Differential effects of platelet-derived growth factor, serum and bombesin on phospholipase D-mediated hydrolysis of phosphatidylethanolamine in NIH 3T3 fibroblasts

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In previous studies, activators of protein kinase C, sphingosine, ATP and various oncogenes were each found to enhance phospholipase D-mediated hydrolysis of phosphatidylethanolamine (PtdEtn) in NIH 3T3 fibroblasts. Here I examined possible stimulation of PtdEtn hydrolysis by various growth-stimulatory agents, including serum, bombesin, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) and insulin. Treatment of NIH 3T3 fibroblasts, prelabelled with [^{14}C]Etn or [^{32}P]PtdEtn, with PDGF-BB resulted in enhanced formation of [^{14}C]Etn or [^{32}P]phosphatidic acid from the respective labelled cellular pools of PtdEtn. A maximal effect (~ 3 -fold stimulation) on PtdEtn hydrolysis was obtained with 50 ng of PDGF/ml after 5 min of treatment. Phosphatidylcholine (PtdCho) was also hydrolysed, although less extensively than PtdEtn, in PDGF-stimulated cells. PDGF-stimulated hydrolysis of both PtdEtn and PtdCho was prevented by prolonged (30 h) treatment of cells with 400 nM-phorbol 12-myristate 13-acetate (PMA). Similar to PDGF, fetal calf serum (1–10 %) also stimulated PtdEtn hydrolysis. However, in contrast to PDGF, the effect of serum on PtdEtn hydrolysis (i) was not diminished by pretreatment with PMA, and (ii) was synergistic with that of PMA after a 1 h incubation. Compared with PDGF and serum, bombesin had less effect on PtdEtn hydrolysis, while FGF and insulin had no effects at all. In contrast to PDGF or serum, bombesin inhibited the effect of PMA on PtdEtn hydrolysis.

INTRODUCTION

Several growth factors, including platelet-derived growth factor (PDGF) [1–4], epidermal growth factor [4–8], fibroblast growth factor (FGF) [9] and bombesin [7,10–12], have been found to stimulate phospholipase D-mediated hydrolysis of phosphatidylcholine (PtdCho) in fibroblasts. In contrast, when examined, growth factors did not appear to stimulate phosphatidylethanolamine (PtdEtn) hydrolysis [5,8]. Thus, although in certain cell lines phorbol 12-myristate 13-acetate (PMA) [13–16], sphingosine [17,18] and ATP [17,19] commonly stimulated the hydrolysis of PtdCho and PtdEtn, it appeared that this may not be true for the growth factors. In view of a recent report [20] that cells contain two different phospholipase D activities, a membrane-bound form acting specifically on PtdCho and a cytoplasmic form acting on both PtdEtn and PtdCho, selective effects of growth factors on PtdCho hydrolysis seemed quite possible. Alternatively, in previous studies the stimulatory effects of growth factors on PtdEtn hydrolysis might have simply gone undetected due to some technical problems of which I have recently become aware [13,21]. For these reasons, it was of interest to re-examine the possible effects of various growth regulators on PtdEtn hydrolysis. Here I show that both PDGF and serum, and to a lesser extent bombesin, but not FGF or insulin, can stimulate PtdEtn hydrolysis in NIH 3T3 fibroblasts.

MATERIALS AND METHODS

Materials

PMA and Dowex-50-WH⁺ were purchased from Sigma; PDGF-BB (human, recombinant), FGF (bovine brain), insulin and bombesin were from Boehringer Mannheim; fetal calf serum was from Flow Laboratories; [$2\text{-}^{14}\text{C}$]Etn (50 mCi/mmol), [$\text{methyl-}^{14}\text{C}$]Cho chloride (60 mCi/mmol) and [^{32}P]P_i (carrier-free

in water) were from Amersham; and tissue culture reagents were from GIBCO. [^{32}P]PtdEtn was prepared as described earlier [17].

Cell culture

NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum, penicillin/streptomycin (50 units/ml and 50 $\mu\text{g}/\text{ml}$ respectively) and glutamine (2 mM). For all experiments, cells were used during the exponential phase of growth.

