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$G\alpha_{q/11}$ and $G\beta\gamma$ Proteins and Membrane Signaling of Calcitriol and Estradiol

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Abstract 17β-estradiol and 1,25-dihydroxyvitamin D_3 (calcitriol) rapidly increase (< 5 sec) the concentration of intracellular calcium by mobilizing Ca^{2+} from the endoplasmic reticulum and forming inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol. Calcitriol increases $InsP_3$ formation via activation of phospholipase C (PLC)-β1 linked to a pertussis toxin (PTX)-insensitive G-protein, and estradiol via activation of PLC-β2 linked to a PTX-sensitive G-protein. Since PLC are effectors of different subunits of various G-proteins, we looked for and identified several G-subunits ($G\alpha_{q/11}$, Gαs, Gαi, Gβ and Gγ) in female rat osteoblasts using Western immunoblotting. The action of calcitriol on $InsP_3$ formation and Ca^{2+} mobilization in Fura-2-loaded confluent osteoblasts involved $G\alpha_{q/11}$. The membrane effects of estradiol involved Gβγ; subunits, and principally Gβ subunits, but not α-subunits. These results may provide additional evidence for membrane receptors of steroid hormones. Since PLC-β1 is the target effector of $G\alpha_{q/11}$, whereas PLC-β2 is only activated by βγ subunits, this specificity may help to generate membrane receptor-specific responses in vivo. J. Cell. Biochem. 75:138–146, 1999.

Key words: G-protein; calcitriol; estradiol; osteoblasts

Most of the known effects of steroid hormones are mediated by intracellular receptors, which are members of the nuclear receptor superfamily [Bagchi et al., 1992; Truss and Beato, 1993]. When free steroid enters the cell by passive diffusion through the plasma membrane, it binds to the receptor, and the steroid-receptor complex is activated as a result of conformational changes. The activated-hormone receptor binds to selective sites on the chromatin, and interacts with specific DNA sequences. The activated steroid-receptor complex acts as a transcription factor to modulate the synthesis of specific mRNA and proteins, leading to the final effect of the hormone. This is the classical, or genomic effect of a steroid.

However, there is now considerable evidence that steroids act very rapidly (within 1 s to a few min) at the membrane in several cells to produce effects that cannot be explained by the classical nuclear receptor machinery, but by the presence of signal-generating steroid receptors on the cell surface [reviewed in Wheling, 1997; Revelli et al., 1998]. Both 17β-estradiol and calcitriol, the hormonally active form of vitamin D_3, increase within 5 sec the concentration of intracellular calcium and trigger the formation of second messengers such as inositol 1,4,5-trisphosphate and diacylglycerol in several cell systems [Lieberherr, 1987; Lieberherr et al., 1989, 1993; Bourdeau et al., 1990; Wali et al., 1990; Morley et al., 1992; Grosse et al., 1993; Benten et al., 1998], and cAMP [Vazquez et al., 1995; Picotto et al., 1996]. This may involve activation of enzymes such as phospholipase C [Le Mellay et al., 1997] or D [Facchinetti et al., 1998), or protein kinase C [De Boland and Norman, 1990; Bisonette et al., 1994; Sylvia et al., 1996; Berry et al., 1996]. The nongenomic action of calcitriol involves a pertussis toxin-insensitive heterotrimeric G-protein [Le Mellay et al., 1997], whereas estradiol acts via a pertussis toxin-sensitive G-protein [Lieberherr

Abbreviations used: $[Ca^{2+}]i$, intracellular calcium concentration; G-protein, guanine nucleotide-binding regulatory proteins; GDPβS, guanosine 5′-O-2′-thio-diphosphate; GTPγS, guanosine 5′-*O*-3′-thiotriphosphate; $InsP_3$, inositol 1,4,5,-trisphosphate; PIP2, phosphatidylinositol 4,5,-diphosphate; PLC, phospholipase C; PTX, pertussis toxin; TBS, Tris-buffered saline.

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et al., 1993]. Although all nongenomic actions of calcitriol and estradiol involve various signaling pathways and different G-proteins, no information is available about the G-protein isoforms that take part in these processes.

Mammals have several types of G-proteins, which 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 [reviewed in Neer, 1995; Hildebrandt, 1997; Exton, 1997; Clapham and Neer, 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 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.

We have recently shown that calcitriol increases the mobilization of Ca^{2+} from the endoplasmic reticulum via activation of a PTX-insensitive G-protein coupled to PLC-β1, whereas estradiol activates a PTX-sensitive G-protein linked to PLC-β2 (21). We have now identified and characterized the subunits of the G-proteins involved in the formation of inositol 1,4,5 trisphosphate and the mobilization of Ca^{2+} in female rat osteoblasts in response to calcitriol and 17β-estradiol.

