Inhibition of Chemotactic Peptide-induced Phosphoinositide Hydrolysis by Phorbol Esters through the Activation of Protein Kinase C in Differentiated Human Leukemia (HL-60) Cells

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ABSTRACT

Phorbol-12,13-dibutyrate (PDBU), a tumor-promoting and protein kinase C-activating phorbol ester, inhibited formylmethionylleucylphenylalanine-induced generation of inositol mono-, bis-, and tris-phosphates from the hydrolysis of phosphoinositides in human leukemia (HL-60) cells, which had been differentiated to polymorphonuclear leukocyte-like cells by pretreatment with dibutyryl cyclic adenosine 3',5'-monophosphate. PDBU did not alter the binding of formylmethionylleucylphenylalanine to the cells. Other protein kinase C-activating substances such as 12-O-tetradecanoylphorbol-13-acetate and 1-oleoyl-2-acetyl-glycerol could substitute for PDBU, but 4a-phorbol-12,13-didecanoate, which is inactive for both tumor promotion and protein kinase C activation, was ineffective in this capacity. Prolonged treatment of the cells with PDBU resulted in the down-regulation and decrease of protein kinase C activity to the level of 30-40% of that in the control cells. In the down-regulated cells, formylmethionylleucylphenylalanine still induced generation of the phosphorylated inositols to the same extent as in the control cells, but the inhibition of this reaction by PDBU was reduced to 30-50% as compared with that in the control cells. These results strongly suggest that tumor-promoting phorbol esters inhibit the agonist-induced phosphoinositide hydrolysis through the activation of protein kinase C in the differentiated HL-60 cells.

INTRODUCTION

It is well established that phosphoinositide turnover plays a role of crucial importance in transmembrane signaling of a wide variety of extracellular signals including growth factors, hormones, neurotransmitters, and other biologically active substances (for reviews, see Refs. 1 and 2). Phosphoinositides are composed of three species of phospholipids; PI, PIP, and PIP2. Upon stimulation of cells by a certain extracellular signal, PIP2 is most rapidly hydrolyzed by the action of phospholipase C, resulting in the generation of diacylglycerol and IP3. Diacylglycerol then serves as a messenger for the activation of protein kinase C (for reviews, see Refs. 3 and 4), whereas IP3 serves as a trigger for the intracellular translocation of Ca2+ (2). Evidence has been accumulated that both protein kinase C and Ca2+ are involved in the regulation of various cell functions elicited by extracellular signals (2-4). Another line of evidence indicates that protein kinase C itself is the receptor for tumor-promoting phorbol esters such as TPA and PDBU and that the diverse effects of the phorbol esters are mediated through the activation of this enzyme (5-7).

In polymorphonuclear leukocytes, fMLP, a chemotactic peptide, elicits rapidly multiple cellular responses including chemotaxis, secretion of lysosomal enzymes, and generation of bactericidal superoxide (8-10). Evidence is available that protein kinase C and Ca2+ may be involved in the regulation of these cellular activities (11, 12). However, it has recently been demonstrated that TPA inhibits Ca2+ mobilization and enzyme secretion which are induced by the chemotactic peptide in polymorphonuclear leukocytes (13). This result has raised the possibility that TPA may inhibit the agonist-induced phosphoinositide hydrolysis. It has been demonstrated that phorbol esters inhibit acetylcholine-induced phosphoinositide hydrolysis in astrocytoma cells (14), this thrombin-induced reaction in human platelets (15, 16), this carbachol-induced reaction in PC 12 cells (17), and this carbachol-induced reaction in rat hippocampal slices (18). Therefore, we examined whether the phorbol esters may inhibit fMLP-induced phosphoinositide hydrolysis in polymorphonuclear leukocyte cells. For this purpose, we used human leukemia (HL-60) cells after the cells were differentiated to polymorphonuclear leukocyte-like cells by treatment with dbcAMP, since the differentiated cells become responsive to the chemotactic peptide to induce phosphoinositide hydrolysis (19) and are easily prepared and handled. This paper describes that PDBU and other phorbol esters inhibit fMLP-induced phosphoinositide hydrolysis through the activation of protein kinase C in the differentiated HL-60 cells.

