors. **C**: Progesterone receptors (PR-A and PR-B) in ethanol-treated female or male osteoblasts; PR -A and PR-B in 1 nM estradiol-treated female cells for 12 h (**1**) or 24 h (**2**); PR-A and PR-B in 1 nM testosterone-treated male cells for 24 h (**1**) or in 1 nM DHT-treated male osteoblasts for 24 h (2). These results are representative of three cultures.

formation in reproductive cells [Murase and Roldan, 1996; Kostellow et al., 1993]. The action of progestins on mobilization of intracellular calcium in both osteoblast types is modulated by PKC, as shown by using staurosporine, which seems to have a negative feedback on the calcium response. Activation of PKC by increasing DAG and intracellular Ca^{2+} may lead to a cascade of phosphorylations of other kinases [Cobb and Golsmith, 1995], that engage in cross-talk with the nucleus of the cell so as to initiate biological responses that depend upon nuclear events. PKC is involved in the phosphorylation of the vitamin D nuclear receptor [Heish et al., 1993], and the binding capacity of estrogen to its nuclear receptor is

TABLE IV. Calcium Response to 1 nM Progesterone in Female and Male Osteoblasts Treated With Estrogens or Androgens[a]

$[Ca^{2+}]i$ nM	
Female osteoblasts	
Ethanol (12 h + 24 h)	358 ± 5
1 nM estradiol (12 h)	360 ± 10
1 nM estradiol (24 h)	348 ± 9
Male osteoblasts	
Ethanol (24 h)	363 ± 8
1 nM testosterone (24 h)	352 ± 11
1 nM DHT (24 h)	358 ± 12

[a]Female osteoblasts were treated for 12 h or 24 h with 1 nM 17β-estradiol or ethanol vehicle before the addition of 1 nM progesterone. Male osteoblasts were treated for 24 h with 1 nM testosterone, 1 nM 5α-dihydrotestosterone (DHT), or ethanol vehicle before the addition of progesterone. Data are the intracellular calcium concentration 10 s after the progesterone addition. Values are the mean ± S.E., n = 3.

regulated by tyrosine phosphorylation [Arnold et al., 1997]. This suggests that activation of PKC by progesterone may lead to a cascade of protein phosphorylations, including phosphorylation of the progesterone nuclear receptor, initiating further biological responses dependent upon nuclear events. But, estradiol may activate the MAPkinase pathway very rapidly leading to direct activation of early genes involved in proliferation and differentiation without interferring with the nuclear receptor [Watters et al., 1997]. These two pathways are now under investigation.

Neither progesterone nor P-CMO BSA affect cAMP, while forskolin increases cAMP formation in both osteoblast types. Progesterone also has no effect in ovarian granulosa cells [Lieberherr et al., 1999], whereas progesterone stimulates cAMP production in *Xenopus* oocytes [Maller et al., 1979] and inhibits membrane-bound adenylate cyclase in *Xenopus laevis* oocytes [Finidori-Lepicard et al.,1981]. This discrepancy needs further investigation.

Although female and male osteoblasts possess classical receptors to both estrogens and androgens (personal data), estradiol only acts at the plasma level of female osteoblasts [Lieberherr et al., 1993] and testosterone at that of male osteoblasts [Lieberherr and Grosse, 1994]. This gender-specificity is also found for the genomic effects [Sömjen et al., 1997]. In contrast, the rapid effects of progesterone are not gender-specific, as are its long-term effects in male osteoblasts [Scheven et al., 1990]. The non gender-specificity of the effect of progesterone may be due to its position in the steroidogenic pathway, since it can be metabolized to testosterone, which is itself metabolized to estradiol.

Both male and female osteoblasts bear classical progesterone receptors (PR), and the receptor concentration is increased by incubating the cells with estradiol and testosterone, but not with DHT. Testosterone undergoes either aromatization, giving rise to 17β estradiol or reduction of the Δ4 bond of testosterone to form 5α-dihydrotestosterone. Since osteoblasts possess both aromatase and 5α-reductase [Shimodaira et al., 1996] and DHT is ineffective, the increase in PR receptors in response to testosterone is probably due to the synthesis of estradiol. The rapid responses to progesterone and P-CMO BSA are independent of the concentration of classical PR. This is not surprising since progesterone may induce rapid responses in cells lacking classical PR [reviewed by Revelli et al., 1998]. The data point to a direct interaction with progesterone-specific membrane steroid-recognizing moieties in female and male osteoblasts. Progesterone immobilized by covalent linkage to BSA, which does not enter the cell, also increases $[Ca^{2+}]i$ via Ca^{2+} influx and mobilization, and $InsP_3$ and DAG formation. Inhibitors of PLC block the responses induced P-CMO BSA. RU-486, which competes with progesterone in the nucleus, does not inhibit the rapid responses induced by free progesterone and P-CMO BSA. Moreover, the mobilization of calcium from the endoplasmic reticulum by progestins involves the activation of a pertussis-toxin-insensitive G-protein, whose effectors are inositide-specific phospholipases C [Exton, 1997]. These results suggest that the progesterone receptor that mediates the rapid effects resides on the outer surface of the osteoblasts, and is distinct from the nuclear receptor in term of agonist and antagonist specificity and the involvement of a G-protein. Although our results provide no conclusive evidence about the nature of the progesterone "membrane" receptor, the time course of the effect on phospholipid metabolism is not compatible with a "liponomic" action of progesterone.

The same ligand can activate, simultaneously or consecutively, a "membrane" recep-

tor and the classical nuclear receptor, and the resulting overall effect of the steroid may be a mixture of rapid and long-term effects. A better understanding of the nature and physiological role of the "membrane" receptor is required before we can evaluate the processes by which cells respond to progesterone in normal and pathological conditions.

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