Measurement of phospholipid hydrolysis in cells prelabelled with ^{14}C -precursors

Cells were grown in 150 mm-diam. plastic dishes (Costar) in the presence of [$2\text{-}^{14}\text{C}$]Etn (0.25 $\mu\text{Ci}/\text{ml}$) or [$\text{methyl-}^{14}\text{C}$]Cho chloride (0.30 $\mu\text{Ci}/\text{ml}$) for 34 h. Cells were washed once with serum-free DMEM and then incubated in serum-free DMEM (containing 1 mg of fatty acid-free BSA/ml) in the presence of the respective labelled precursors for 14 h. Finally, cells were washed twice with serum-free DMEM and then incubated in serum-free medium (containing BSA as above) for 3 h in the absence of labelled precursors. This latter procedure decreases the background levels of water-soluble ^{14}C -labelled degradation products of phospholipids, as discussed elsewhere [21]. Cells were harvested by gentle scraping from the dish. Cells collected from 3–5 dishes were washed with serum-free DMEM. At this step, 95–98 % of cells were viable, determined by the Trypan Blue dye exclusion assay, and contained > 1000 d.p.m. of [^{14}C]Etn/ 10^6 cells. Scraped cells also retained sensitivity to each activator of phospholipase D examined previously [13,17,19]. Aliquots (0.2 ml) of cell suspension [$(1.1\text{--}1.3) \times 10^6$ cells in 1 ml of DMEM] were incubated (final volume 0.25 ml) in polypropylene tubes in an incubator at 37 °C in the presence of 2 mM unlabelled Etn (to prevent further metabolism of newly formed [^{14}C]Etn; see [13,21]) and BSA (1 mg/ml). Incubations were terminated

and ^{14}C -labelled water-soluble products of phospholipids were separated on Dowex-50-WH⁺ resin according to the method of Cook & Wakelam [10], with modifications indicated elsewhere [19,21].

For the determination of effects of the PDGF on the degradation of cellular [^{32}P]PtdEtn, cells prelabelled with [^{32}P]PtdEtn-containing liposomes (2.2 $\mu\text{Ci}/\text{ml}$) for 10 h (in the absence of serum) were used as described earlier [17,22]. Phosphatidic acid and PtdEtn were separated from other phospholipids on silica gel H plates as indicated earlier [14].

RESULTS

Treatment of [^{14}C]Etn-prelabelled cells with PDGF-BB for 15 min resulted in enhanced formation of [^{14}C]Etn from the prelabelled PtdEtn pool (Fig. 1). Half-maximal (~ 1.6 -fold stimulation) and maximal (~ 2.2 -fold) stimulatory effects were observed with 5 and 50 ng of PDGF/ml respectively. PDGF had no effect on the formation of Etn phosphate (results not shown). PDGF also stimulated the formation of [^{14}C]Cho from the prelabelled cellular PtdCho pool (Fig. 1). Although the fold stimulatory effects of 50 ng of PDGF/ml on the hydrolysis of PtdEtn and PtdCho were of similar magnitude (2.2- and 2.7-fold stimulation respectively), these values do not reflect the absolute amounts of phospholipids hydrolysed in PDGF-treated cells. After subtraction of values for phospholipid-derived Etn and choline obtained in the absence of PDGF, it is apparent that PDGF (50 ng/ml) stimulated the degradation of about 0.6% and 0.3% of the labelled cellular PtdEtn and PtdCho pools respectively (Fig. 1). This calculation is based on the assumption that the different metabolic pools of PtdEtn and PtdCho derivatives are in near isotopic equilibrium (because of the 48 h labelling period); therefore formation of water-soluble products reflects inverse changes in the phospholipid mass.

The most effective activator of phospholipase D in various cellular systems (including NIH 3T3 cells) is the protein kinase C activator PMA. Since PDGF also stimulates protein kinase C [10–12], it was of interest to determine the relationship between the actions of PMA and PDGF on phospholipid hydrolysis. As shown in Fig. 2, treatment of [^{14}C]Etn-labelled cells with PDGF (50 ng/ml) for 2.5 min resulted in greater than 2-fold stimulation of [^{14}C]Etn formation, with a maximal effect (~ 3 -fold stimulation) being detected after 5 min of incubation. Except at the earliest (2.5 min) incubation time, PMA was more effective than PDGF in stimulating PtdEtn hydrolysis, and its effect was not enhanced further by PDGF during the 15 min incubation period. These data suggested that PMA and PDGF might utilize a common mechanism, involving activation of protein kinase C, to stimulate phospholipid degradation.