MATERIALS AND METHODS

Materials and Chemicals. fMLP, HEPES, and BSA (fraction V) were purchased from Sigma. dbcAMP was obtained from Yamasa Chemicals. [3H]Inositol (specific activity, 14.2 Ci/mmol), [3H]PDBU (specific activity, 10.5 Ci/mmol), [3H]fMLP (specific activity, 51 Ci/mmol), and [γ-32P]ATP (specific activity, 3000 Ci/mmol) were obtained from Amer sham. All media and fetal calf serum were from GIBCO. Ultroser G was purchased from Reactifs IBF. TPA and PDBU were purchased from C.C.R., Inc. 4a-Phorbol-12,13-didecanoate was obtained from P. Barchert, Eden Prairie, MN. OAG was synthesized as described previously (20). Calf thymus H1 histone was prepared as described previously (21). Other materials and chemicals were obtained from commercial sources.

Cell Culture. HL-60 cells were a generous gift from Dr. S. Murao (Kobe University School of Medicine), who originally obtained the cells from Dr. R. C. Gallo (NIH, Bethesda, MD), (22) and were routinely passed as described (23). For differentiation of the cells by dbcAMP to polymorphonuclear leukocyte-like cells, the cells were seeded at a concentration of 7.5 x 10⁶ cells/ml in Ham's F-10 medium containing 2% Ultroser G, 10 mM HEPES (pH 7.4), and 500 µM dbcAMP. After a 36-h incubation at 37°C in an atmosphere of 95% air:5% CO2, the cells were collected by low speed centrifugation and resuspended at a concentration indicated in each experiment in the indicated medium. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP2, phosphatidylinositol 4,5-biphosphate; IP3, inositol 1,4,5-trisphosphate; TPA, 12-O-tetradecanoylphorbol-13-acetate; PDBU, phorbol-12,13-dibutyrate; fMLP, formylmethionylleucylphenylalanine; dbcAMP, 3',5'-diguanylate cyclic AMP; HEPES, 2-hydroxyethylpiperazine-N,N'-ethane-sulfonic acid; BSA, bovine serum albumin; OAG, 1-oleoyl-2-acetyl-glycerol; IP3, inositol 1-monophosphate; IP3, inositol 1,4-bisphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid.
viable as judged by a trypan blue exclusion test. Before differentiation, the cells did not bind [3H]fMLP but the differentiated cells showed [3H]fMLP-binding activity and responded to this agonist to induce phosphoinositide hydrolysis as described previously (19).

Measurement of [3H]Phosphorylated Inositol. For measurement of the generation of phosphorylated inositols, the cells preabeled with [3H]inositol were used. This labeling was performed during the differentiation of the cells with dbcAMP. The cells were labeled with [3H]inositol (4 µCi/ml) under the differentiation conditions described above except that inositol-free Ham's F-10 medium was used. The cells labeled with [3H]inositol were collected by low speed centrifugation and resuspended in Ham's F-10 medium containing 0.025% BSA. This procedure was repeated twice. The suspended cells were washed by the same medium twice and finally suspended at a concentration of 5 x 10^6 cells/ml in 20 ml of 150 mM NaCl, 5 mM KCl, 5.5 mM glucose, 0.8 mM MgSO_4, 1 mM CaCl_2, and 0.1% BSA. Under these conditions, 4 µCi of [3H]inositol were taken up by 1 x 10^6 cells, and about 305, 8, and 9 nCi of [3H]inositol were incorporated into PI, IP_2, and IP_3, respectively. [3H]inositol-labeled HL-60 cells (2 x 10^6 cells) were pretreated for 10 min at 37°C with or without the indicated concentrations of PDBU in the presence of 10 mM lithium chloride in a volume of 0.5 ml. The cells were then stimulated by fMLP for 1 min at 37°C. The reaction was stopped by the addition of 1.88 ml of chloroform: methanol:HCI (100:200:2), and 0.62 ml of chloroform and 0.62 ml of water were added to separate the phases. The tubes were mixed and then centrifuged for 5 min at 1200 x g. The phosphorylated inositols were separated as described by Berridge (24). A 1.5-ml aliquot of the upper aqueous phase was mixed with 2.5 ml of water and subjected to a column containing 1 ml of Dowex AG 1-XS-formate. The column was washed with 8 ml of water and then with 20 ml of 60 mM ammonium formate/5 mM disodium tetraborate. [3H]Inositol and [3H]glycerophosphorylinositol were eluted with water and 60 mM ammonium formate/5 mM disodium tetraborate, respectively. These fractions were discarded. After this washing, [3H]IP_1 was eluted with 8 ml of 200 mM ammonium formate/100 mM formic acid. The column was washed with 8 ml of the same buffer and [3H]IP_1 was eluted with 8 ml of 400 mM ammonium formate/100 mM formic acid. The column was washed with 8 ml of the same buffer and [3H]IP_2 was eluted with 4 ml of 1 M ammonium formate/100 mM formic acid. Fractions of 2 ml each were collected. The radioactivity of each fraction containing IP_3, IP_4, and IP_5 was determined.

