Calcitriol transmembrane signalling: regulation of rat muscle phospholipase D activity

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Abstract In rat skeletal muscle, calcitriol, the hormonal form of vitamin D_3, rapidly stimulates the biphasic formation of diacylglycerol (DAG), the second phase being independent of phosphoinositide hydrolysis driven by phospholipase C. In this work we showed that the effect of calcitriol on the second phase of DAG formation was totally inhibited in the absence of extracellular Ca^{2+} and by the Ca^{2+}-channel blockers nifedipine and verapamil, whereas the Ca^{2+} ionophore A23184, similar to calcitriol, increased DAG formation by 100%. GTPγS, which activates G protein-mediated signals, mimicked the effects of the hormone while GDPβS, an inhibitor of G proteins, suppressed calcitriol-induced DAG formation. To elucidate the metabolic pathway of the late phase of DAG production, we examined the contribution of phospholipase D (PLD), which acts on phosphatidylcholine (PC) generating phosphatidic acid that is converted to DAG by a phosphatidate phosphohydrolase. In [^{3}H]arachidonate-labeled muscle, calcitriol increased [^{3}H]phosphatidylethanol (PEt) formation in the presence of ethanol, a reaction specific for PLD. The effects of the hormone were time- and dose-dependent with maximum PEt levels achieved at 10^{-9} M. The phorbol ester TPA also stimulated PEt formation. The combination of calcitriol and TPA was more effective than either compound alone. In rat muscle, calcitriol increased PKC activity in a time-dependent fashion. Bisindolymaleimide, a selective inhibitor of the enzyme, completely suppressed TPA-induced PEt and attenuated the effects of the hormone. These results provide the first evidence concerning calcitriol stimulation of the hydrolysis of PC in a mammalian tissue through a phospholipase D catalyzed mechanism involving Ca^{2+}, protein kinase C, and G proteins.—**Facchinetti, M. M., R. Boland, and A. R. de Boland.** Calcitriol transmembrane signalling: regulation of rat muscle phospholipase D activity. *J. Lipid Res.* 1998. **39:** 197–204.

Supplementary key words rat skeletal muscle • calcitriol • diacylglycerol • phospholipase D • phosphatidylethanol • protein kinase C • G proteins

Calcitriol, the hormonally active metabolite of vitamin D_3 [1,25$(OH)_2D_3$], exerts its biological activities through specific intracellular receptors which are nuclear transcription factors of the steroid–thyroid receptor gene superfamily (1). Interaction of calcitriol with its nuclear receptor results in binding of the complex to specific promoter regions of DNA, which in turn regulates gene transcription (2). Regulation of gene expression by calcitriol involves a growing number of genes and gene products that include genes that encode proteins that regulate Ca^{2+} transport such as calbindins D28K (3) and D9K (4), calcitonin (5), preparathyroid hormone (6), calmodulin-binding proteins (7), protein kinase C (8), and Ca^{2+}-ATPase (9).

Recent studies suggest that several effects of calcitriol in a variety of tissues occur very rapidly and are not blocked by inhibitors of transcription (10–12). In skeletal muscle, calcitriol exerts direct effects on Ca^{2+} fluxes independent of gene activation. In chick and rat cultured muscle cells (13) and chick skeletal and cardiac muscle (14, 15) the hormone has been shown to induce a rapid (within 1 min) stimulation of Ca^{2+} uptake through voltage-operated calcium-channels, effects that are mimicked by agonists of cAMP- and PKC-dependent pathways (16–18). Recent evidence shows that the hormone increases cAMP levels, adenylyl cyclase activity, and translocates PKC activity from cytosol to membranes (17, 18), suggesting that phosphorylation through PKA- and PKC-dependent pathways would be the mode by which calcitriol modulates Ca^{2+}-channel activity in muscle. Moreover, evidence for an interaction or cross-talk between the calcitriol-modulated PKA and PKC pathways was obtained (19). In various cell

Abbreviations: DAG, diacylglycerol; PLD, phospholipase D; PEt, phosphatidylethanol; PI-PLC, phosphoinositide phospholipase C; IP_3, inositol trisphosphate; PC, phosphatidylcholine; TPA, 12-O-tetradecanoyl phorbol-13-acetate; PA, phosphatidic acid; PKC, protein kinase C; PKA, protein kinase A; PLA_2, phospholipase A_2; GTPγS, guanosine 5′-O-(3-thio)triphosphate; GDP βS, 5′-O-(3-thio)diphosphate; KHG, Krebs-Henseleit buffer containing 2% glucose; PMSF, phenylmethylsulfonyl fluoride.

