Phospholipase C β and Membrane Action of Calcitriol and Estradiol*

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We have shown that estrogens and calcitriol, the hormonally active form of vitamin D, increase the concentration of intracellular calcium ([Ca²⁺]ᵢ) within 5 s by mobilizing calcium from the endoplasmic reticulum and the formation of inositol 1,4,5-trisphosphate and diacylglycerol. Because the activation of effectors as phospholipase C (PLC) coupled to G-proteins is the early event in the signal transduction pathway leading to the inositol 1,4,5-trisphosphate formation and to [Ca²⁺]ᵢ increase, we described different PLC isoforms (β1, β2, γ1, and γ2, but not β4) in female rat osteoblasts using Western immunoblotting. The data showed that phospholipase C β was involved in the mobilization of Ca²⁺ from the endoplasmic reticulum of Fura-2-loaded confluent osteoblasts by calcitriol and 17β estradiol, and PLC γ was ineffective. The data also showed that only a PLC β1 linked to a Pertussis toxin-insensitive G-protein and a PLC β2 coupled to a Pertussis toxin-sensitive G-protein are involved in the effects of calcitriol and 17β estradiol on the mobilization of Ca²⁺ from intracellular Ca²⁺ stores. In conclusion, these results may be an important step toward understanding membrane effects of these steroids and may be an additional argument in favor of membrane receptors to steroid hormones.

An increase in the turnover of inositol lipids in response to receptor is one of the most important molecular mechanisms used by cells for transmembrane signaling. The initial event is the hydrolysis of phosphatidylinositol 4,5-bisphosphate, a reaction catalyzed by a phosphoinositide-specific phospholipase C (PLC), which generates two intracellular second messengers, inositol 1,4,5-trisphosphate and 1,2-diacylglycerol (1–4). Inositol 1,4,5-trisphosphate binds to specific receptors on the endoplasmic reticulum (5) and mobilizes intracellular calcium, whereas diacylglycerol activates protein kinase C (6), which results in increased phosphorylation of cellular proteins.

Molecular cloning has revealed at least three major families of PLC, β, γ, and δ (7–9). Each of these families occurs in a number of isoforms. The enzymes are classified on the basis of their size and their immunological and structural similarities. The PLC isoforms have two highly conserved domains, X and Y, which form the active site of the protein. PLC δ and PLC γ proteins differ from PLC β in that they have shorter C-terminal extensions past the end of the Y domain (9). This diversity among the PLC isoforms also extends to distinct mechanisms of regulation and function for the three PLC families. PLC γ is regulated via the phosphorylation of tyrosine residues between the X and Y domains by receptor tyrosine kinases (10–12). PLC β enzymes, of which there are four isoforms, PLC β1–4, are regulated via heterotrimeric G-proteins in response to an agonist binding to a receptor (13–15). The way in which PLC γ is regulated is not yet known, but enzyme activity is not affected by either the G-protein subunits or by receptor tyrosine kinases (16).

The activation of molecules as PLC is an early event in the signal transduction pathways leading to a variety of cellular responses, including metabolism, proliferation, secretion, and motility. We have shown that estrogens (17) and calcitriol (18, 19), the hormonally active form of vitamin D, increase the concentration of intracellular calcium within 5 s by mobilizing calcium from the endoplasmic reticulum and the formation of inositol 1,4,5-trisphosphate and diacylglycerol. This process involves the activation of a phospholipase C linked to a Pertussis toxin-sensitive G-protein for estradiol (17) and an as yet uncharacterized G-protein for calcitriol (20).

However, no information is presently available on the PLC present in osteoblasts, the cells responsible for osteogenesis, or about the PLC isoforms involved in the membrane effects of calcitriol and estradiol.

We have therefore described PLC isoforms in the osteoblasts of female rats. We have also identified the PLC isoenzymes involved in the rapid actions of calcitriol and 17β estradiol on the mobilization of calcium from the endoplasmic reticulum.