MATERIALS AND METHODS

Materials

ECL kit, Fura-2/AM, and L-3-phosphatidyl [2-^{3}H]inositol 4,5-diphosphate were purchased from Amersham Corp (Les Ulis, France). Polyclonal rabbit anti-G antibodies to Gαq/α11, Gαs, Gαi1→3, Gβ1–4, Gγ1, and control peptide for each G-protein subunit were obtained from Santa Cruz Biotechnology, Inc. (Tebu, Le Perray-en-Yvelines, France). Peroxidase-conjugated goat anti-rabbit IgG from BioRad (Ivry sur Seine, France). Calcitriol (1,25[OH] $_2D_3$) was a gift from Hoffman-La-Roche (Basel, Switzerland), and 17β-estradiol was purchased from Sigma (St. Quentin, Fallavier, France). Dulbecco's modified Eagle's essential medium (DMEM) without phenol red and fetal calf serum (FCS) were supplied by Eurobio (Paris, France). Calcitriol and 17β-estradiol were dissolved in ethanol; the final concentration of ethanol never exceeded 0.01%. This concentration of ethanol was without effect on intracellular calcium concentration and $InsP_3$ formation [Lieberherr, 1987; Grosse et al., 1993; Lieberherr et al., 1993].

Isolation and Culture of Female Rat Osteoblasts

Two-day-old female 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 for 4 days on rectangular glass coverslips or in Petri dishes (150 cm^2) in phenol red-free DMEM 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.

Preparation of Crude Plasma Membranes, Protein Separation, and Immunoblotting

Cells were washed three times 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 sec) at 40 KHz, and the homogenate was centrifuged for 10 min at 600*g* to remove nuclei. The supernatant was centrifuged at 100,000*g* for 60 min, and the supernatant was saved. The resulting membrane pellet was suspended in extraction buffer containing 0.3% Triton X-100 (w/w), left on ice for 60 min, and centrifuged again at 100,000*g* for 60 min. The resulting supernatant (solubilized membrane fraction) was collected. All the fractions (homogenate, cytosol, and membrane) were stored at -80°C. Protein was determined by the method of Bradford [1970].

Proteins were separated by SDS-polyacrylamide gel electrophoresis (13% resolving gel) in 25 mM Tris base pH 8.3, 192 mM glycine, 0.1% SDS, or by Tricine/SDS-PAGE to identify Gγ subunit. 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 the membrane was prevented by incubating the membranes in 10 mM Tris buffer, pH 7.5, containing 150 mM NaCl, 0.1% Tween-20, 2% bovine serum albumin, and 5% skim powder milk (TN-TBM buffer) with 1% normal goat serum for 1 h at room temperature. Membranes were incubated overnight at 4°C with polyclonal rabbit antibodies against $G\alpha_{q/11}$, $G\alpha i_{1\rightarrow 3}$, Gαs, Gβ, and Gγ1. The $G\alpha_{q/11}$ antibody was raised to a carboxy terminal peptide common to $G\alpha_q$ and $G\alpha_{11}$. The anti-$G\alpha i_{1->3}$ antibody was raised to a carboxy terminal peptide common to $G\alpha i_1$, $G\alpha i_2$, and $G\alpha i_3$, and the anti-Gβ antibody was directed against the amino terminus domain that is common to all four isoforms, Gβ1, GβGβ3, and Gβ4. The anti-Gαs antibody was raised to a specific amino terminal domain of the αs subunit. The anti-Gγ1 antibody was directed against a specific amino terminal peptide. The antibodies were used at: 0.2 µg/ml for Gαq/11, Gαi1→3 and Gαs, 0.3 µg/ml for Gβ and 0.25 µg/ml for Gγ. Unbound antibodies were removed by four washes with PBS; the antibodies bound to membrane were detected with peroxidase-conjugated goat anti-rabbit IgG (1 µg/ml) diluted in TN-TBM plus 0.2% normal goat serum, and revealed by enhanced chemiluminescence. The specificity of the antibodies was evaluated by incubating them with 100-fold excess of their control peptide for 3 h prior to use.