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types, membrane effects of calcitriol include activation of phospholipases C (20–22) and A_2 (23). We have recently shown in rat skeletal muscle that the hormone stimulates the hydrolysis of phosphoinositides by phospholipase C (PI-PLC) with the resultant production of the second messengers inositol trisphosphate (IP_3) and diacylglycerol (DAG) (24).

The formation of DAG was shown to be biphasic, the latter phase being independent of PI-PLC activation. It is now accepted that the second elevation of DAG in response to various agonists results from signal-induced hydrolysis of phosphatidylcholine (PC) (25). The purpose of this work was to identify the mechanism by which calcitriol induces a second phase of DAG production in mammalian muscle.

MATERIALS AND METHODS

Materials

Calcitriol, $25OHD_3$ and $24,25\ (OH)_2D_3$ were kindly donated by Dr. M. Uskokovic (Hoffmann La Roche Co., Nutley, NJ). [^{3}H]arachidonate (76 Ci/mmol) was obtained from New England Nuclear (Chicago, IL). GTPγS, GDPβS, and other reagents were from Sigma Chemical Co. (St. Louis, MO).

Animals

Wistar rats (3-months-old) were fed with standard rat food (1.2% Ca; 1.0% phosphorous), given water ad libitum, and maintained on a 12-h light–12-h dark cycle. Animals were killed by cervical dislocation. Thigh muscle from back legs were excised, cleaned from fat and connective tissue, cut in slices (1.5–2 mm thickness, 20 mm length), and placed in cold Krebs-Henseleit buffer containing 2% glucose (KHG) (26).

Muscle labeling

To evaluate the action of calcitriol on the formation of DAG, muscle slices were labeled with [^{3}H]arachidonate (1 μCi/ml) in KHG for 2 h at 37°C under O_2/CO_2 (95%/5%) with shaking; the tissue was then extensively washed with KHG and preincubated in the same medium for 10 min at 37°C before treatments or subjected to homogenization for membrane isolation as described below.

Measurement of diacylglycerol

Muscle prelabeled with [^{3}H]arachidonate was incubated in KHG containing calcitriol (10^{-9} m, dissolved in ethanol) for 2 min at 37°C. Ethanol alone (<0.1%) was added to the control samples. To terminate incubations, muscle slices were placed in 3 ml ice-cold chloroform–methanol 2:1 (v/v). After homogenization, lipids were extracted according to Folch, Lees, and Sloane Stanley (27). DAG was isolated by one-dimensional TLC on silica gel G plates developed using hexane–diethyl ether–acetic acid 75:25:4 (v/v) as solvent system. Lipids were visualized by iodine staining and identified by comparison with standards. Labeled DAG was scraped off and its radioactivity was measured by liquid scintillation spectrometry with Aquasol.

To investigate the involvement of guanine nucleotide binding proteins (G-proteins) in calcitriol-dependent DAG production, [^{3}H]arachidonate prelabeled muscle was homogenized in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 250 mm sucrose, 2 mM dithiothreitol, 0.3 mM paramethylsulfonylfluoride, leupeptin (20 μg/ml), and aprotinin (20 μg/ml) followed by centrifugation for 20 min at 12000 *g*. Aliquots of the supernatant (cytosol + microsomes; 0.8–1 mg protein), were incubated at 37°C in a final assay volume of 200 μl containing 0.5 μM $CaCl_2$ (200 nM free calcium), 1 μM $MgCl_2$, 100 GTPγS or 100 μM GDPβS in the presence or absence of calcitriol (10^{-9} M) for 2 min. The assay was stopped by addition of 3 ml ice-cold chloroform–methanol 2:1 (v/v). DAG was determined as described above. Protein content was determined by the procedure of Lowry et al. (28).

