Activation of Phospholipase D by Endothelin-1 in Rat Myometrium. Role of Calcium and Protein Kinase C

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ABSTRACT
In rat myometrium labeled with [3H]myristic acid, endothelin (ET)-1 via ETA receptors stimulated, in the presence of 0.3% butanol, the formation of [3H]phosphatidylbutanol ([3H]PBut) as a result of phospholipase D activity. Fluoroaluminates increased [3H]PBut generation, which indicated that a heterotrimeric G protein was involved. The ET-1 effect was insensitive to pertussis toxin and was rapidly desensitized. The calcium ionophore ionomycin as well as 4β-phorbol 12-myristate-13-acetate and 4β-phorbol 12,13-dibutyrate also stimulated [3H]PBut production. Protein kinase C (PKC) inhibition, particularly with Ro-31–8220, and down-regulation of PKC by 4β-phorbol 12-myristate-13-acetate, abrogated 4β-phorbol 12,13-dibutyrate responses but partially reduced (50%) ET-1 and ionomycin stimulatory effects. [3H]PBut production induced by ionomycin depended on Ca2+ influx, whereas that induced by 4β-phorbol 12,13-dibutyrate did not. Decrease of extracellular Ca2+ partially reduced (60%) ET-1 stimulation that was additionally attenuated (75%) by chelerythrine, a PKC inhibitor. The data indicate that in myometrium, phospholipase D was activated by PKC and Ca2+, which both contribute at least partially to ET-1-mediated phospholipase D activation.

Endothelins are a family of 21 amino acid peptides that includes ET-1, ET-2 and ET-3. In addition to their vasoconstrictive activity, ETs induce various physiological effects that are mediated by distinct receptors with different specificities for the three peptides. The ETA receptor is characterized by the rank order of potency ET-1 > ET-2 > ET-3, the ETB receptor displays equal affinities for the three ET isoforms, whereas ETC receptor preferentially binds ET-3. Molecular characterization of ET receptors has revealed that they belong to the G-protein-coupled receptor superfamily. The mechanisms of ET action involve second messenger generation via different signal transduction processes. These include activation of the PLC degrading PtdInsP2 with an increased production of InsP3 and diacylglycerol, inhibition or activation of adenyl cyclase and activation of phospholipase A2. Recent observations also concern ET-1 interaction with the PLD pathway (Huggins et al., 1993; Masaki et al., 1994; Sokolovsky, 1994).

Evidence is accumulating that receptor-mediated hydrolysis of phospholipids by PLD is an important signaling pathway in many biological systems. PLD hydrolyzes PC with the production of PA. PA may serve as a potential lipid second messenger and/or constitute a source for diacylglycerol or lysophosphatic acid (for reviews, see Cockcroft, 1992; Exton, 1994). The molecular details of the mechanisms involved in receptor signaling to PLD are incompletely defined. Besides heterotrimeric G proteins, a modulatory role for PKC and Ca2+ has been reported in the regulation of PLD activity (Cockcroft, 1992; Exton, 1994; Liscovitch et al., 1993). Activation of PLD has also been mediated by small G proteins (Bourgoin et al., 1995; Cockcroft et al., 1994; Malcolm et al., 1994), cytosolic factors (Bourgoin et al., 1995; Lambeth et al., 1995; Singer et al., 1996) and tyrosine kinases (Bourgoin and Grinstein, 1992).

The ability of ET-1 to contract isolated rat myometrium has recently been documented and binding sites for ET/Sarafotoxin have been identified in myometrial preparations from different species (Bousso-Mittler et al., 1989; Breuiller-Fouché et al., 1994). We have previously reported that in estrogen-treated rat myometrium ET-1 interacts with a specific ETA receptor, which results in both activation of the PLC/PtdInsP2 transducing system through a pertussis toxin-insensitive G protein, and inhibition of the adenyl cyclase system through a pertussis toxin-sensitive G protein (Dokhac et al., 1994). Preliminary data (Dokhac et al., 1995) indicated that in the myometrium ETA receptors stimulated, in the presence of 0.3% butanol, the formation of [3H]phosphatidylbutanol ([3H]PBut) as a result of phospholipase D activity. Fluoroaluminates increased [3H]PBut generation, which indicated that a heterotrimeric G protein was involved. The ET-1 effect was insensitive to pertussis toxin and was rapidly desensitized. The calcium ionophore ionomycin as well as 4β-phorbol 12-myristate-13-acetate and 4β-phorbol 12,13-dibutyrate also stimulated [3H]PBut production. Protein kinase C (PKC) inhibition, particularly with Ro-31–8220, and down-regulation of PKC by 4β-phorbol 12-myristate-13-acetate, abrogated 4β-phorbol 12,13-dibutyrate responses but partially reduced (50%) ET-1 and ionomycin stimulatory effects. [3H]PBut production induced by ionomycin depended on Ca2+ influx, whereas that induced by 4β-phorbol 12,13-dibutyrate did not. Decrease of extracellular Ca2+ partially reduced (60%) ET-1 stimulation that was additionally attenuated (75%) by chelerythrine, a PKC inhibitor. The data indicate that in myometrium, phospholipase D was activated by PKC and Ca2+, which both contribute at least partially to ET-1-mediated phospholipase D activation.