EXPERIMENTAL PROCEDURES

Materials—The ECL kit and Fura-2/AM were from Amersham Corp. Polyclonal rabbit anti-PLC antibodies to PLC β1, PLC β2, PLC β3, PLC β4, PLC γ1, and PLC γ2 and antigens raised against PLC β1, PLC β2, PLC β3, PLC γ1, and PLC γ2 were from Santa Cruz Biotechnology, Inc., and Tebu (Le Perray en Yvelines, France), and peroxidase-conjugated goat anti-rabbit IgG was from Bio-Rad (Ivry sur Seine, France). 1,25(OH)₂D₃ was from Hoffman-La Roche (Basel, Switzerland), and 17β estradiol was from Sigma, α-Minimum essential medium without phenol red and fetal calf serum were from Eurobio (Paris, France).

Isolation and Cell Culture—Two-day-old female Wistar rats were from Charles River Breeding Laboratories (St Aubin les Elbeufs, France). Osteoblasts were isolated from parietal bones of the newborn rats by sequential enzymatic digestion (21). These cells had the following osteoblast characteristics: high alkaline phosphatase activity, high type I collagen synthesis, a CAMP and intracellular calcium response to parathyroid hormone, and an osteoclastin response to 1,25-dihydroxyvitamin D₃.

Cells were grown on rectangular glass coverslips or in Petri dishes (100 cm²) for 4 days in phenol red-free α-minimum essential medium supplemented with 10% heat-inactivated fetal calf serum. Cells were then incubated for 72 h in phenol red-free medium containing 1% heat-inactivated fetal calf serum and transferred to serum-free medium 24 h before use.

Preparation of Plasma Membranes—Cells were washed three times with ice-cold phosphate-buffered saline, pH 7.4, then 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, 0.125 mM aprotinin, and 1 mM diithiothreitol). Cells were sonicated on ice (twice for 20 s at 40 kHz) and the homogenate was centrifuged for 10 min at 600 × g to remove nuclei. The remaining supernatant (cytosolic fraction) was stored at −80 °C. The supernatant (cytosol fraction) was saved. The pellet containing the plasma membranes was resuspended in extraction buffer containing 0.3% Triton X-100 (w/v), 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 fractions (homogenate without nuclei, cytosolic fraction, and membrane) were stored at −80 °C.

The brain was used as a positive control, because the cerebellum is rich in PLC β3, PLC β4 and PLC γ2, whereas the cerebral ventricles are rich in PLC β1, PLC β2 and PLC γ1 (22). Extracts of these two tissues were treated in the same way as the osteoblast homogenate.

Protein was determined by the method of Bradford (23) with bovine serum albumin as standard. Alkaline phosphatase activity, as an enzyme marker of the plasma membrane, was assayed as described by Lieberher et al. (24).

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 (25). They were then electrophoretically transferred to nitrocellulose membranes (Immobilon P). The membranes were blocked with 20% ethanol a 70°C for 2 h at 100 V (26). Specific binding to nitrocellulose was prevented by incubating the membranes in 50 mM Tris-buffered saline (TBS), pH 7.5, containing 150 mM NaCl, 5% skim milk powder, and 0.05% Tween 20 for 12 h at 4 °C. The membranes were then incubated with TBS with 0.1% Tween 20 and were then washed twice to remove saponin and incubated with the anti-PLC antibody or nonimmune rabbit serum, used at 1:100 dilutions (1:10 or 1:100 for Santa Cruz Biotechnology antibodies, according to the specifications of the manufacturer) prior to use.

Western Immunoblotting of the PLCs—The films were scanned with a Scanjet II CXT (Hewlett Packard) densitometer. The linear range of protein concentrations on ECL Western blots was 5–50 μg of proteins. All Western blots were done with 35 μg of proteins of each subcellular fraction for each tissue or cell. Western blotting showed a 150-kDa immunoreactive band in both tissues. Immunoblots probed with the PLC β2 antibody showed a 163-kDa immunoreactive band in osteoblasts and cerebellum (Fig. 1), which was mainly in the osteoblast and cerebellum membrane fractions (Fig. 2). There was a 153-kDa immunoreactive band in soluble and particulate fractions of brain and osteoblasts using the PLC β3 antibody (Fig. 1), mainly in the cytosolic fractions in both brain and osteoblasts (Fig. 2C). PLC β4 with an apparent molecular mass of 160 kDa was mainly in the membrane fraction of brain, but no immunoreactive band for PLC β4 was found in osteoblasts whatever the protein (35–100 μg) concentration and the anti-PLC antibody (0.5-5 μg/ml) concentration (Fig. 1) (data shown for 35 μg in Fig. 2). Immunoblots probed with the PLC γ1 antibody showed a 156-kDa immunoreactive band mainly in the cytosolic fractions of brain and osteoblasts (Figs. 1 and 2). Immunoblots with the PLC γ2 antibody revealed a 145-kDa immunoreactive band in both tissues. Although the signal intensities for this isoenzyme in the two subcellular fractions of the brain were the same, the greatest signal was obtained from the membrane fraction of osteoblasts (Figs. 1 and 2).