Assay of PLD activity

Rat muscle slices or membranes (to evaluate the effects of GDPβS) prelabeled with [^{3}H]arachidonate and washed with KHG containing 0.1% bovine serum albumin were incubated in the same medium in the presence of 1.5% ethanol for 20 min prior to the addition of calcitriol (10^{-8}–10^{-11} m; 1–10 min). To stop treatment, muscle slices were placed in chloroform–methanol 2:1 (v/v) followed by homogenization. Lipids were extracted according to Folch (27); aliquots were taken to measure total [^{3}H]lipids and spotted on silica gel G plates. The plates were developed according to Listcovich (29) using a solvent system consisting of ethyl acetate–isooctane–acetic acid–water 13:2:3:10 (v/v). This solvent system allowed separation of PA (phosphatidic acid, R_f = 0.24), PEt (phosphatidylethanol, R_f = 0.34), neutral lipids (R_f = 0.95–1.0), and phospholipids (R_f = 0). The lipids were visualized and processed as described above.

Protein kinase C activity

Rat muscle slices were pre-equilibrated 10 min in KHG, followed by treatments. The tissue was homogenized for 40 sec with an Ultraturrax (Jank and Kunkel, Staufen, FRG) homogenizer using 500 μl of homogenization buffer (20 mM Tris-HCl, pH 7.4, 0.33 M sucrose, 1 mm EGTA, 0.7 mm $CaCl_2$, 20 mm NaF, 0.5 nm

phenylmethylsulfonyl fluoride (PMSF), 1 mm dithiothreitol, 40 μg/ml leupeptin and aprotinin). Protein kinase C activity was determined in muscle homogenates by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into the threonine group of a specific peptide sustrate (PRO-LEU-SER-ARG-THR-LEU-SER-VAL-ALA-ALA-LYS-LYS) for PKC (30). PKC activity was assayed in 80 μl of a solution containing 50 mm Tris-HCl, pH 7.5, 15 mm $MgCl_2$, 0.7 mm $CaCl_2$, 1 mm EGTA, 12.5 mm NaF, 50 μg/ml leupeptine, 0.2 mm PMSF, 75 μm binding peptide, 20 μm [γ-^{32}P]ATP and sample. Incubations were carried out at 30°C for 5 min. The reaction was terminated by transference of radioactive material to an ion-exchange-chromatography paper (Whatman-P81), and then washed three times in 30% (v/v) acetic acid containing 1% H_3PO_4 for 10 min, and once with ethanol. The paper was dried, immersed in Aquasol, and the bound radioactivity was measured in a scintillation counter.

Statistical analysis

The statistical significance of the data was evaluated using Student's *t*-test (31).