Phosphatidylinositol 4,5-Diphosphate Hydrolysis Assay

Phospholipid vesicles were prepared according to Hofmann and Majerus [1982], and assays were done essentially as described by Wu et al. [1992]. Diluted membranes (10 µl, 10 µg protein) in 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 40µl 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 preincubated for 2 h with 10µl 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 after adding 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 cells were washed with 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 $MgSO_4$.7H2O, 27.7 mM glucose, 1.25 mM $CaCl_2$, and 25 mM HEPES), and loaded with 1 µM Fura-2/AM for 30 min in the same buffer at room temperature. The glass coverslip carrying the confluent cells was inserted into a cuvette containing 2.5 ml Hanks' HEPES, pH 7.4 [Lieberherr, 1987]. The cuvette was placed in a thermostatted (37°C) Hitachi F-2000 spectrofluorimeter. Drugs and reagents were added directly to the cuvette under 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 [Grynkiewicz et al., 1985]. The dissociation constant for the Fura-2-Ca^{2+} complex was taken as 224 nM. The values for R_{max} and R_{min} were calculated from measurements made using 25 µM digitonin and 4 mM EGTA and enough Tris base to raise the pH to 8.3 or higher. Each measurement on Fura-2-loaded cells was followed by a parallel experiment under the same conditions with cells not loaded with Fura-2. We determined whether various subunits of different G-proteins were involved in the effects of calcitriol and 17β-estradiol on [Ca^{2+}]i by incubating rat osteoblasts for 5 min with 20 µg/ml saponin plus excess anti-G-protein antibody,

used at 10× higher than for Western blot, or nonimmune rabbit serum [Le Mellay et al., 1997]. Cells were washed thoroughly with Hanks' HEPES to remove saponin, and incubated with anti-G-protein antibody or nonimmune rabbit serum for 1 h at 37°C. Fura-2/AM was then added for 30 min. In some experiments, 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-G-protein antibodies at room temperature (antibody:peptide 1:100, according to the specification of the manufacturer) prior to use. Calcitriol and 17β-estradiol were used at 100 pM as this gave the greatest increase in $[Ca^{2+}]i$ in osteoblasts [Lieberherr, 1987; Lieberherr et al., 1993].

Statistical Analysis

The data were analysed 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

Western Blots of the G-Proteins in Osteoblasts

We looked for various G-protein subunits in the subcellular fractions (homogenate, membranes, and cytosol) of female rat osteoblasts (Fig. 1). The anti-carboxyl terminus $G\alpha_q/\alpha_{11}$ antibody detected two proteins which corresponded to $G\alpha_q$ and $G\alpha_{11}$ subunits in the membrane fraction. The anti-Gαs antibody detected a single 43-kDa band. The anti-Gαi1→3 antibody, which recognizes αi1, αi2, αi3 subunits, revealed a 40-kDa immunoreactive band. Western immunoblotting showed a 35-kDa immunoreactive band using the anti-$G\beta_{1->4}$ antibody, and a 8 kDa immunoreactive band using the anti-Gγ1 antibody.

G-Proteins Involved in the Effects of Calcitriol and Estradiol on Phosphatidylinositol 4,5-Diphosphate Hydrolysis

We used a cell-free membrane system to test the activity of the antibodies against G-proteins on the effect of calcitriol and 17β-estradiol on phosphatidylinositol 4,5 diphosphate (PIP_2) hydrolysis. Both steroids were used at 100 pM as this gave the greatest increase in 1,4,5 trisphosphate formation in osteoblasts [Grosse et al., 1993; Lieberherr et al., 1993]. Osteoblast membranes containing endogenous G-subunits

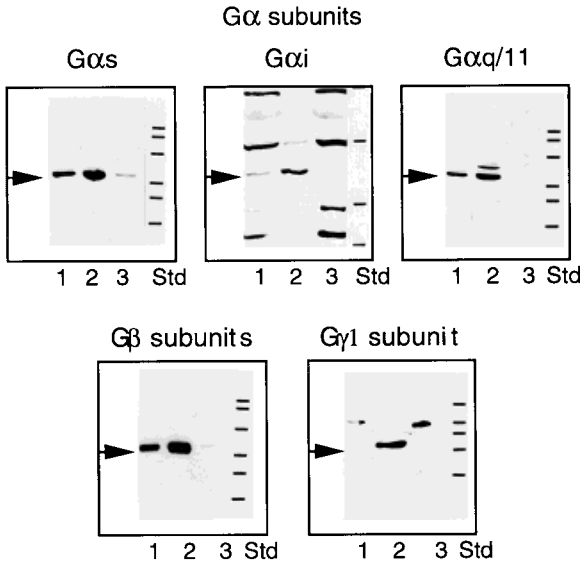

Fig. 1. Western blots of heterotrimeric G-proteins of female rat osteoblasts. Aliquots of subcellullar fractions, homogenate (1), membrane (2) and cytosol (3), were separated by electrophoresis on SDS-polyacrylamide gel, transferred to PVDF membranes, and probed with antibodies against subunits of G-proteins (arrows). 35-µg aliquots were used for $G\alpha_{q/11}$, Gαi, Gβ1→4, and Gγ1, and 80-µg aliquot for Gαs. Lines indicate the molecular masses of the standards from top to bottom: triosephosphate isomerase, 28 kDa; myoglobulin, 18 kDa; α-lactalbumin, 15.6 kDa; aprotinin, 7.6 kDa, and insulin, 3.6 kDa for Gγsubunits; phosphorylase B, 105 kDa; bovine serum albumin, 82 kDa; ovalbumin, 49 kDa; carbonic anhydrase, 33.3 kDa; soybean trypsin inhibitor, 28.6 kDa, and lysosyme, 19.4 kDa, for Gα and Gβ subunits. These results are representative of at least three cultures.