ABBREVIATIONS: PLD, phospholipase D; ET, endothelin; PBut, phosphatidylbutanol; PMA, 4β-phorbol 12 myristate-13-acetate; PDBu, 4β-phorbol 12,13-dibutyrate; PKC, protein kinase C; OAG, oleoyl-2-acetyl-sn-glycerol; PLC, phospholipase C; PtdInsP2, phosphatidylinositol 4,5-bisphosphate; PA, phosphatic acid; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; InsP3, inositol 1,4,5 trisphosphate; EGTA, ethyleneglycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid.
also displays stimulatory effects on the PLD pathway. We now extend these observations by studying in more detail the receptor-mediated events at the level of PLD activation. The data further revealed that the two major signals which originated from the breakdown of PtdInsP$_2$ by PLC, namely increased [Ca$^{2+}$], and PKC activation, are important regulators of PLD activity and that both signals largely contribute to ET-1-mediated PLD activation.

Materials and Methods

Chemicals. [3H]Myristic acid (30–40 Ci/mmol) was provided by N.E.N. (Les Ulis, France); endothelin-1, endothelin-3, and BQ123 were provided by Neosystem (Strasbourg, France); β-estradiol 3-benzoate, bovine serum albumin, ionomycin, PC, PS, PI, PE, PMA, PDBu, OAG, chelerythrine, calphostin C and H7 were from Sigma (St. Louis, MO); PBut was from Avanti Polar Lipids (Alabaster, AL), pertussis toxin from List Biological Laboratories (Campbell, CA) and Silica Gel 60 plates from Merck (Darmstadt, Germany). Ro-31–8220 was generously provided by Dr. Bradshaw (Roche Ltd. Hertfordshire, United Kingdom). The ethanolic solution of [3H]myristic acid was evaporated under N$_2$ and the residue was dissolved in NH$_4$OH. All other chemicals were of the highest grade available.

Animals and tissue processing. Immature Wistar female rats (4–5 weeks of age) were treated with 30 μg of estradiol for 2 days and were sacrificed by decapitation the following day. Uteri were removed, and the myometrium was prepared free of endometrium as described previously (Amiot et al., 1993; Dokhac et al., 1994).

PLD assay. The activity of PLD was determined in [3H]myristic acid-labeled myometrium by measuring the formation of [3H]PA and [3H]P-But in the absence and presence of 0.3% butanol, respectively. [3H]P-But, which is formed exclusively through the PLD-catalyzed transphosphatidylation reaction, is considered to be a specific marker of PLD activity (Billah and Anthes, 1990; Liscovitch et al., 1993). Myometrial strips (about 25 mg) were equilibrated for 20 min in 5 ml of Krebs-Ringer bicarbonate buffer (pH 7.4) containing (in mM): NaCl, 117; KCl, 4.7; MgSO$_4$, 1.1; KHPO$_4$, 1.2; NaHCO$_3$, 2.4; CaCl$_2$, 0.8 and glucose, 1 (gas phase 95% O$_2$–5% CO$_2$) under constant agitation. The tissues were then incubated with 8 μCi/ml of [3H]myristic acid in 600 μl of fresh buffer for 5 h by which time the incorporation of [3H]myristic acid into phospholipids had reached a plateau (data not shown). Tissues were then washed once with 5 ml of nonradioactive buffer containing 1 mg/ml of serum albumin, then twice with buffer without serum albumin. After another 20-min incubation in 5 ml of buffer, myometrial strips were transferred to 1 ml of fresh buffer and incubated with 0.3% butanol for 10 min before exposure to the indicated agents. The reactions were stopped at the time indicated in the legends to figures by the rapid immersion of myometrial strips in liquid nitrogen, and lipids were extracted by a modification of the method of Bligh and Dyer (1959). The lipids were homogenized in 1.8 ml of chloroform/methanol/HCl (50:100:1, v/v/v) and left at room temperature for 30 min. One-half milliliter of H$_2$O was then added and, after a brief homogenization, the monophasic step by the addition of 0.6 ml of 2 M KCl and 0.6 ml of chloroform. After a vigorous mixing, phases were separated by a 10-min centrifugation at 1000 × g and the aqueous phase was removed. The chloroform extract was dried with a speed vacuum concentrator and was then reconstituted in 50 μl of methanol/chloroform (95:5, v/v). Relevant standards were added to each sample before thin-layer chromatography on heat-activated precoated 20 × 20 cm silica gel plates. To determine the incorporation of radiolabeled myristic acid into various phospholipids, plates were developed by three consecutive solvent systems with intermittent drying (Irvine et al., 1984): the first run with petrol ether/acetone (90:30; v/v), with use of the full length of the plate (16 cm); the second run in CHCl$_3$/methanol/acetone/H$_2$O (75:60:9:0.9; v/v/v/v) up to 11.5 cm; and the third run in ethyl acetate/acetone/H$_2$O/acetic acid (40:40:2:1; v/v/v/v), up to 13 cm. PC, (R$_f$, 0.01), PI (R$_f$, 0.32), PS (R$_f$, 0.42), PE (R$_f$, 0.78) were well separated from both neutral lipids and free myristic acid which comigrated at the solvent front. To analyze the production of [3H]P-But and [3H]PA, plates were developed in the organic phase of a mixture of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (13:2:3:10; v/v/v/v). In this solvent system (Van Blitterswijk et al., 1991), PBut (R$_f$, 0.5) and PA (R$_f$, 0.26) were separated from each other and from both PC (R$_f$, 0.01) and neutral lipids, the latter compounds migrating at the solvent front. PC was separated from PE but comigrated with two other phospholipids, PS and PI. After the appropriate runs, the plates were stained with iodine vapor and were analyzed, after iodine sublimation, by a computerized radioactivity scanner. The production of [3H]PA and [3H]P-But was expressed as the percentage of the radioactivity in PC obtained from the same sample.