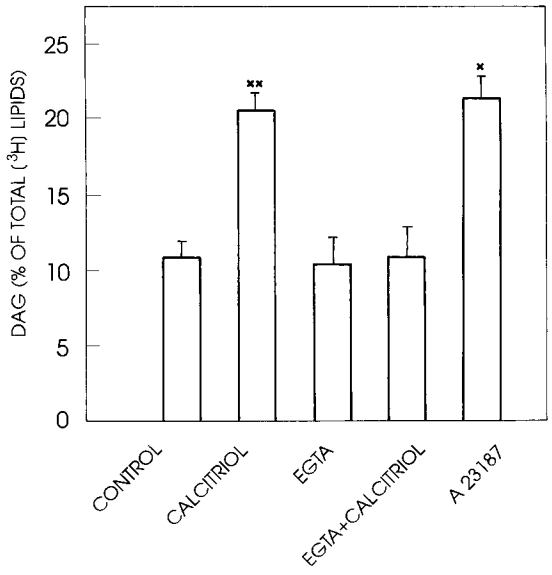

Fig. 1. Effects of the calcium chelator EGTA on the second phase of calcitriol-induced DAG formation. [^{3}H]arachidonate-labeled rat skeletal muscle slices preincubated in Krebs-Henseleit-0.2% glucose were exposed to calcitriol (10^{-9} M) in the presence or absence of EGTA (5 mM) or the Ca^{2+}-ionophore A23187 (1 μM) for 2 min. The treatment was terminated by placing the muscle tissue in ice-cold methanol. Lipids were extracted with Cl_3CH–CH_3OH 2:1 (v/v), and [^{3}H]DAG was separated by TLC as indicated in Methods. Values are the means ± SD (n = 4) of three separate experiments. *$P < 0.005$; **$P < 0.001$, with respect to control.

RESULTS

This study was carried out to further examine phospholipid metabolism in rat skeletal muscle treated with calcitriol. In a previous study we demonstrated that the hormone induced a biphasic formation of DAG peaking at 15 sec and 2 min, the early phase only being abolished by neomycin, a specific inhibitor of phosphoinositide hydrolysis (24). We now show that the second phase of hormone-generated DAG (2 min) in rat skeletal muscle is dependent on extracellular Ca^{2+}, and is potentially inhibited by the Ca^{2+} chelator EGTA and is mimicked by the Ca^{2+} ionophore A23187 (**Fig. 1**). In the absence of extracellular Ca^{2+}, the ionophore failed to generate the second peak of DAG (data not shown). **Figure 2** shows that hormone generation of DAG at 2 min was also effectively abolished by the Ca^{2+}-channel blockers nifedipine (5 μm) and verapamil (50 μm), thus demonstrating dependency on a rise in intracellular calcium levels by the sustained increase of DAG induced by calcitriol.

To investigate the participation of a guanine nucleotide binding protein (G protein) in the calcitriol-induced DAG delayed phase, we examined the effects of an activator and an inhibitor of G proteins on hormone-dependent DAG release. To that end, we used muscle membranes (microsomes + cytosol) prelabeled with [^{3}H]arachidonate. As shown in **Fig. 3,** GTPγS (100 μm), a non-hydrolyzable analogue of GTP that leads to a permanent activation of G proteins, increased DAG production by 150% over basal values. The non-

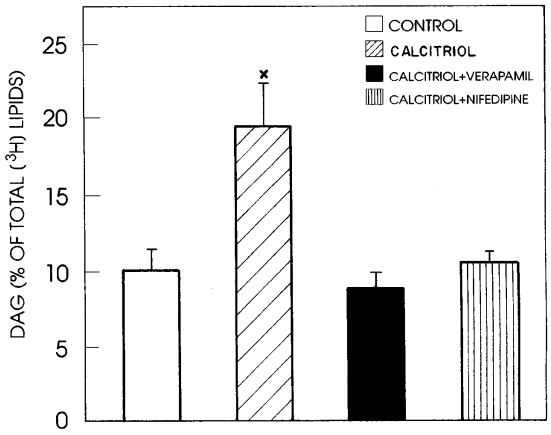

Fig. 2. Effects of Ca^{2+}-channel blockers on the second phase of calcitriol-induced muscle DAG formation. [^{3}H]arachidonate-labeled rat skeletal muscle was treated with calcitriol (10^{-9} M, 2 min) in the presence or absence of nifedipine (5 μM) or verapamil (10 μM). [^{3}H]DAG was measured as described in the legend to Fig. 1. Values are the means ± SD (n = 4) of three separate experiments. *$P < 0.01$.