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. 2A,B). One hundred µM GDPβS inhibited the increase caused by 10 µM GTPγS (data not shown). GTPγS effects on $InsP_3$ formation induced by both steroids were dose-dependent (Fig. 2A,B). Osteoblast membranes were incubated with antibodies prior to stimulation with 100 µM GTPγS, the maximal active concentration on PIP_2 hydrolysis. Figure 2C shows the specificity and the activity of the various antibodies on InsP formation induced by 100 µM GTPγS. Anti-$G\alpha_{q/11}$, anti-Gβ, and anti-Gβ plus anti-Gγ1 antibodies inhibited the PIP_2 hydrolysis induced by GTPγS, whereas anti-Gαs and anti-Gαi antibodies did not. The $[^3H]$ $InsP_3$ concentration produced when membranes were incubated with both the anti-$G\alpha_{q/11}$ antibody and the blocking peptide or the anti-Gβ antibody and the blocking peptide was the same as that

obtained with preimmune serum (Fig. 2C). Figure 2D shows the inhibition of calcitriol-induced $InsP_3$ formation by anti-$G\alpha_{q/11}$ antibody and of estradiol-induced $InsP_3$ formation by anti-Gβ antibody or anti-Gβ antibody plus anti-Gγ1 antibody in the presence of 10 μM GTPγS (Fig. 2D). Anti-Gαi and anti-Gαs antibodies did not modify $InsP_3$ response to calcitriol or estradiol (Fig. 2E).

G-Proteins Involved in the Effects of Calcitriol and Estradiol on Intracellular Calcium Mobilization

The basal intracellular calcium concentration ($[Ca^{2+}]i$) in confluent female rat osteoblasts was 108 ± 5 nM, mean ± S.E., n = 12. Treatment of the cells with saponin for 5 min followed by incubation for 60 min with the anti-G protein in the absence of saponin did not alter the basal $[Ca^{2+}]i$. Nonimmune serum did not alter the basal $[Ca^{2+}]i$ or the $[Ca^{2+}]i$ response to the steroids. The transient increase in $[Ca^{2+}]i$ caused by 100 pM calcitriol or estradiol formed a sharp peak which fell rapidly after 15 s, but remained above the basal level (plateau phase) (25 ± 3%, mean ± S.E., n = 12) (Figs. 3 and 4). The calcitriol-induced increase in $[Ca^{2+}]i$ was reduced by anti-Gαq/11 antibody (−81 ± 2%, mean ± S.E., n = 6, $p < 0.001$) (Fig. 3). The residual increase was due to Ca^{2+} influx from the extracellular medium because this was totally blocked by incubating the cells for 30 s with 2 mM EGTA, a chelator of extracellular calcium (Fig. 3). The other antibodies had no effect (Fig. 3). The estradiol-induced increase in $[Ca^{2+}]i$ was reduced by anti-$G\beta_{1\rightarrow4}$ antibody, and anti-$G\beta_{1\rightarrow4}$ antibody plus anti-