The competitive Western blot using polyclonal PLC β1, PLC β2, PLC β3, PLC γ1, and PLC γ2 antibodies and the antigens were raised against showed that the immunoreactivity
was completely abolished only when the antigen was used at 100 times the concentration used for the corresponding antibody (data not shown). This also showed that each of the antibodies was using its intended target isoform.

**PLC Isoenzymes Involved in the Membrane Effects of Calcitriol and Estradiol**—The basal intracellular calcium concentration ([Ca$^{2+}$]) in confluent female rat osteoblasts was 135 ± 5 nM (mean ± S.E.; n = 6). Pretreatment 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 alter the basal [Ca$^{2+}$]. Nonimmune serum did not alter the basal [Ca$^{2+}$], or the [Ca$^{2+}$] response to the steroids.

Fig. 3 shows the transient increase (Δ[Ca$^{2+}$]) = 160 ± 5 nM, mean ± S.E.; n = 6; p < 0.001) in [Ca$^{2+}$]i induced by 100 pm calcitriol, which is the concentration of calcitriol that has the most effect on this parameter (18). [Ca$^{2+}$i] dropped rapidly after 15 s but remained above the basal level (21 ± 2%, mean ± S.E.; n = 6; p < 0.001). The calcitriol-induced increase in [Ca$^{2+}$i] was partly inhibited by anti-PLC β1 antibody (Fig. 3A). The residual increase was due to a Ca$^{2+}$ influx from the extracellular medium because this remaining increase was totally blocked by preincubating the cells for 30 s with 2 mM EGTA (18). On the other hand, the antibodies to PLC β2, PLC β3, or PLC γ1 did not block the effect of calcitriol on [Ca$^{2+}$i] (Fig. 3, B and C).

Fig. 4 shows the transient increase (Δ[Ca$^{2+}$]) = 130 ± 4 nM, mean ± S.E.; n = 6; p < 0.001) in [Ca$^{2+}$i], induced by 100 pm β estradiol, which is the concentration of estradiol that has the most effect on this parameter (17). [Ca$^{2+}$i] dropped rapidly after 15 s but remained above the basal level (19 ± 1%, mean ± S.E.; n = 6; p < 0.001). The estradiol-induced increase in [Ca$^{2+}$i] was partly inhibited by the anti-PLC β2 antibody (Fig. 4A). The residual increase was due to a Ca$^{2+}$ influx from the extracellular medium because this remaining increase was totally blocked by preincubating the cells for 30 s with 2 mM EGTA (17). But anti-PLC β1, anti-PLC β3, anti-PLC γ1, and anti-PLC γ2 antibodies did not block the effect of estradiol on [Ca$^{2+}$i] (Fig. 4, B and C).

Polyclonal anti-PLC β1 and anti-PLC β2 antibodies were incubated for 2 h with their corresponding antigens or with the antigens used for producing the anti-PLC β3, anti-PLC γ1, and anti-PLC γ2 antibodies (antibody:antigen ratio, 1:10 or 1:100) before use. The inhibition of the calcitriol-induced increase in [Ca$^{2+}$i] due to the anti-PLC β1 antibody totally disappeared only when the anti-PLC β1 antibody was co-incubated with its antigen but not with the antigens corresponding to PLC β2, PLC β3, PLC γ1, or PLC γ2. Only the co-incubation with the PLC β2 antigen blocked the inhibition observed with the anti-PLC β2 antibody on the estradiol-induced increase in [Ca$^{2+}$i]. This occurred when the antigen was used at 100 times the concentration used for the corresponding antibody (data not shown).