[3H]fMLP-binding Assay. A [3H]fMLP-binding assay was performed by the procedure described by Okajima and Ui (25) with slight modifications. The differentiated HL-60 cells were suspended at a concentration of 5 x 10^6 cells/ml in 10 mM HEPES (pH 7.4) containing 150 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM MgSO_4, 2.5 mM CaCl_2, 5 mM Tris-HCl, 0.1% BSA, and 5.5 mM glucose, and 0.1% BSA. The cells were finally resuspended at a concentration of 5 x 10^6 cells/ml in 20 ml oflow speed centrifugation and resuspended in Ham's F-10 medium containing 0.025% BSA. This procedure was repeated twice. The cells were finally resuspended at a concentration of 5 x 10^6 cells/ml in 20 ml of 150 mM NaCl, 5 mM KCl, 5.5 mM glucose, 0.8 mM MgSO_4, 1 mM CaCl_2, and 0.1% BSA. Under these conditions, 4 µCi of [3H]inositol were taken up by 1 x 10^6 cells, and about 305, 8, and 9 nCi of [3H]inositol were incorporated into PI, IP_2, and IP_3, respectively. [3H]inositol-labeled HL-60 cells (2 x 10^6 cells) were pretreated for 10 min at 37°C with or without the indicated concentrations of PDBU in the presence of 10 mM lithium chloride in a volume of 0.5 ml. The cells were then stimulated by fMLP for 1 min at 37°C. The reaction was stopped by the addition of 1.88 ml of chloroform: methanol:HCI (100:200:2), and 0.62 ml of chloroform and 0.62 ml of water were added to separate the phases. The tubes were mixed and then centrifuged for 5 min at 1200 x g. The phosphorylated inositols were separated as described by Berridge (24). A 1.5-ml aliquot of the upper aqueous phase was mixed with 2.5 ml of water and subjected to a column containing 1 ml of Dowex AG 1-XS-formate. The column was washed with 8 ml of water and then with 20 ml of 60 mM ammonium formate/5 mM disodium tetraborate. [3H]Inositol and [3H]glycerophosphorylinositol were eluted with water and 60 mM ammonium formate/5 mM disodium tetraborate, respectively. These fractions were discarded. After this washing, [3H]IP_1 was eluted with 8 ml of 200 mM ammonium formate/100 mM formic acid. The column was washed with 8 ml of the same buffer and [3H]IP_1 was eluted with 8 ml of 400 mM ammonium formate/100 mM formic acid. The column was washed with 8 ml of the same buffer and [3H]IP_2 was eluted with 4 ml of 1 M ammonium formate/100 mM formic acid. Fractions of 2 ml each were collected. The radioactivity of each fraction containing IP_3, IP_4, and IP_5 was determined.