Data analysis. The results are expressed as the mean ± S.E. and were analyzed statistically by the Student’s t test. P < .05 was considered significant.

Results

Distribution of [3H]myristic acid in total lipid extracts of myometrium. Analysis of [3H]labeled lipid extracts obtained from myometrial strips incubated with [3H]myristic acid revealed (table 1) that 80% of [3H]myristic acid was associated with neutral lipids versus 20% of the label incorporated into phospholipids. The major labeled phospholipid was PC, which accounted for 97% of the total labeled phospholipids. PE (contaminated with PA) was next but represented at most 2% of the label, whereas less than 0.5% of the label was associated with PS plus PI. The results are consistent with observations made for other tissues (Cockcroft, 1992; Exton, 1994; Liscovitch et al., 1993) and clearly indicate that in the myometrium [3H]myristic acid preferentially labeled the lipid domain of PC and that this fatty acid was not readily incorporated into phosphoinositides. In subsequent experiments, with thin-layer chromatography with a developing system (Van Blitterswijk et al., 1991) that allowed an adequate separation of PA from PC, it was found that the label associated with PA represented 0.56 ± 0.07% of label in PC (see legend to fig. 1).

Time-dependent accumulation of [3H]PA and [3H]P-But induced by ET-1. Figure 1A shows that addition of 0.2 μM ET-1 to [3H]myristic acid-labeled myometrial strips rapidly increased [3H]PA formation. A significant increase was detected within 1 min and reached a maximum at 5 min with a formation of [3H]PA amounting to 0.8% label in PC. The

<table>
<thead>
<tr>
<th>Cellular Lipids</th>
<th>[3H]Myristic Acid Incorporated</th>
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<tbody>
<tr>
<td>A. Neutral lipids</td>
<td>1,222,160</td>
</tr>
<tr>
<td>B. Phospholipids</td>
<td>305,540</td>
</tr>
<tr>
<td>PC</td>
<td>296,374 (97%)</td>
</tr>
<tr>
<td>PI + PS</td>
<td>1,450 (&lt;0.5%)</td>
</tr>
<tr>
<td>PE + PA</td>
<td>7,640 (2%)</td>
</tr>
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</table>
Dose-dependent effects of ET-1 and ET-3 on [3H]P-But accumulation. Effect of BQ123. ET-1-evoked [3H]P-But accumulation, as determined by 10 min [3H]P-But accumulation, was dose dependent with EC₅₀ = 50 ± 5.2 nM and a maximal response at 0.2 μM ET-1 (fig. 2). ET-3 also stimulated [3H]-P-But accumulation, but with a rightward shift of its concentration-response curve and a stimulatory effect at 10 μM that did not exceed 50% of the response triggered by ET-1. The rank order of potency ET-1 >> ET-3 was consistent with an ETₐ receptor (Huggins et al., 1993; Masaki et al., 1994) coupled to PLD activation. This interpretation was confirmed by the ability of BQ123 (Eguchi et al., 1992), a selective ETₐ receptor antagonist, to suppress ET-1-mediated [3H]P-But accumulation.

Desensitization of the [3H]P-But response to ET-1. As shown above (fig. 1), after a linear increase, the formation of [3H]P-But caused by ET-1 rapidly ceased. Considering that [3H]P-But is metabolically stable, the plateau phase of its formation in response to ET-1 tended to indicate that PLD was transiently activated. To address the possibility of a desensitization phenomenon, myometrial strips were exposed to 0.2 μM ET-1 for various times in the absence of butanol so that no [3H]P-But was formed. The alcohol was then added during the last 10 min of ET-1 treatment to determine any subsequent ET-1-mediated PLD activation. It was previously verified that during the 5- to 30-min pretreatment, ET-1 was not degraded in the medium (Dokhac et al., 1994). Data in figure 3 clearly show that, compared with the nontreated preparation, pretreatment with ET-1 caused a progressive reduction in the stimulatory effect of the peptide in terms of [3H]P-But accumulation. After a 5-min pretreatment with ET-1, the formation of [3H]P-But was reduced by

![Image](https://via.placeholder.com/150)

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**Fig. 1.** Time course of ET-1-induced [3H]PA and [3H]P-But accumulation. [3H]Myristic acid-labeled myometrial strips were stimulated with 0.2 μM ET-1 in the absence (A) or presence (B) of 0.3% butanol, added 10 min before ET-1. Incubations were stopped at the indicated times. [3H]P-But (●) and [3H]PA (○) production was estimated as described under “Materials and Methods” and expressed as % of label in PC. Values were obtained after subtraction of basal values: 0.65% and 0.57% of label in PC for [3H]PA in (A) and (B), respectively, and 0.2% of label in PC for [3H]P-But. Data represent the mean of six independent experiments, each done in duplicate, and the standard error did not exceed 10% of mean.

[3H]PA level triggered by ET-1 remained elevated for at least 10 min and then declined progressively with time. With this procedure, the source of PA is ambiguous because it could have been generated by the combined actions of PLC and diacylglycerol kinase rather than PLD (Billah and Anthes, 1990). We therefore took advantage of the unique PLD-catalyzed transphosphatidylation reaction (Billah and Anthes, 1990; Liscovitch et al., 1993), which results in the formation of [3H]P-But in the presence of butanol. Thus, 0.3% butanol was added 10 min before exposure of myometrium to 0.2 μM ET-1. At this concentration, butanol had no effect on inositol phosphate accumulation induced by ET-1 (data not shown).