**Effects of Pertussis Toxin on the Intracellular Calcium Response to Calcitriol and Estradiol**—Preincubation of the cells
for 16 h with Pertussis toxin did not alter the basal $[\text{Ca}^{2+}]_i$ or the $[\text{Ca}^{2+}]_i$ response to 1 pM-10 nM calcitriol (data not shown). In contrast, PTX partly blocked the response to 100 pM estradiol (Fig. 5). The residual increase disappeared when cells were incubated with 2 mM EGTA.

**DISCUSSION**

This is, to our knowledge, the first study showing a direct involvement of phospholipase C $\beta$ in the membrane actions of calcitriol or $17\beta$ estradiol in confluent female rat osteoblasts; PLC $\gamma$ is ineffective. The data also indicate that only PLC $\beta_1$ linked to a Pertussis toxin-insensitive G-protein and a PLC $\beta_2$ coupled to a Pertussis toxin-sensitive G protein are involved in the effects of calcitriol and $17\beta$ estradiol, respectively, on the mobilization of $\text{Ca}^{2+}$ from intracellular $\text{Ca}^{2+}$ stores.

Female rat osteoblasts possess several isoforms of PLC: $\beta_1$, $\beta_2$, $\beta_3$, $\gamma_1$, and $\gamma_2$, as shown by Western immunoblotting. Confluent female rat osteoblasts do not possess PLC $\beta_4$, which is present in the cerebellum (22) (see Fig. 3). This suggests that this isoenzyme may have a tissue and cellular specificity or that the gene encoding PLC $\beta_4$ is posttranslationally regulated. The gene may be expressed during the logarithmic phase of proliferation and not when the cells are confluent, corresponding to the differentiation of osteoblasts, or the gene may not be expressed in cells during proliferation and differentiation. These possibilities can be checked by *in situ* hybridization and Northern blot analysis of osteoblasts at different stages of maturation.

Most of the PLC $\beta_1$ and PLC $\beta_2$ in mature confluent osteoblasts is linked to the plasma membrane, whereas most PLC $\beta_3$ is in the cytosol. PLC $\beta_1$ and PLC $\beta_3$ are predominantly in the cytosol of brain, whereas PLC $\beta_2$ is in the plasma membrane. The relationship between membrane-bound and cytosolic PLC $\beta$ is controversial (29–31). It is not clear whether they are distinct enzymes or whether the same enzyme is distributed between the two intracellular pools. It is generally believed that only the membrane-bound enzyme is involved in receptor-mediated phosphatidylinositol metabolism. Our finding that PLC $\beta$ enzymes are distributed between soluble and mem-

**FIG. 3.** Responses of osteoblast intracellular calcium to calcitriol after pretreatment of the osteoblasts with antibodies against the PLC isoforms. Cells were cultured, loaded with Fura-2/AM, and incubated with PLC antibody as described under “Experimental Procedures.” In some experiments, calcitriol was added 30 s after 2 mM EGTA. These results are representative of at least six different cultures for each experimental case.

**FIG. 4.** Responses of osteoblast intracellular calcium to estradiol after pretreatment of the osteoblasts with antibodies against the PLC isoforms. Cells were cultured, loaded with Fura-2/AM, and incubated with PLC antibody as described under “Experimental Procedures.” In some experiments, $17\beta$ estradiol was added 30 s after 2 mM EGTA. These results are representative of at least six different cultures for each experimental case.

**FIG. 5.** Effects of Pertussis toxin on intracellular calcium responses to calcitriol and estradiol. Osteoblasts were incubated for 16 h with 100 ng/ml Pertussis toxin (PTX). Fura-2/AM loading and calcium measurements were carried out in the presence of PTX. In some experiments, $17\beta$ estradiol was added 30 s after 2 mM EGTA. These results are representative of at least six different cultures for each experimental case.
brane-bound subcellular fractions in osteoblasts indicates that the membrane-bound and cytosolic PLC \( \beta \) is the same enzyme. This is corroborated by our immunocytochemical data showing that PLC \( \beta 1 \) and \( \beta 2 \) are mostly membranous, whereas PLC \( \beta 3 \) is cytosolic in osteoblasts.\(^2\) The differential distribution of the enzymes may be a way of regulating enzyme activity that is comparable to the regulation of protein kinase C (6, 32) rather than an artifactual redistribution of the enzyme due to cell homogenization. The presence of PLC \( \gamma \) in osteoblasts is not surprising because these osteogenic cells respond to growth factors that possess tyrosine kinase domains (33).