[3H]fMLP-binding Assay. A [3H]fMLP-binding assay was performed by the procedure described by Okajima and Ui (25) with slight modifications. The differentiated HL-60 cells were suspended at a concentration of 5 x 10^6 cells/ml in 10 mM HEPES (pH 7.4) containing 134 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM MgSO_4, 2.5 mM CaCl_2, 5 mM Tris-HCl, 0.1% BSA, and 5.5 mM glucose, and 0.1% BSA. The cells (2 x 10^6 cells) were pretreated with or without 200 nM PDBU in 0.1 ml of the same buffer for 10 min at 37°C and then incubated with the indicated concentrations of [3H]fMLP for 30 min at 37°C. The incubation was terminated by pouring the same cold buffer which contained no CaCl_2 and 2 mM HEPES instead of 10 mM HEPES. It was followed by immediate filtration through a Whatman GF/B glass microfiber filter and rapidly washed three times with this cold buffer. Filters were placed in liquid scintillation vials containing 5 ml of scintillator and their radioactivities were measured. Nonspecific binding was determined by incubating the cells with [3H]fMLP in the presence of 50 µM unlabeled PDBU. Nonspecific counts represented less than 25% of the total counts.

Determinations. The radioactivity of [3H]fMLP in fluid scintillation spectrometer. Protein was determined by the method of Lowry et al. (26).

RESULTS

Incubation of the differentiated HL-60 cells with 0.1 µM fMLP caused a marked generation of IP_1, IP_2, and IP_3 as shown in Fig. 1. The concentrations of fMLP necessary for these reactions were nearly the same and the maximum activities were obtained with 1 µM fMLP. Fig. 2 shows the time courses for these reactions. The generation of IP_2 was observed rapidly, reached to the maximum level within 30 s, and declined. IP_2 was also produced rapidly, accumulated to the maximum level within 1 min, and then gradually decreased. In contrast, IP_3 was most slowly produced.

Pretreatment of the differentiated HL-60 cells with PDBU for 10 min resulted in the marked reduction of fMLP-induced generation of IP_1, IP_2, and IP_3 as shown in Fig. 2. This inhibition by PDBU was dose dependent and the doses of PDBU necessary for the inhibition of fMLP-induced generation of the three phosphorylated inositols were nearly identical as shown in Fig. 3. Treatment of the cells with 200 nM PDBU for 10 min increased the pool sizes of PIP and PIP_2 from 8 and 9 nCi/1 x 10^6 cells to 12 and 11 nCi/1 x 10^7 cells, respectively. The pool size of PI was not altered by this treatment. These results were
consistent with the earlier observation made in platelets (15) that the phorbol ester stimulates the phosphorylation of PI and PIP to produce PIP and PIP₂, respectively. However, the inhibition by PDBU of fMLP-induced generation of IP₁, IP₂, and IP₃ was not simply due to the change of the pool sizes of PIP and PIP₂, since treatment of the cells with PDBU increased the pool sizes of PIP and PIP₂ 50 and 22%, respectively, of the control levels and did not change that of PI but inhibited fMLP-induced generation of IP₁, IP₂, and IP₃ more than 50% of the control levels. The inhibitory action of PDBU was observed also with other protein kinase C-activating substances. Table 1 shows that TPA and OAG were also effective for inhibiting fMLP-induced generation of the three phosphorylated inositols. However, 4α-phorbol-12,13-didecanoate, which was shown not to activate protein kinase C (5), was ineffective in this capacity. Under these conditions, PDBU did not alter the fMLP binding to the cells as shown in Fig. 4.