In the absence of ET-1, the percentage of conversion to [3H]-P-But was somewhat small (0.15–0.3% of the radioactivity in PC). Addition of ET-1 markedly enhanced [3H]P-But formation with a time course that paralleled that of [3H]PA synthesis. As illustrated in figure 1B, the formation of [3H]P-But triggered by ET-1 increased at the expense of [3H]PA. Thus, at 10 min stimulation, [3H]P-But and [3H]PA formation caused by ET-1 amounted to 1.0 ± 0.1% and 0.3 ± 0.05% of label in PC, respectively. These findings are consistent with the notion that [3H]P-But synthesis stimulated by ET-1 occurred by a PLD-catalyzed transphosphatidylation reaction. Considering that PC appears to be the preferred substrate for PLD (Billah and Anthes, 1990; Exton, 1994), it is reasonable to assume that in rat myometrium PC was the major source for [3H]P-But generated through PLD activated by ET-1.

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**Fig. 2.** Dose-dependent effects of ET-1 and ET-3 on [3H]P-But accumulation. Inhibitory effect of BQ123. [3H]Myristic acid-labeled myometrial strips were exposed to the indicated concentrations of ET-1 (●) and ET-3 (▲) for 10 min in the presence of 0.3% butanol added 10 min before the agonist. BQ123 (1 μM), when tested, was added 5 min before the addition of 0.2 μM ET-1. [3H]P-But production was estimated as described under “Materials and Methods” and expressed as % label in PC. Values represent the mean ± S.E. of three independent experiments, each done in duplicate.
Completely ADP-ribosylated (Tanfin and Harbon, 1987), did which the pertussis toxin-sensitive G proteins in the cells are with 300 ng/ml pertussis toxin for 6 h, conditions under Table 2 further shows that pretreatment of myometrium protein contributed to the activation of PLD in myometrium. Enhanced. These results indicate that a heterotrimeric G adenylyl cyclase pathways (Amiot activation of G proteins which are coupled to the PLC and

Enhanced. These results indicate that a heterotrimeric G adenylyl cyclase pathways (Amiot activation of G proteins which are coupled to the PLC and yet-1 evoked [3H]PBut accumulation was an homologous process. Stimulation of myometrium with AlF4 stimulatory effect on PLD insensitive to pertussis toxin [3H]myristic acid-prelabeled myometrial strips were incubated either with 0.2 mM AlCl3 (10 μM) and bombesin (100 nM) in the presence of butanol. [3H]PBut production was estimated as described under “Materials and Methods.” Results are expressed as a percentage of the control [3H]PBut responses to the indicated agonists (1.15, 1.26 and 0.6% of label in PC, for ET-1, AlF4, and bombesin, respectively. Values represent the mean ± S.E. of three independent experiments, each done in duplicate. * P < .01 vs. ET-1 alone. NS, no significant difference with ET-1 alone. Values for AlF4− and BN were not significantly different from their respective controls that were not pretreated with ET-1.

Almost 80%. Under these conditions of ET-1-induced self-desensitization, there was no loss of PLD activation triggered by AlF4− and by bombesin, two activators of PLD (see fig. 4). Thus, during the first 5 min, desensitization of ET-1-evoked [3H]PBut accumulation was an homologous process.

Stimulatory effect of fluoroaluminates on [3H]PBut accumulation. Insensitivity to pertussis toxin of ET-1-induced production of [3H]PBut. ET receptors have been characterized as members of the G protein-coupled receptor superfam (Huggins et al., 1993; Masaki et al., 1994; Sokolovsky, 1994). On the other hand, several studies implicated a heterotrimeric G protein in the activation of PLD (Cockcroft, 1992; Exton, 1994; Liscovitch et al., 1993). We previously demonstrated that treatment of myometrium with AlF4− (20 mM NaF + 10 μM AlCl3) for 20 min caused the activation of G proteins which are coupled to the PLC and adenyl cyclase pathways (Amiot et al., 1993; Dokhac et al., 1994). Data in table 2 show that, under these conditions of fluoroaluminate treatment, the production of [3H]PBut was enhanced. These results indicate that a heterotrimeric G protein contributed to the activation of PLD in myometrium. Table 2 further shows that pretreatment of myometrium with 300 ng/ml pertussis toxin for 6 h, conditions under which the pertussis toxin-sensitive G proteins in the cells are completely ADP-ribosylated (Tanfin and Harbon, 1987), did not affect the production of [3H]PBut caused by ET-1.

<table>
<thead>
<tr>
<th>Additions</th>
<th>[3H]PBut</th>
<th>cpm % PC</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>0.32 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>AlF4−</td>
<td>1.58 ± 0.14</td>
<td></td>
</tr>
<tr>
<td>ET-1</td>
<td>1.51 ± 0.16</td>
<td></td>
</tr>
<tr>
<td>PTX-treated tissue</td>
<td>0.30 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>ET-1</td>
<td>1.55 ± 0.15∗</td>
<td></td>
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</table>

∗NS, pertussis toxin (PTX)-treated + ET-1 vs. ET-1.