We have previously shown that \( 17 \beta \) estradiol and calcitriol rapidly (within 5 s) increase the intracellular calcium concentration by mobilizing \( \text{Ca}^{2+} \) from the endoplasmic reticulum (17, 18) and by forming inositol 1,4,5-trisphosphate via a phospholipase C (17, 19, 20). Anti-PLC \( \beta 1 \) and anti-PLC \( \beta 2 \) antibodies inhibit the steroid-induced increase in \( [\text{Ca}^{2+}] \) in much the same way as do direct or indirect inhibitors of PLC (17, 19). Anti-PLC antibodies, like PLC inhibitors (17, 19), block only the part of the increase in \( [\text{Ca}^{2+}] \) that is due to the mobilization of \( \text{Ca}^{2+} \) from the endoplasmic reticulum. Moreover, the inhibition of the enzyme activity by anti-PLC antibodies totally disappears in competitive experiments when polyclonal PLC \( \beta 1 \) and PLC \( \beta 2 \) antibodies and the antigens they were raised against are used but not when the antigens corresponding to the other PLCs are used. This type of enzyme inhibition by selective antibodies against phosphoinositide-specific PLC has also been demonstrated in fresh bovine erythrocytes (34). One explanation of the noninhibiting effects of the other anti-PLC antibodies on the increase in \( [\text{Ca}^{2+}] \) induced by the steroids may be that most antibodies do not classically inhibit enzyme activity. On the other hand, because the inhibition by the anti-PLC \( \beta 1 \) and anti-PLC \( \beta 2 \) antibodies can be reversed only by their antigens, the possible blockade of the activity of the other isoenzymes by their respective antibodies cannot be excluded. The inhibitory effect of polyclonal antibodies against PLC \( \beta 1 \) or PLC \( \beta 2 \) may be best explained by one of two hypotheses: (i) inhibition of the enzyme activity may result from a stereochemical effect of the binding of the antibody to an epitope at or near the active site of the enzyme; or (ii) alternatively, binding of the antibody to a site remote from the active site in three-dimensional space may lead to a conformational change of the enzyme, rendering it incapable of substrate binding or hydrolysis. It is clear, however, that the anti-PLC \( \beta \) antibody binds to a site of the enzyme that is critical to maintaining the correct geometry of the active site. Further studies are needed to distinguish between these hypotheses, and it will be of interest to identify the site on the PLC protein recognized by the inhibitory antibodies.

It is very likely that only PLC \( \beta \) are involved in the membrane action of these steroids because only PLC \( \beta \) types may be regulated via heterotrimeric G-proteins in response to agonists binding to receptors (1, 7, 8). This study shows that the PLC \( \beta 1 \) involved in the membrane action of calcitriol is linked to a Pertussis toxin-insensitive G-protein, whereas the PLC \( \beta 2 \) implicated in the membrane effect of estradiol is coupled to a Pertussis toxin-sensitive G-protein. The heterotrimeric G-proteins, which transduce a signal from a hormone-bound receptor to a variety of downstream effectors, form a large family of homologous proteins classified according to the amino acid sequence of their \( \alpha \)-subunits (35–37). G-proteins have been divided into two types based on their sensitivity to the Bordella PTX (7, 38). The PTX-sensitive G-proteins are substrates for growth factor receptor protein-tyrosine kinases (45).

Finally, these results, showing that both calcitriol and estradiol use PLC \( \beta \) to increase the intracellular calcium concentration via inositol 1,4,5-trisphosphate formation (17, 19), may be an important step toward understanding the membrane effects of these steroids. These PLC \( \beta \) enzymes are also linked to two classes of G-proteins, one insensitive to Pertussis toxin, as for calcitriol, the other linked to a Pertussis toxin-sensitive G-protein, as for estradiol. These findings may be an additional argument in favor of membrane receptors for steroid hormones (46–52).

Acknowledgments—We gratefully acknowledge Dr. Owen Parkes for careful review of the English in the manuscript.

REFERENCES


\(^2\) V. Le Mellay, B. Grosse, and M. Lieberherr, unpublished data.