In another set of experiments, the differentiated cells were treated with either 400 nM PDBU in RPMI 1640 or the medium alone as a control for 24 h. After this treatment, the Triton X-100 extract of the cells was prepared and subjected to gel filtration on a Toyo Soda TSK G3000SW column under the conditions described in “Materials and Methods.” When each fraction of the control cells was assayed for protein kinase C activity in the presence of Ca²⁺, TPA, and phospholipid, a single peak appeared as shown in Fig. 5A. The enzymatic activity of this peak was dependent on the presence of these three activators. The molecular weight of this peak was estimated to be about 77,000, which is the same as that of well characterized rat brain protein kinase C (27). However, when each fraction of the cells treated with PDBU was assayed for protein kinase C, a small single peak appeared at the same position as that of the control cells, but the enzymatic activity of this peak was reduced to the level of about 30% of that of the control levels. The radioactivities of IP₁, IP₂, and IP₃, which were obtained in the absence of PDBU pretreatment, were 2950 ± 86, 3400 ± 102, and 520 ± 38 cpm, respectively. Each point of IP₁, IP₂, and IP₃ generation is expressed as a percentage of these respective values. The background values for IP₁, IP₂, and IP₃ were 1750 ± 62, 90 ± 12, and 10 ± 1 cpm, respectively, and were obtained from the cells which were not stimulated by fMLP. From all experimental values, the respective background values were subtracted. All experimental values are the means of four independent experiments.

Table 1 Effect of various phorbol derivatives and OAG on fMLP-induced generation of phosphorylated inositols

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IP₁</th>
<th>IP₂</th>
<th>IP₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>11,420 ± 205²</td>
<td>1,620 ± 59</td>
<td>510 ± 43</td>
</tr>
<tr>
<td>PDBu</td>
<td>5,680 ± 174²</td>
<td>160 ± 19²</td>
<td>240 ± 28²</td>
</tr>
<tr>
<td>TPA</td>
<td>5,230 ± 158²</td>
<td>150 ± 24 ²</td>
<td>190 ± 24²</td>
</tr>
<tr>
<td>4α-Phorbol-12,13-didecanoate</td>
<td>12,290 ± 236²</td>
<td>1,510 ± 68²</td>
<td>490 ± 51²</td>
</tr>
<tr>
<td>OAG</td>
<td>6,010 ± 122²</td>
<td>110 ± 11²</td>
<td>210 ± 16²</td>
</tr>
</tbody>
</table>

² Incubation time with fMLP was 15 min.
² Incubation time with fMLP was 1 min.
² Mean ± SE.
PHORBOL ESTERS AND PHOSPHOINOSITIDE HYDROLYSIS

Fig. 4. Effect on PLDII on fMLP binding to the differentiated HL-60 cells. The differentiated cells, which were pretreated with or without 200 nM PDBU for 10 min at 37°C, were incubated with the indicated concentrations of [3H]fMLP for 30 min at 25°C. •, total binding without PDBU pretreatment; D, total binding with PDBU pretreatment; •, specific binding without PDBU pretreatment; O, specific binding with PDBU pretreatment; A, nonspecific binding without PDBU pretreatment; A, nonspecific binding with PDBU pretreatment. All experimental values are the mean of three independent experiments.

Fig. 5. Down-regulation of protein kinase C by prolonged treatment with PDBU. After the differentiated cells were treated with or without 400 nM PDBU for 24 h at 37°C, the Triton X-100 extract of the cells was prepared and subjected to a Toyo Soda TSK G3000SW column under the conditions described under “Materials and Methods.” Each fraction was assayed for protein kinase C activity as described under “Materials and Methods.” A, control cells; B, down-regulated cells. O, in the presence of Ca++, TPA, and phospholipid; •, in the presence of EGTA. The data shown are typical of three independent experiments.

the control cells as shown in Fig. 5B. This result clearly indicates that protein kinase C was markedly down-regulated and reduced by prolonged treatment of the cells with PDBU. This phenomenon was confirmed by using another assay method for protein kinase C. Fig. 6 shows that [3H]PDBU bound to the control cells in a dose-dependent manner and this binding was reduced by prolonged treatment of the cells with PDBU. The binding affinity of PDBU shown in Fig. 6 was 3- to 4-fold lower than that described previously by Solanki et al. (28), but the exact reason for this difference is not clear. The decrease of the radioactive PDBU-binding to the down-regulated cells was not simply due to the presence of nonradioactive PDBU which might remain in the cells after washing, since PDBU was almost completely removed by washing as estimated by using the radioactive compounds. In these cells, in which the enzymatic and [3H]PDBU-binding activities of protein kinase C were markedly decreased, the inhibitory effect of PDBU on the fMLP-induced generation of phosphorylated inositols was also reduced to the same extent as that of protein kinase C as shown in Fig. 7.