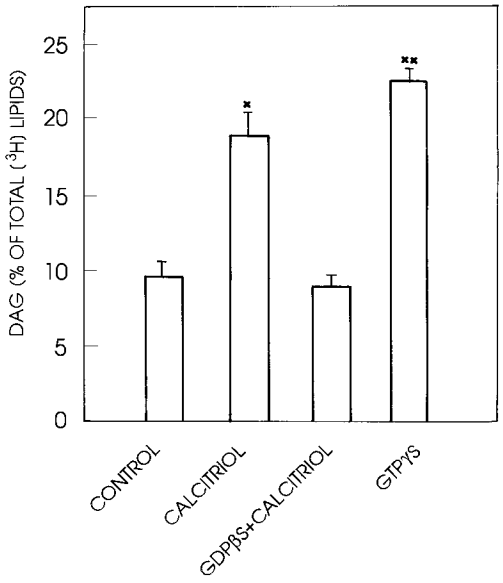

Fig. 3. Effects of guanine nucleotide analogs on the DAG-delayed phase induced by calcitriol in rat skeletal muscle membranes. Membranes isolated from rat muscle prelabeled with [^{3}H]arachidonate were exposed to GTPγS (100 μM) or 10^{-9} m calcitriol in the presence or absence of GDPβS (100 μM) for 2 min. [^{3}H]DAG was determined as described in the legend to Fig. 1. Values are the mean ± SD (n = 3) of three separate experiments. *$P < 0.05$; **$P < 0.01$, with respect to control.

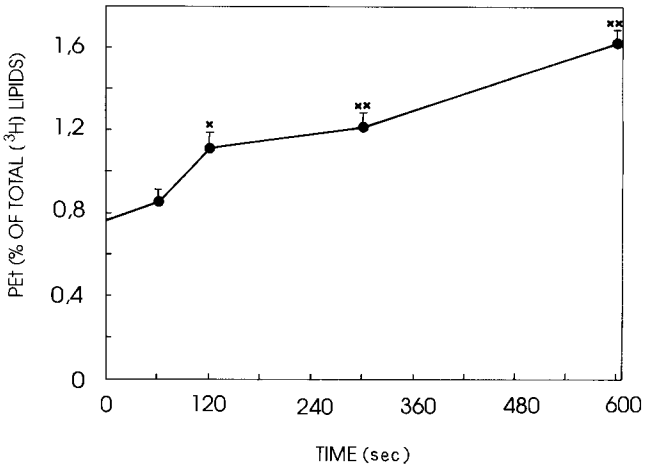

Fig. 4. Time-course of calcitriol-stimulated PEt formation in rat skeletal muscle. Muscle slices prelabeled with [^{3}H]arachidonate were preincubated with Krebs-Henseleit-0.2% glucose containing 1.5% ethanol for 20 min at 37°C. Calcitriol (10^{-9} M) was then added for the indicated times. Muscle slices were placed in ice-cold methanol, followed by lipid extraction and measurement of [^{3}H]PEt as indicated in Methods. Each point represents the mean ± SD (n = 3) of three separate experiments. *$P < 0.005$; **$P < 0.001$, with respect to control.

hydrolyzable analogue of GDP, GDPβS (100 μm), known to inhibit G protein-mediated events, completely abolished calcitriol-dependent DAG release at 2 min.

To investigate the involvement of PLD on calcitriol-induced sustained phase of DAG production, PLD activity was measured, based on the unique ability of the enzyme to catalyze transphosphatidylation in which the phosphatidyl group can be transferred to a nucleophile, such as ethanol, thus producing phosphatidylethanol [PEt] (32). In [^{3}H]arachidonate-labeled rat skeletal muscle, incubated in the presence of 1.5% ethanol, calcitriol induced a time-dependent accumulation of [^{3}H]PEt. As shown in **Fig. 4**, [^{3}H]PEt increased in a linear manner up to 10 min. The dose–response relationship of hormone-stimulated [^{3}H]PEt accumulation was also examined (**Fig. 5**). Calcitriol induced a concentration-dependent production of PEt, with maximal stimulation achieved at 10^{-9} m (115% of control values). The effect of calcitriol on PLD activity was specific, as other physiological metabolites of vitamin D_3, $25OHD_3$, $24,25(OH)_2D_3$, and $1\alpha OHD_3$ failed to produce PEt accumulation (**Table 1**).