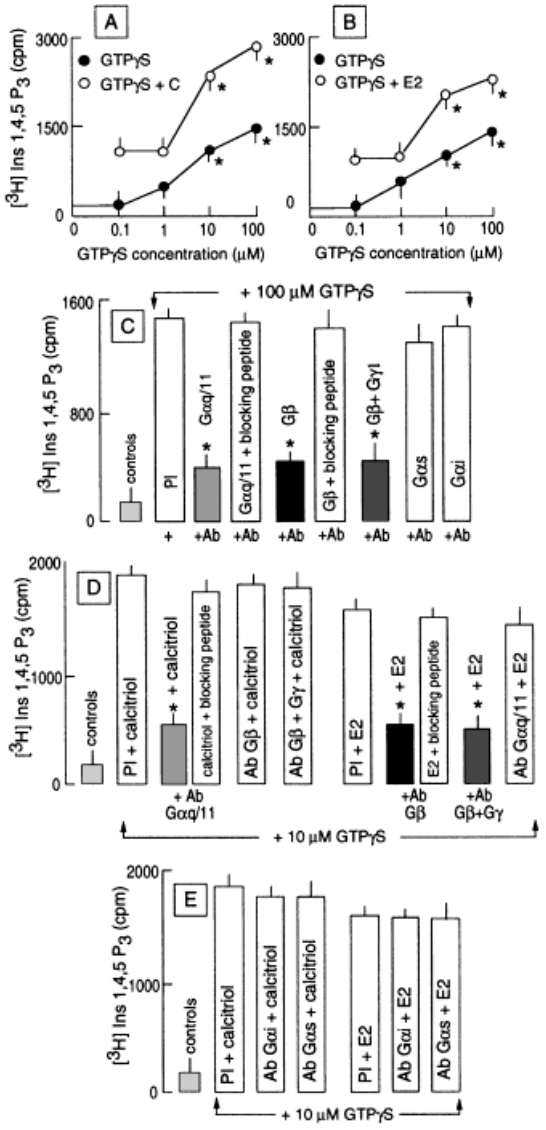

Fig. 2. Effects of anti-G-protein antibodies (Ab) on PIP_2 hydrolysis induced by calcitriol (C) and estradiol (E2) and/or GTPγS in osteoblast membranes. **A:** Effect of GTPγS on the hydrolysis of PIP_2 in the absence or presence of 100 pM calcitriol. **B:** Effect of GTPγS on the hydrolysis of PIP_2 in the absence or presence of 100 pM estradiol. **C:** 5 μg of osteoblast membranes were incubated for 2 h with 2 μg/ml anti-$G\alpha_{q/11}$ antibody, 3μg/ml anti-Gβ1→4 antibody, 2.5 μg/ml anti-Gγ1 antibody, anti-Gβ antibody plus anti-Gγ antibody, 2 μg/ml anti-Gαs antibody, 2 μg/ml anti-Gαi1→3-antibody or preimmune serum (PI). PIP2 hydrolysis was performed in the absence (controls) or presence 100 μM GTPγS. In some experiments, 5 μg of osteoblast membranes were incubated for 2 h with either 2 μg/ml anti-$G\alpha_{q/11}$ or the antibody plus 20 μg/ml blocking peptide or with either 3μg/ml anti-Gβ antibody or anti-Gβ antibody plus 30 μg/ml blocking peptide. **D:** 5 μg of osteoblast membranes were incubated for 2 h with 2 μg/ml anti-$G\alpha_{q/11}$ antibody, 2 μg/ml anti-$G\alpha_{q/11}$ antibody plus 20 μg/ml blocking peptide, 3 μg/ml anti-Gβ1→4 antibody, 3 μg/ml anti-Gβ antibody plus 30 μg/ml blocking peptide, 3 μg/ml anti-Gβ antibody plus 2.5 μg/ml anti-Gγ antibody or preimmune serum. PIP2 hydrolysis was performed in the presence 10 μM GTPγS and either 100 pM calcitriol or 100 pM estradiol. **E:** 5 μg of osteoblast membranes were incubated for 2 h with 2 μg/ml anti-Gαs antibody, 2 μg/ml anti-Gαi1→3-antibody or preimmune serum. PIP2 hydrolysis was performed in the presence 10 μM GTPγS and either 100 pM calcitriol or 100 pM estradiol. The PIP2 hydrolysis in control incubation with GTPγS and without membranes was subtracted from all values. Values are means ± S.E., n = 4, and are significantly different: A and B, from the basal level in the presence of GTPγS or GTPγS plus calcitriol or estradiol, $*P <$ 0.001; C, from the level of preimmune serum, $*P < 0.001$; D and E, from the level of 100 pM calcitriol plus PI or 100 pM estradiol plus PI, $*P < 0.001$, or from the level of anti-G-protein antibody plus control peptide in the presence of calcitriol or estradiol, $*P < 0.001$.

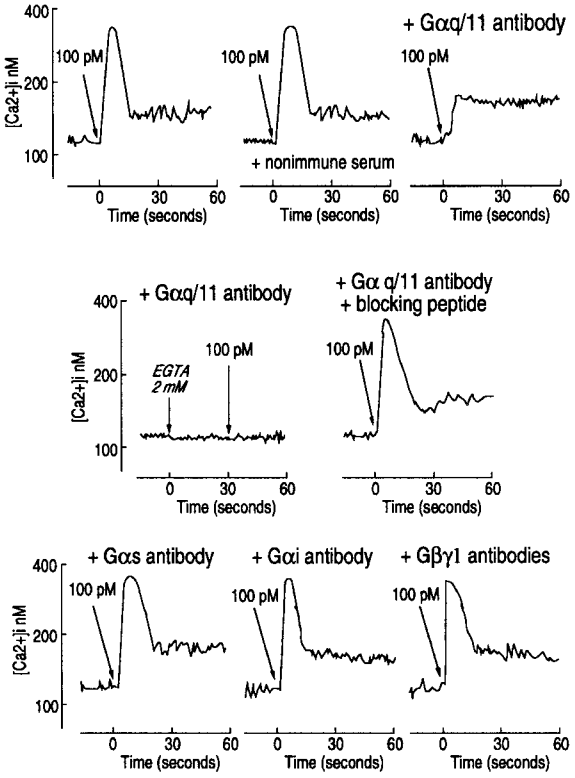

Fig. 3. Responses of female rat osteoblasts treated with antibodies against G-protein subunits to calcitriol. Cells were cultured, incubated with the G-protein antibodies, and loaded with Fura-2/AM as described. The concentrations of anti-G-protein antibodies were 2 μg/ml for Gαq/11, Gαi1→3 and Gαs, 3 μg/ml for Gβ1→4, and 2.5 μg/ml for Gγ1. Preimmune serum was used at concentrations corresponding to those of each anti-G-protein antibody. Only anti-$G\alpha_{q/11}$antibody blocked Ca^{2+} mobilization induced by 100 pM calcitriol. In some experiments, calcitriol was added 30 sec after 2 mM EGTA. These results are representative of at least six cultures for each experimental case.