Effect of bombesin on [3H]PBut accumulation. It has been observed for various cellular systems that agonists which elicit PtdInsP2 hydrolysis also stimulate PLD activity (Cockcroft, 1992; Exton, 1994; Liscovitch et al., 1993). Figure 4 shows that, in addition to ET-1, bombesin, another Ca+2-mobilizing agonist, when tested at 100 nM, a concentration which has been observed to maximally stimulate inositol phosphate generation (Amiot et al., 1993), also stimulated [3H]PBut accumulation. Simultaneous addition of bombesin to ET-1 did not result in any further enhancement of [3H]P-But accumulation induced by ET-1 alone, which suggested that both agonists share a common pathway in the activation of PLD. These observations raise the possibility that in myometrium activation of PLC and PLD may be interrelated responses to the stimulatory agonists.
Role of PKC on \[^{[3]H}\text{PBut}\] accumulation. The activation of PLD by tumor-promoting phorbol esters has been reported in a wide variety of cells (Cockcroft, 1992; Exton, 1994; Liscovitch et al., 1993). Figure 5A shows that a 10-min exposure of myometrium to various concentrations of PDBu resulted in a dose-dependent accumulation of \[^{[3]H}\text{PBut}\]. Half and maximal responses were obtained at 0.5 \(\mu\)M and 2 \(\mu\)M, respectively, in close agreement with the responses obtained for PDBu activation of PKC in several tissues. The response to PDBu was slower in onset than ET-1, becoming apparent by 1 to 2 min, then continuing to accumulate for up to 30 min after addition of PDBu (fig. 5B). After a 10-min stimulation, PDBu was found to be more effective than ET-1 at eliciting \[^{[3]H}\text{PBut}\] formation. Data in figure 5 (A and B) show that two other activators of PKC, the phorbol ester PMA and the permeable analog of diacylglycerol, OAG, both at 1 \(\mu\)M, also increased \[^{[3]H}\text{PBut}\] accumulation. In contrast, the inactive 4\(\alpha\)-phorbol (1 \(\mu\)M) was without effect (not shown). To further determine the role of PKC in the generation of \[^{[3]H}\text{PBut}\] induced by PDBu, we tested the effects of three PKC inhibitors. It can be seen (fig. 6, upper panel) that calphostin C and chelerythrine (Herbert et al., 1990), at 1 and 10 \(\mu\)M, respectively, inhibited by 40\% \[^{[3]H}\text{PBut}\] accumulation induced by PDBu. Higher concentrations of chelerythrine were not used because of the possibility of chelerythrine reducing cell viability. However, the most potent PKC inhibitor, Ro-31–8220 (Davis et al., 1989), greatly attenuated the generation of \[^{[3]H}\text{PBut}\] promoted by PDBu. More than 80\% inhibition was achieved at 5 \(\mu\)M Ro-31–8220, which suggested that almost the entire stimulatory effect of the phorbol ester on the accumulation of \[^{[3]H}\text{PBut}\] was mediated by activation of PKC.

In additional experiments, a prolonged (5-h) exposure of the tissue to 1 \(\mu\)M PMA led to decrease (down-regulation) in the amounts of PKC determined by immunoreactivity (data not shown). This procedure similarly abolished about 80\% of the PDBu-promoted \[^{[3]H}\text{PBut}\] response (fig. 6, upper panel).

The participation of PKC in ET-1-induced activation of PLD was further explored by use of the PKC inhibitors and depletion of the enzyme by prolonged exposure to PMA. Figure 6 (center panel) shows that calphostin C and chelerythrine inhibited by 35\% and 45\%, respectively, \[^{[3]H}\text{PBut}\] accumulation induced by ET-1. A similar degree of inhibition (45\%) was found with Ro-31–8220, when used at its maximal concentration (5–10 \(\mu\)M). It has been verified that at 10 \(\mu\)M, Ro-31–8220 did not affect the generation of inositol phosphates triggered by a 10-min stimulation with ET-1 (520,000 ± 35,000 cpm/100 mg of tissue for 10 \(\mu\)M Ro-31–8220 + 0.2 \(\mu\)M ET-1 versus 490,000 ± 30,000 cpm/100 mg of tissue for 0.2 \(\mu\)M ET-1 alone), excluding the possibility that the attenuation demonstrated for the peptide-mediated \[^{[3]H}\text{PBut}\] response could be related to a toxic effect of the
inhibitor. Furthermore, pretreatment of myometrium with 1 μM PMA for 5 h, which abrogated the stimulation of PLD by PDBu, similarly attenuated by 50% the ability of ET-1 to promote the generation of [3H]PBut. These results support the conclusion that in myometrium the stimulatory effect of ET-1 on the generation of [3H]PBut is dependent, albeit partially, on PKC activation.

**Role of Ca**++ **in the generation of [3H]PBut.** To determine whether activation of PLD in the myometrium could arise simply by increasing [Ca**++**]i, the ability of the calcium ionophore ionomycin to stimulate [3H]PBut formation was examined. Figure 4 shows the increased production of [3H]PBut promoted by a 10-min incubation with 10 μM ionomycin. The amount of accumulated [3H]PBut was similar to that induced by a maximally effective concentration of ET-1. Simultaneous addition of ET-1 to ionomycin did not result in any further enhancement of [3H]PBut accumulation triggered by each agent alone. Hence, both ET-1 and ionomycin appear to share a common pathway in the activation of PLD. As shown in figure 6 (lower panel), treatment of myometrial preparations with chelerythrine (10 μM) and with the most potent PKC inhibitor, Ro-31-8220, at its maximal effective concentration, caused 30% and 70% reduction, respectively, of the ionomycin response. Also, the ability of ionomycin to stimulate the generation of [3H]PBut was similarly attenuated (45%) in tissue preparations depleted of PKC. The data demonstrate that a major part of the effect of Ca**++** entry on PLD activity was mediated by PKC activation.