Direct evidence that the neomycin-resistant second phase of DAG production induced by calcitriol was not derived from PLC-mediated hydrolysis of PC was obtained from measurements of [^{3}H]arachidonate-labeled DAG release in the presence of 0.5 μm neomycin and 1.5% ethanol (to block conversion of PA to DAG). Under these conditions, calcitriol did not modify basal levels of [^{3}H]DAG at 2 min (11.20 ± 0.70 and 12.14 ± 0.97% of total [^{3}H]lipids for control and 10^{-9} m calcitriol, respectively) ruling out the involvement of PC-PLC activity in the second phase of hormone-induced DAG formation. Furthermore, in mammalian tissues, most PLC enzymes have been shown to be reactive only with phosphoinositides, not with PC (25).

Additional proof of the involvement of G proteins in the calcitriol-dependent late phase of DAG associated to PLD was furnished by the observation that in muscle membranes prelabeled with [^{3}H]arachidonate, GDPβS blocks the formation of PEt in response to the hormone (0.85 ± 0.10, 1.98 ± 0.05, and 0.92 ± 0.07% total [^{3}H]lipids for control, 1 nM calcitriol, and 1 nM calcitriol + 100 μM GDPβS-treated membranes, respectively).

To investigate the role of PKC in calcitriol-induced PLD activity, rat muscle was exposed to the phorbol ester TPA (12-O-tetradecanoyl phorbol-13-acetate). As shown in **Fig. 6**, TPA (100 nM) and calcitriol (10^{-9} M) each stimulated PEt accumulation (140% and 170% above basal values/10 min, respectively). The response to the combined stimulation with TPA and calcitriol was significantly greater (+260%) than each individual response.

A protein kinase C assay, utilizing the in vitro phosphorylation of a specific PKC peptide substrate was

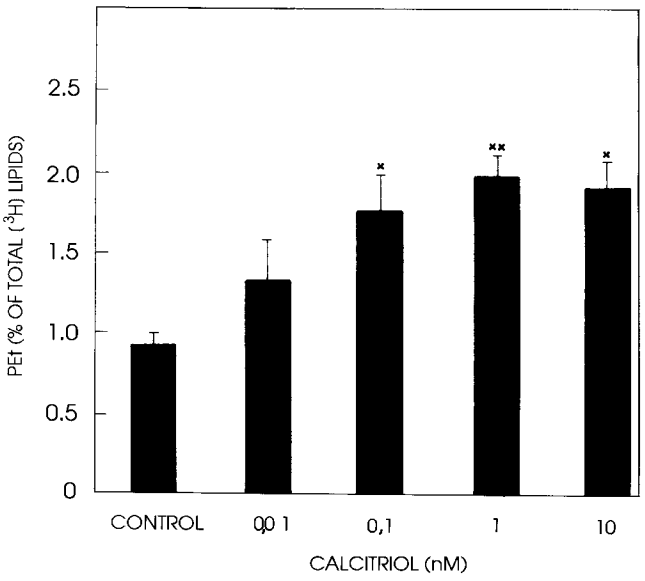

Fig. 5. Concentration-dependent stimulation of PEt formation by calcitriol in rat skeletal muscle. Muscle slices prelabeled with [^{3}H]arachidonate were preincubated with Krebs-Henseleit-0.2% glucose containing 1.5% ethanol for 20 min at 37°C. Calcitriol was then added at the indicated doses for 10 min. [^{3}H]PEt was measured as described in the legend to Fig. 4. Each point represents the mean ± SD (n = 3) of three separate experiments. * $P <$ 0.005; ** $P < 0.0025$, with respect to control.