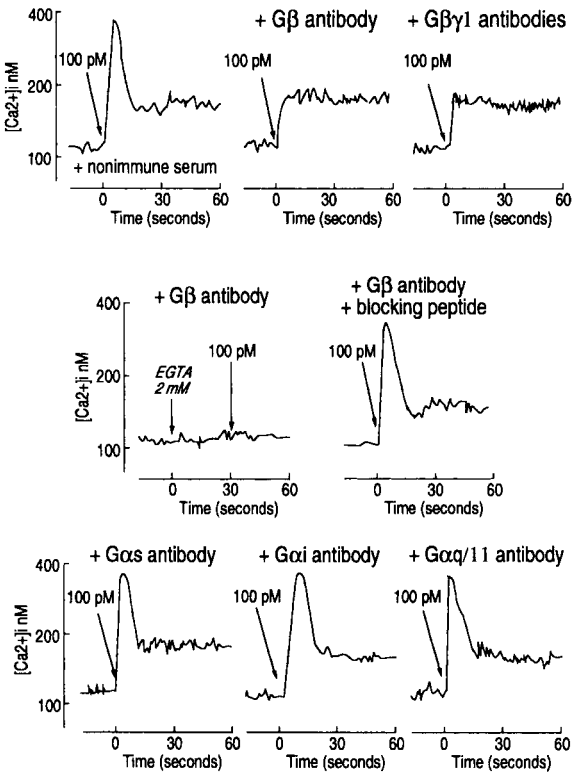

Fig. 4. Responses of female rat osteoblasts treated with antibodies against G-protein subunits to 17β-estradiol. Cells were cultured, incubated with the G-protein antibodies, and loaded with Fura-2/AM as described. The concentrations of anti-G-protein antibodies were 2 μg/ml $G\alpha_{q/11}$, Gαi1→3, and Gαs, 3 μg/ml for Gβ1→4, and 2.5 μg/ml for Gγ1. Preimmune serum was used at concentrations corresponding to those of each anti-G-protein antibody. Only anti-Gβγ1 and anti-Gβ antibodies blocked Ca^{2+} mobilization induced by 100 pM estradiol. In some experiments, estradiol was added 30 sec after 2 mM EGTA. These results are representative of at least six cultures for each experimental case.

Gγ1 antibody, but adding anti-Gγ1 antibody did not modify the response to anti-Gβ antibody: $-83 \pm 2\%$, mean ± S.E., $n = 6$, $p < 0.001$ for Gβ, and $-80 \pm 3\%$, mean ± S.E., $n = 6$, $p < 0.001$ for Gβ plus Gγ1 (Fig. 4). The residual increase was due to Ca^{2+} influx because this was totally blocked by incubating the cells for 30 sec with 2 mM EGTA. In contrast, anti-Gαi1→3, anti-Gαs, and anti-$G\alpha_q/\alpha_{11}$ antibody did not block the effect of 17β-estradiol on $[Ca^{2+}]i$ (Fig. 4).

DISCUSSION

The rapid actions of calcitriol and estradiol on $InsP_3$ formation and Ca^{2+} mobilization involve specific subunits of G-proteins. These are $G\alpha_{q/11}$ for calcitriol, and Gβor Gβγ for estradiol.

Female rat osteoblasts possess several subunits of heterotrimeric G-proteins: $G\alpha_{q/11}$ coupled to the PLC pathway, Gαs which are activators of cAMP pathway, Gαi which are involved in the inhibition of the adenylyl cyclase pathway and modulate several ion channels, Gβ and Gγ1 (see Fig. 1). We started with γ1 subunit as no information was available on the distribution of γ subunits in osteoblasts. Moreover, an anti-Gγ antibody broadly reactive with all γ subtypes did not exist because of the apparent heterogeneity in γ-subunit sequences. Our data show that osteoblasts possesss the γ1 subtype that has only been found in retina [Cali et al., 1992]. Further investigation is needed to identify other γ subtypes present in osteoblasts.