Results presented in figure 7 further revealed the role of extracellular Ca**++** in the activation of PLD triggered by ET-1. A progressive reduction of Ca**++** in the incubation medium resulted in a progressive attenuation of both ET-1- and ionomycin-mediated responses, albeit to a differential extent. Compared with a maximal stimulation (Ca**++** = 800 μM), the responses to ET-1 and ionomycin were reduced by 50% and 70% respectively, at Ca**++** = 100 μM. There was no further attenuation of the ET-1 effect when Ca**++** was decreased to a concentration as low as 1 μM, whereas under these conditions the ionophore-mediated generation of [3H]PBut was totally abrogated, as expected. These results are consistent with the notion that the ionomycin stimulatory effect on [3H]PBut accumulation depended entirely on the presence of extracellular Ca**++**. They also demonstrate that a sustained rise in [Ca**++**]i, was playing a role, although partial, in the activation of PLD by ET-1. Similarly to other agonists, such as carbachol and oxytocin (Arnaudeau et al., 1994; Dokhac et al., 1996), which activate the phospholipase C pathway, ET-1 has been reported (Molnar and Hertelendy, 1995) to transiently elevate [Ca**++**]i in myometrial cells by stimulating the release of Ca**++** from the InsP_3-sensitive intracellular store. It was further shown that this myometrial Ca**++** store could be emptied by the addition of thapsigargin (Arnaudeau et al., 1994), an inhibitor of the Ca**++**-ATPase, that prevents Ca**++** reuptake (Thastrup et al., 1990). Data in table 3 show that treatment of myometrial strips in a normal Ca**++**-containing medium for 10 or 30 min with 1 μM thapsigargin resulted in marginal rises in basal level of [3H]PBut. Also, a 30-min pretreatment with thapsigargin failed to affect the ability of ET-1 to stimulate the accumulation of [3H]PBut, precluding a major role for the InsP_3-sensitive Ca**++** pool. The findings suggested that an increased Ca**++** influx contributed to the activation of PLD caused by ET-1. Figure 7 further shows that addition of nifedipine, a selective antagonist of voltage-dependent Ca**++** channels, 1 min before ET-1, resulted in an inhibition of [3H]PBut accumulation induced by ET-1. The extent of inhibition was 30% as compared with 60% inhibition noted in a Ca**++**-poor medium. Therefore, it appears that a significant contribution of the Ca**++** influx to the ET-1-evoked [3H]PBut
response involved L-type voltage-sensitive Ca\(^{2+}\) channels. As expected, nifedipine did not affect \(^{3}H\)PBut response to ionomycin. Results in figure 7 further demonstrate that there was no significant alteration in the PDBu- and PMA-triggered PDBu responses when extracellular Ca\(^{2+}\) was greatly reduced, down to 1 \(\mu\)M. At the most, a modest inhibition, averaging 20 and 5\% for PDBu and PMA, respectively, could be detected. Thus, in contrast to ionomycin and to a lesser extent ET-1, the phorbol ester-mediated PKC-dependent production of \(^{3}H\)PBut did not display a critical requirement for Ca\(^{2+}\) entry. It should be noted that an almost total inhibition of \(^{3}H\)PBut accumulation triggered by ET-1, ionomycin as well as phorbol esters was obtained under conditions of total Ca\(^{2+}\) depletion (in a Ca\(^{2+}\)-deprived medium supplemented with 1 mM EGTA). Hence, the expression of PLD activity apparently was dependent on a certain minimum content of Ca\(^{2+}\).

The observations described above (figs. 6 and 7) clearly established that PLD activation by ET-1 appears to be regulated in a manner which displays a conditional requirement for both extracellular Ca\(^{2+}\) and PKC activation. Experiments were next aimed at evaluating the combined effect of a reduction in extracellular Ca\(^{2+}\) and the inhibition of PKC on the ET-1 response (fig. 8). Treatment of myometrium with chelerythrine in a Ca\(^{2+}\)-poor medium resulted in an enhanced inhibition (75\%) of \(^{3}H\)PBut accumulation induced by ET-1, compared with the attenuation of the peptide effect caused by either a reduction of Ca\(^{2+}\) (62\% inhibition) or the presence of chelerythrine in a normal Ca\(^{2+}\) medium (32\% inhibition). However, it should be to noted that under these inhibitory conditions, a significant amount (25–30\%) of \(^{3}H\)PBut response triggered by ET-1 was still maintained.

**Discussion**

The results presented in this study provide evidence for the expression in rat myometrium of a PLD pathway that can be stimulated through receptor as well as heterotrimeric G protein activation. The data demonstrate that the potent contractile agonist, ET-1, was able to enhance the accumulation of PBut, the unambiguous transphosphatidylation product of PLD, via the ET\(_A\) receptor subtype involving a pertussis toxin-insensitive G protein. Moreover, we have demonstrated that in the myometrium, PLD was activated by pathways involving PKC and Ca\(^{2+}\) and that the two signals make a major, although not exclusive, contribution to ET-1-mediated PLD activation.