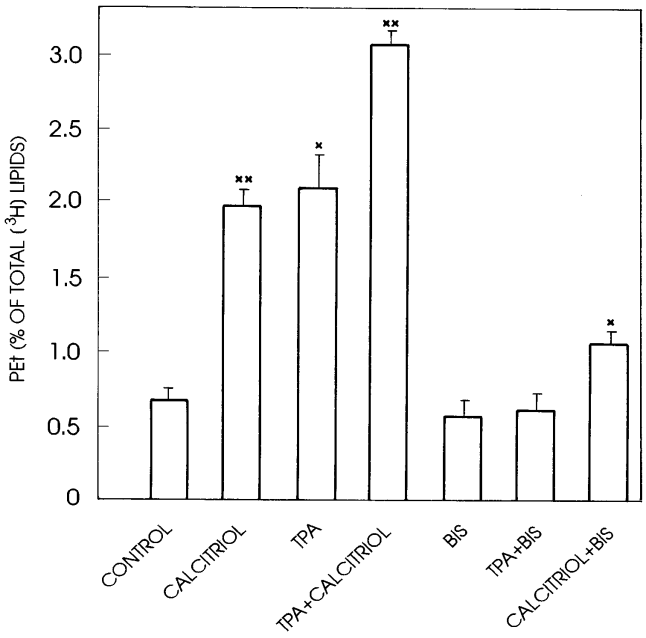

Fig. 6. Effects of TPA, calcitriol, and bisindolylmaleimide on [^{3}H]PEt formation in rat skeletal muscle. Muscle slices prelabeled with [^{3}H]arachidonate were treated for 10 min with TPA (100 nM), calcitriol (10^{-9} M), and bisindolylmaleimide (70 nM) alone or in combination in the presence of 1.5% ethanol. [^{3}H]PEt was measured as described in the legend to Fig. 4. Each point represents the mean ± SD (n = 3) of three separate experiments. * $P <$ 0.01; ** $P < 0.005$, with respect to control.

used to investigate the influence of calcitriol on muscle PKC activity. As shown in **Table 2,** stimulation with calcitriol (10^{-9} m) increased muscle PKC activity in a time-dependent fashion with maximal stimulation achieved after 1 min of hormone exposure (+2-fold). To determine the extent of PKC involvement in hormone-induced PLD activation, the PKC specific inhibitor bisindolylmaleimide (70 nm) was tested for its ability to inhibit TPA and calcitriol PEt production. Bisindolymaleimide completely suppressed TPA effects but only decreased hormone-dependent accumulation of PEt by approximately 70%.

TABLE 1. Specificity of calcitriol effects on PEt formation in rat skeletal muscle

Treatment	[^{3}H]PEt
	% total [^{3}H]lipids
Control	0.91 ± 0.04
$25OHD_3$	0.90 ± 0.11
$1\alpha OHD_3$	0.87 ± 0.15
$24,25(OH)_2D_3$	0.99 ± 0.13
$1,25(OH)_2D_3$	2.02 ± 0.08[a]

Muscle slices were labeled and treated as in the legend to Fig. 4. $25OHD_3$ (5×10^{-8} m, $1\alpha OHD_3$ (5×10^{-9} m), $24,25(OH)_2D_3$ (calcitriol, 10^{-9} m) were then added for 10 min in the presence of 1.5% ethanol. Each point represents the mean ± SD (n = 3) of three separate experiments.

[a] $P < 0.005$, with respect to control.

DISCUSSION

The regulation of the production of diacylglycerol, the endogenous activator of protein kinase C, is critical to the mechanism by which the secosteroid hormone calcitriol induces calcium channel activation (18) and cell proliferation (33). Many cells produce DAG in a biphasic manner, involving an initial and usually transient rise in DAG correlating with phosphoinositide-specific phospholipase C activation followed by a sustained increase in DAG derived from phosphatidylcholine (PC) hydrolysis (24, 34, 35). In the present study we showed that the sustained increase in DAG induced by calcitriol in rat skeletal muscle is dependent on the influx of extracellular calcium and is mediated by a GTP-binding protein that was mimicked by a calcium ionophore and GTPγS and was suppressed by EGTA, calcium-channel blockers, and GDPβS.