DISCUSSION

The present paper shows that PDBU and other protein kinase C-activating substances inhibit fMLP-induced generation of IP1, IP2, and IP3 in the differentiated HL-60 cells. 4α-Phorbol-
12,13-didecanoate, which is inactive for protein kinase C, is inactive in this capacity. These results are in good agreement with the earlier observations that the phorbol esters inhibit acetylcholine-induced generation of IP$_1$, IP$_2$, and IP$_3$ in astrocytoma cells (14), thrombin-induced generation of these phosphorylated inositols in human platelets (15, 16), carbachol-induced generation of these phosphorylated inositols and rise of cytosolic Ca$^{2+}$ in PC 12 cells (17), and carbachol-induced generation of IP$_3$ in rat hippocampal slices (18). Moreover, evidence is presented in this paper that the decrease of protein kinase C activity down-regulated by treatment of the cells with PDBU roughly parallels with the reduction of inhibition by PDBU of fMLP-induced generation of IP$_1$, IP$_2$, and IP$_3$. These results strongly suggest that protein kinase C is involved in the inhibitory effect of the phorbol esters on fMLP-induced generation of the phosphorylated inositols.

It has been described that IP$_2$ is generated from the hydrolysis of PIP$_2$ by the action of phospholipase C (2). IP$_2$ is produced either from the hydrolysis of PIP by the action of phospholipase C or from the dephosphorylation of IP$_3$ by its phosphatase (29). IP$_3$ is produced from the hydrolysis of PI or the dephosphorylation of IP$_2$. Berriedge has proposed that the primary event of receptor stimulation is probably the hydrolysis of IP$_2$ and that IP$_3$ is formed by a two-stage phosphorylation of PI, which is first phosphorylated at position 4 of its inositol head group by a specific kinase to form PIP; this is in turn further phosphorylated at position 5 to give PIP$_2$. In his proposal, IP$_3$ is produced from the hydrolysis of PIP$_2$, but IP$_2$ and IP$_3$ are produced from the dephosphorylation of IP$_2$, and not from PIP and PI. However, Wilson et al. (30) have demonstrated in platelets that IP$_2$ is most rapidly hydrolyzed and subsequently PIP and PI are hydrolyzed by the action of the same phospholipase C, resulting in the production of IP$_3$, IP$_2$, and IP$_1$, respectively. It is not known which of the above mechanisms operates upon stimulation by fMLP in the differentiated HL-60 cells. However, our results indicate that PDBU and other protein kinase C-activating substances inhibit fMLP-induced hydrolysis of PIP$_2$ and possibly of PIP and PI in HL-60 cells.

Recently, it has been demonstrated that fMLP-induced hydrolysis of phosphoinositide is inhibited by pertussis toxin in the differentiated HL-60 cells (19). Moreover, it has been shown that the generation of IP$_2$ and IP$_3$ is stimulated by GTP and its stable analogues such as guanyl-5'-yl imidodiphosphate and guanosine-5'-(3-O-thio)triphosphate (31, 32). Based on these observations, it has been suggested that G$_i$ (N$_i$) protein, which originally has been shown to be an inhibitory transducer for the adenylate cyclase system (for a review, see Ref. 33), may also be involved in the transduction from the receptor to phospholipase C-catalyzing phosphoinositide hydrolysis (19, 34–36). Another line of evidence indicates that protein kinase C phosphorylates the $\alpha$ subunit of G$_i$ protein in a cell-free system (37) and that TPA interferes with thrombin-induced inhibition of the platelet adenylate cyclase (38). It has been examined whether the phorbol esters may stimulate the phosphorylation of this protein also in the differentiated HL-60 cells, and the mode of action of the phorbol esters in the inhibition of fMLP-induced hydrolysis of phosphoinositides has not yet been clarified. However, it is most likely that the phorbol esters may disturb the coupling between the receptor and phospholipase C through the activation of protein kinase C and thereby inhibit this reaction, since the phorbol esters do not affect the binding of fMLP to the receptor.


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