Numerous studies have demonstrated that PA can be generated through the action of PLD that preferentially hydrolyzes PC (Cockcroft, 1992; Exton, 1994; Liscovitch et al., 1993). In rat myometrium labeled with \(^{3}H\)myristic acid, with PC constituting the major (97\%) labeled phospholipid, there was a rapid production of \(^{3}H\)PA in response to ET-1. The observations that, in the presence of butanol, the production of \(^{3}H\)PA was diverted largely to the accumulation of \(^{3}H\)PBut, a specific product of the transphosphatidyltransferase activity of PLD, support the contention that the PLD/PC pathway is the predominant route used by ET-1 to accumulate PA in the myometrium. In view of the relatively higher metabolic stability of PBut, compared with PA, the production of \(^{3}H\)PBut appeared a suitable indicator for PLD activity in the myometrium.

The components and mechanisms involved in receptor signaling to PLD are not yet totally defined and may even be distinct for different receptors and cellular systems. Besides the receptors themselves, Ca\(^{2+}\) and/or PKC have been shown to modulate cellular PLD activity. As far as the many cases of stimulation of the PLC/PtdInsP\(_{2}\) pathway by receptors in which PLD activation has also been reported to occur, it has been proposed that receptor-mediated activation of PLD may be secondary to prior activation of PLC degrading PtdInsP\(_{2}\). In the myometrium, in addition to its stimulatory effect on PLD activity, ET-1 has enhanced the PLC/PtdInsP\(_{2}\) pathway (Dokhac et al., 1994). Examination of the rank order of potencies of ET-1 and ET-3, both in inducing the accumulation of inositol phosphates (Dokhac et al., 1994) and the production of PBut, indicated that both signaling pathways responded in complete agreement with the ET peptide selectivity for ET\(_A\) receptors. Thus, ET-1 stimulated inositol phosphate accumulation and PBut formation with very similar EC\(_{50}\) values (70 ± 5.7 and 50 ± 5.2 nM, respectively). ET-3 similarly increased the production of both inositol phosphates and PBut but was less potent. Also, BQ123, a selective antagonist of the ET\(_A\) receptor subtype, significantly abolished both ET-1-mediated effects. The findings clearly illustrate that both the PLC and the PLD signaling pathways were triggered by the same ET\(_A\) subclass receptors. Furthermore, the stimulatory effects of ET-1, both on the accumulation of inositol phosphates (Dokhac et al., 1994) and on the production of PBut, were insensitive to pertussis toxin and were susceptible to self-induced desensitization involving a receptor-mediated homologous process. Such a close relationship between the two phospholipid signaling pathways stimulated by ET-1, added to the ability of another Ca\(^{2+}\)-mobilizing agonist, bombesin (Amiot et al., 1993), to stimulate

![Fig. 8. Combined effects of PKC and Ca\(^{2+}\) on \(^{3}H\)PBut accumulation induced by ET-1. After the \(^{3}H\)myristic acid prelabeling step, myometrial strips were transferred to a fresh medium containing 800 \(\mu\)M of 1 \(\mu\)M Ca\(^{2+}\) and were allowed to equilibrate for 10 min. 0.3\% butanol was then added and incubations were continued for an additional 10 min in the presence and absence of 10 \(\mu\)M chelerythrine before exposure of the tissues to 0.2 \(\mu\)M ET-1. \(^{3}H\)PBut accumulation was estimated 10 min later, as described under "Materials and Methods" and expressed as \% of labeled PC. Values represent the mean ± S.E. of three independent experiments, each done in duplicate. * P < .01 versus control.](image-url)
PLD, suggest that ET-1 may activate PLD via the intracellular signals generated by its stimulatory effect on the PLC/PtdInsP2 pathway.

It is well established that activation of PKC by phorbol esters can stimulate PLD activity (for reviews, see Cockcroft, 1992; Exton, 1994; Liscovitch et al., 1993). The present results in myometrium show that two phorbol esters, PMA and PDBu, enhanced the production of PBut, whereas the inactive 4α-phorbol ester was without effect. PDBu-mediated PBut production was markedly attenuated (80%) by inhibitors of PKC (Davis et al., 1989; Herbert et al., 1990), particularly Ro-31–8220, as well as after a prolonged treatment of the myometrium with PMA, conditions that led to PKC depletion, which indicated that PDBu exerted its effect on PLD via PKC. The demonstration that PKC serves as an upstream regulator of PLD in the ET-1 signal transduction cascade was provided by the findings that different PKC inhibitors (chelerythrine, Ro-31–8220), as well as PKC down-regulation, attenuated the production of PBut caused by ET-1. However, ET-1-stimulated PLD activity was inhibited by no more than 40% under conditions that abrogated the PDBu response. It clearly appeared that the PKC-triggered process accounted only for a part of the ET-1 effect on PLD, which suggested an additional PKC-independent mechanism(s).