The relative contributions of PC-phospholipase C (PC-PLC) and phospholipase D (PLD) to delayed DAG forma-

TABLE 2. Time course of the calcitriol effects on PKC activity in rat skeletal muscle

Time	PKC Activity
min	*pmol/mg protein*
0	1.40 ± 0.15
0.5	2.21 ± 0.11[a]
1	4.15 ± 0.28[b]
2	2.68 ± 0.13[a]
3	2.30 ± 0.18[a]
5	2.95 ± 0.21[a]

Muscle slices from 3-month-old rats were incubated with or without (control) calcitriol (10^{-9} M) during the indicated times. The tissue was then homogenized and PKC activity was determined by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into a specific peptide substrate as described in Materials and Methods. Each point represents the mean ± SD (n = 4) of three separate experiments.

[a] $P < 0.05$; [b] $P < 0.025$, with respect to control.

tion vary greatly among cells. Phospholipase D-mediated hydrolysis of PC predominates in some cell types (32, 36) whereas in other cells, DAG is formed directly by PLC-mediated hydrolysis of PC (37, 38). Phospholipase D catalyzes hydrolytic cleavage of the terminal diester bond of phospholipids, resulting in the direct formation of phosphatidic acid (PA) and the respective base (39). The phosphatidic acid produced may act directly in downstream functions or serve as a precursor for the production of DAG by a PA phosphohydrolase. The major finding of this study was that calcitriol stimulated PC cleavage by PLD in rat skeletal muscle. The hormone increased in a time- and dose-dependent manner PEt formation, in the presence of ethanol, a reaction specific of PLD. In avian myoblasts, calcitriol also stimulates the biphasic formation of DAG, the first phase of DAG production coinciding with hormone-stimulated inositol trisphosphate formation (20) and the second phase arising from PC hydrolysis driven by phospholipase D (22).

The biochemical mechanism underlying signal-induced activation of PLD remains unclear. Various intracellular components such as PKC, tyrosine kinases, and small molecular weight G proteins, particularly ARF and Rho family proteins, have been implicated as regulators of PLD activity (34, 35, 39–42). Accordingly, in the present study, the effects of guanine nucleotide analogues described above were observed in muscle membrane preparations containing soluble factors. Our results also indicate that PKC activation is needed, at least in part, for hormone activation of PLD in rat muscle. We found that calcitriol-induced PEt response was partially suppressed by the PKC inhibitor bisindolylmaleimide. Furthermore, an increase in PKC activity was observed in response to calcitriol in rat muscle. Stimulation of PLD by phorbol esters has been involved in providing a second phase of increased DAG levels by PKC-dependent as well as independent mechanisms (43). The results presented here show that treatment of muscle with TPA increases PLD activity and suggest that this effect is mediated by PKC as PEt production is completely abolished by bisindolylmaleimide. There is evidence suggesting that multiple PLD enzymes with different kinetic properties and substrate specificities are present in various tissues in different intracellular components (25). It is therefore possible that simultaneous activation of PKC-dependent and PKC-independent isoforms of PLD by calcitriol takes place in rat skeletal muscle. Alternatively, different PKC isozymes with different sensitivity to inhibition by bisindolylmaleimide might be involved in PLD activation by TPA and calcitriol. Because the delayed phase of calcitriol-induced diacylglycerol production is exclusively dependent on calcium, it seems likely that both Ca^{2+} and the DAG produced by hormone activation of phosphoinositide-specific PLC (24) might act cooperatively to activate PLD in rat skeletal muscle.

Early work from our laboratory has shown that calcitriol also activates PLA_2 in muscle cells (23), generating arachidonic acid which serves as the rate-limiting precursor for the synthesis of prostaglandins and leukotrienes. Stimulation of PLD activity by prostaglandins $F_2\alpha$ and D_2 has been reported in various cell types (44–46). The possibility that calcitriol response to PLD activation is due to arachidonic acid metabolites with their potential contribution to cellular regulation in rat skeletal muscle remains to be investigated.

In summary, these results provide the first demonstration of PLD activation by calcitriol in a mammalian tissue, namely skeletal muscle. Specifically, the data indicate that the hormone stimulates muscle PLD through a mechanism that is dependent on extracellular Ca^{2+}, partially dependent on PKC activation and involves a G protein.

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