We have shown that calcitriol mobilizes intracellular Ca^{2+} from the intracellular Ca^{2+} stores by activating PLC-β1 linked to a PTX-insensitive G protein [Le Mellay et al., 1997]. The PTX-insensitive G-proteins that regulate phosphoinositide-specific PLC are members of the Gq family, which includes G_q, G_{11}, G_{14}, and $G_{15/16}$. The α subunits of this family show less than 50% sequence identity with the other G-protein α-subunits. $G_{q/11}$ are widely distributed in the tissues of vertebrates and invertebrates [Strathmann and Simon, 1990]. Although PLC-β isoenzymes (β1–>β4) may be activated by α subunits of the Gq family [Lee et al., 1992], $\alpha_{q/11}$ subunits seem to be the strongest activators of PLC-β1 [Blank et al., 1991; Taylor et al., 1991]. The calcitriol-induced increase in $InsP_3$ and $[Ca^{2+}]i$ is blocked by antibodies directed against the C terminal region of $G\alpha_{q/11}$, but not by anti-Gαs, anti-Gαi or anti-Gβγ antibody. Thus, calcitriol activates PLC-β1 via $G\alpha_{q/11}$, leading to an increase in $InsP_3$ formation and mobilization of Ca^{2+} from the endoplasmic reticulum in female rat osteoblasts.

The selective activation of PLC-β2 by 17β-estradiol involves a pertussis toxin-sensitive G-protein [Le Mellay et al., 1997]. Anti-$G\beta_{1->4}$ antibody and anti-$\beta_{1->4}$ antibody plus anti-γ1 antibody block the mobilization of Ca^{2+} from the endoplasmic reticulum and the formation of $InsP_3$ caused by 17β-estradiol in female rat osteoblasts. But neither anti-$G\alpha i_{1->3}$ nor $G\alpha_{q/11}$ antibody can do so. PLC are activated by PTX-sensitive G-proteins of the Gi and Go subtypes. However, attemps to demonstrate stimulation of PLC-β isoenzymes by the α-subunits of Go and Gi have generally been unsuccessful. Recent evidence indicates that the βγ-subunits of these proteins are responsible for PLC activation [Camps et al., 1992; Boyer e al., 1994]. But this does not rule out a possible effect of βγ-subunits released from members of the Gq family. Our results support the idea that the PLC-β2 linked to PTX-sensitive G-proteins is activated by βγ-subunits rather than by α subunits, and the βγ-subunits of PTX-sensitive G proteins preferentially activate PLC-β2 and, to a less extent, PLC-β1 [Camps et al., 1991]. The existence of five different forms of G-protein β-subunits and up to eight forms of γ-subunits [Cali et al., 1992] makes this a potentially complex system, since βγ-subunit dimers formed from different combinations of β and γ may differentially stimulate the PLC isoenzymes [reviewed in Hildebrandt, 1997; Clapham and Neer, 1997]. Whereas the β-subunits are very similar, with 80–90% sequence identity, the γ-subunits differ from each other in the prenyl group modifying the C-terminal cysteine [Casey, 1995]. Prenylation of γ is not necessary for βγ formation, but is necessary for membrane attachment of the βγ dimer and, in some cases, for association with the α-subunit [Casaey, 1995]. Yan and Gautam [1997] recently suggested that the βγ dimer effector specificity resides in their β-subunits. Our data suggest that γ1-subunit is not involved in the activation of βγ dimer by estradiol or GTPγS as InsP3 and calcium responses to estradiol were inhibited in a same extend by either anti-Gβ antibody or anti-Gβ antibody plus anti-Gγ1 antibody. But we used an anti-Gβ antibody raised against an amino terminus domain that is the same in all four forms of Gβ and an antibody raised against only one kind of γ-subunit in this study. Further investigation is needed to identify the β-subunit or the βγ dimer involved in the membrane effect of estradiol.

In conclusion, this finding that heterotrimeric G-proteins are directly involved in the membrane effects of calcitriol and estradiol may be an important step towards understanding the process, thus the likelihood of membrane receptors for steroid hormones. Since PLC-β1 is the target effector of $G\alpha_{q/11}$, whereas PLC-β2 is only activated by βγ-subunits, this may endow membrane receptor-specific responses with specificity in vivo. Thus, steroid ligand may activate a membrane-bound receptor and, simultaneously or consecutively, modulate the classic nuclear receptor. The resultant overall effect on a target cell may be the superimposition of the two receptor-mediated events, one with a rapid onset and the other more long-term. In other respects, calcitriol [Grosse et al., 1993] and estradiol [Lieberherr et al., 1993] increase DAG formation and $[Ca^{2+}]i$ in rat osteoblasts, suggesting that both steroids activate protein kinase C. Calcitriol and estradiol translocate within 5 sec PKC α, β and γ, which are activated by both DAG and Ca^{2+}, from the cytosol to the membrane in female rat osteoblasts (personal data). Morerover, calcitriol could rapidly activate mitogen-activated protein kinase (MAPK) via PKC activation leading to phosphorylation of transcription factors involved in cell differentiation and mitosis [Beno et al., 1995; Song et al., 1998]. Estradiol could stimulate the phosphorylation of cAMP response element