In addition to PKC, Ca$^{2+}$ has also been reported to modulate PLD activity in different intact cell preparations (for reviews, see Billah and Anthes, 1990; Exton, 1994; Liscovitch et al., 1993). Our present findings illustrate that the Ca$^{2+}$ ionophore, ionomycin, enhanced the accumulation of PBut in the myometrium, to an extent similar to that displayed by ET-1. The stimulation of PLD activity produced by ionomycin was highly dependent on extracellular Ca$^{2+}$ and was almost completely inhibited in the presence of the EGTA used to buffer extracellular Ca$^{2+}$ to 1 μM. Thus, increases in [Ca$^{2+}$]i are able to activate PLD. Similarly to previous reports (Cook et al., 1991; Llahi and Fain, 1992), an important attenuation of ionomycin effect was noted with Ro-31–8220 and in PKC-depleted myometrial preparations, which suggested that at least part of ionomycin-stimulated PLD activity was perhaps caused by Ca$^{2+}$, acting as a cofactor in activation of PKC. In contrast, the PDBu stimulatory effect on PLD was not modified in the Ca$^{2+}$-poor medium, consistent with PDBu exerting its effect solely by activation of PKC. The dependence on the rise of Ca$^{2+}$ for the activation of PLD mediated by receptor agonists has been reported previously (Billah and Anthes, 1990; Exton, 1994; Liscovitch et al., 1993). The observation that, in the Ca$^{2+}$-poor medium, ET-1-stimulated PLD activity was attenuated by about 60%, suggested that a sustained rise in [Ca$^{2+}$]i, was partly involved in the peptide-mediated activation of PLD. The findings that thapsigargin failed to alter the accumulation of [3H]PBut in response to ET-1 precluded the contribution of the intracellular InsP$_3$-sensitive Ca$^{2+}$ pool (Arnaudeau et al., 1994; Molnar and Hertelendy, 1995) in the activation of PLD caused by the peptide. Similar findings were reported for the activation of PLD by bombesin (Cook et al., 1991) and by bradykinin (Pyne and Pyne, 1995). Of interest, the inhibition noted with nifedipine supports the notion that, similar to other Ca$^{2+}$-mobilizing agonists (Dokhac et al., 1996), ET-1 is capable of activating an ion channel which elicits specific Ca$^{2+}$ influx in the myometrium and thus contributes to the regulation of PLD activity.

Furthermore, when the PKC inhibitor, chelerythrine, was added to a poor Ca$^{2+}$ medium, an additive attenuation of ET-1-mediated PBut accumulation was observed, consistent with the interpretation that both Ca$^{2+}$- and PKC-mediated mechanisms contributed to the peptide stimulation. Nevertheless, inhibitions obtained by the combined effects of decreased Ca$^{2+}$ and PKC inhibition were not completely additive, which supported the interpretation that some of the effects of increased [Ca$^{2+}$], can be attributed to activation of PKC. Our data are similar to those previously reported for bombesin (Cook et al., 1991)- and norepinephrine (Llahi and Fain, 1992)-mediated PLD activation. They are in variance with recent reports demonstrating that prostaglandin F$_{2\alpha}$-stimulated PLD in osteoblast-like cells via a Ca$^{2+}$-calmodulin process which was totally independent of PKC activation (Imamura et al., 1995). Despite many studies, the exact mechanism for the implication of PKC in PLD activation has not yet been defined. It may be possible that PKC interacts directly with PLD in membranes or that PKC interacts with other membrane-associated proteins that in turn activate PLD (Conricode et al., 1992; Singer et al., 1996).

Our observations that AlF$_4^-$ enhanced the production of PBut support the contention that a heterotrimERIC G protein is contributing to the modulation of PLD activity in the myometrium. The ET-1 stimulatory effect on PLD is shown to be insensitive to pertussis toxin. Because pertussis toxin does not prevent PtdInsP$_2$ degradation caused by ET-1 (Dokhac et al., 1994), it remains unclear as to whether the pertussis toxin insensitivity at the level of PLD activation may reflect a potential link between the PLC and PLD pathways, via Ca$^{2+}$ and PKC, or may be suggestive of a pertussis toxin-insensitive G protein that directly couples to PLD. Although the activation of PLD by ET-1 in the myometrium appears to be determined by the two major signals derived from PtdInsP$_2$ breakdown, increased [Ca$^{2+}$], and PKC activation, it is worth noting that with simultaneous inhibition of both PKC activity and Ca$^{2+}$ entry into the cell, ET-1 still retained the ability to trigger a small (30%) but consistent production of PBut. The possibility that receptor-mediated up-regulation of PLD activity in the myometrium could be mediated by other components such as small G proteins (Cockcroft et al., 1994; Malcolm et al., 1994), tyrosine phosphorylation (Bourgoignie and Grinstein, 1992) and an yet undefined cytosolic factor (Bourgoignie et al., 1995; Lambeth et al., 1995; Singer et al., 1996) should be worth considering.

A large body of evidence emphasizes the important role of ET-1 in the regulation of different functions of smooth muscle cells (Huggins et al., 1993). We previously reported that in the myometrium, activation of ET$_A$ receptors are coupled to both the stimulation of the Ca$^{2+}$/PtdInsP$_2$ pathway and the inhibitory arm of the adenyl cyclase. The resulting increase in Ca$^{2+}$ and the decline in cAMP provide major determinants of ET-1-induced uterine contractions (Dokhac et al., 1994). The present findings demonstrate that in the myometrium, activated ET$_A$ receptors are associated with an additional signaling system, namely the PLD pathway which degrades PC and leads to an increased production of PA. The functional significance of PA in the myometrium remains to be delineated. PA has been proposed to mediate a variety of cellular processes, including promotion of entry and mobili-
zation of Ca$$^{2+}$$, as well as cell proliferation (Cockcroft, 1992; Exton, 1994; Liscovitch et al., 1993). Such potentially regulatory processes are particularly interesting in view of the key role of both motility and cell proliferation in the physiology of the myometrium.

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References


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