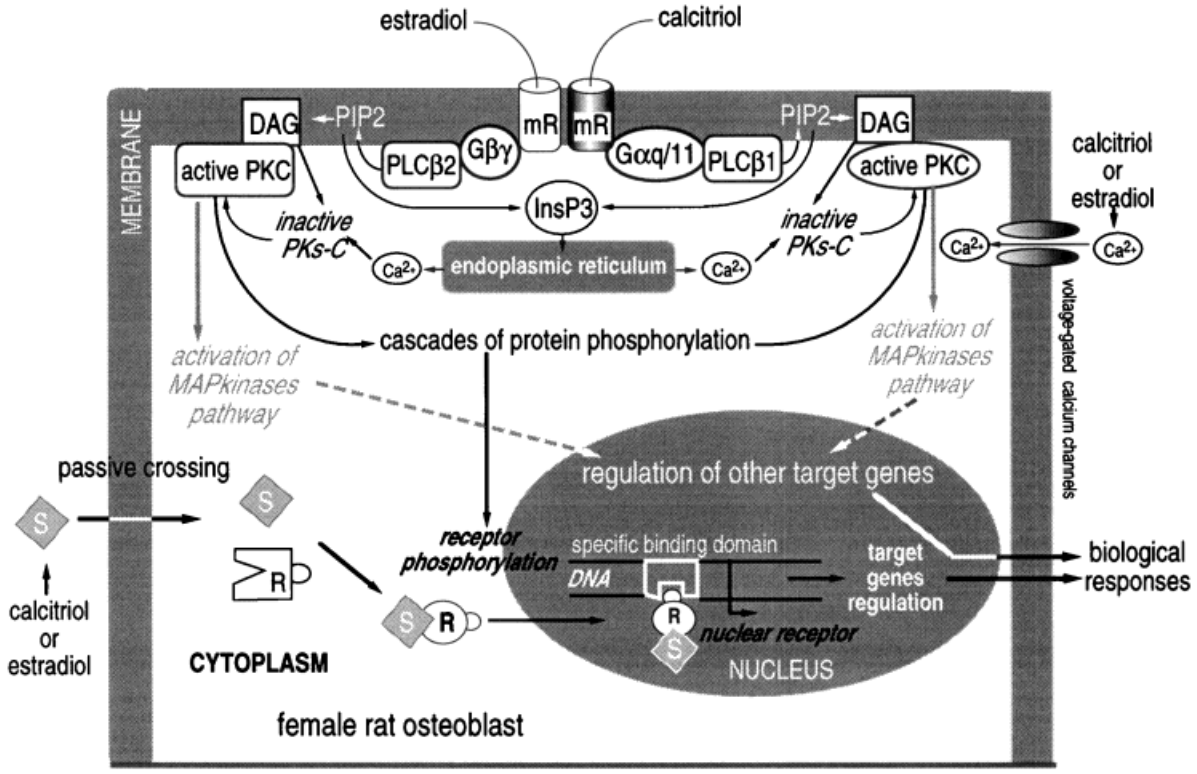

Fig. 5. Cross-talk between classical and non classical pathways of calcitriol and estradiol action in female rat osteoblast. Rapid nonclassical effects (within 5 sec), mediated through membrane receptor systems, include activation of Gαq/11 coupled to phospholipase C (PLC) β1 for calcitriol and Gβγ subunits linked to PLC β2 for estradiol leading to Ca^{2+} mobilization from the endoplasmic reticulum and inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) formation. Both steroids activate voltage-gated Ca^{2+} channels and translocate protein kinases C (PKC) α, β, and γ from cytosol to membrane. Interactions mediated through binding of calcitriol and estradiol to nuclear receptors (classical effects) modulate transcription specific target genes and require longer times (many hours to days). Cross-talk between both pathways may occur via either phosphorylation of various protein kinases (PKC and mitogen-activated protein kinases, MAPKinases) leading to transcriptional effects on early genes which do not require the nuclear receptors or direct phosphorylation of the nuclear receptors by PKC.

binding protein (CREBP) in rat brain and enhance transcription of genes which do not contain EREs, but do have CREs [Zhou et al., 1996]. $G\alpha_{q/11}$ and Gβγ subunits are known to activate the MAPK pathway in several systems [Della Roca et al., 1997]. Thus, knowledge of whether calcitriol and estradiol stimulate MAPK activity via $G\alpha_{q/11}$ or Gβγ subunits should identify two independent pathways for the effects of steroids: a classical pathway involving a nuclear receptor, and a membrane and intracellular pathway involving a membrane receptor (Fig. 5).

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