To explore the possible role of protein kinase C in the mediation of the effects of PDGF on phospholipid hydrolysis, I used prelabelled cells in which this enzyme was down-regulated by prolonged (30 h) treatment with 400 nM-PMA [23,24]. In PMA-pretreated cells, the formation of both [^{14}C]Etn and [^{14}C]Cho was slightly increased, probably reflecting the presence of residual protein kinase C activity (Table 1). Importantly, neither newly added PMA nor PDGF stimulated the hydrolysis of PtdEtn and PtdCho in the pretreated cells (Table 1). These data are consistent with a role for protein kinase C in mediating the effects of PDGF on phospholipid hydrolysis.

Similar to PDGF, fetal bovine serum (10%) also stimulated the hydrolysis of both PtdEtn and PtdCho (Table 1); half maximal effects were observed with 2% serum (results not shown). Interestingly, pretreatment of cells with PMA had little effect on serum-stimulated hydrolysis of PtdEtn, while it com-

pletely blocked the effect of serum (in both the absence and the presence of PDGF) on PtdCho hydrolysis (Table 1).

To further explore the relationship between the effects of serum, PDGF and activated protein kinase C, I also examined the effects of serum and PMA on PtdEtn hydrolysis over an extended time scale. As shown in Fig. 3(a), serum alone had

Fig. 1. Concentration-dependent effects of PDGF-BB on the hydrolysis of PtdEtn and PtdCho in NIH 3T3 cells

Cells were prelabelled with [^{14}C]Etn (●) or [^{14}C]Cho (▲) for 48 h, followed by incubation of suspended cells for 15 min in the presence of various concentrations of PDGF-BB, as described in the Materials and methods section. [^{14}C]Etn and [^{14}C]Cho were separated by ion-exchange chromatography. The mean ^{14}C content of PtdEtn and PtdCho was 1.62×10^6 and 1.14×10^6 d.p.m./ 10^6 cells respectively. Each point represents the mean $\pm$ S.E.M. of four determinations. Similar results were obtained in two other experiments.

Fig. 2. Time-dependent effects of PDGF-BB and PMA on the hydrolysis of PtdEtn in NIH 3T3 cells

Cells were prelabelled with [^{14}C]ethanolamine for 48 h, followed by incubation of suspended cells in the absence (●) or presence of 50 ng of PDGF-BB/ml (▲), 100 nM-PMA (■) or PDGF-BB plus PMA (◆), as described in the Materials and methods section. The mean ^{14}C content of PtdEtn was 1.71×10^6 d.p.m./ 10^6 cells. Each point represents the mean $\pm$ S.E.M. of four determinations. Similar results were obtained in two other experiments.

Table 1. Effects of PDGF-BB, PMA and fetal bovine serum on the hydrolysis of PtdEtn and PtdCho in untreated and PMA-pretreated NIH 3T3 cells

Cells were prelabelled with [¹⁴C]Etn or [¹⁴C]Cho for 48 h as described in the Materials and methods section. Where indicated, 400 nM-PMA was added for the last 30 h of the prelabelling period (PMA-pretreated cells). Prelabelled cells were further incubated for 15 min in the absence or the presence of PDGF-BB (50 ng/ml), PMA (100 nM) or fetal bovine serum (10 %, v/v). Data are the means ± S.E.M. of three determinations. Similar results were obtained in two other experiments.

Addition	Hydrolysis of [¹⁴ C]phospholipids (d.p.m. of [¹⁴ C]product/15 min per 10 ⁶ d.p.m. of [¹⁴ C]phospholipid) to:			
	[¹⁴ C]Etn		[¹⁴ C]Cho	
	Untreated	PMA-pretreated	Untreated	PMA-pretreated
None	4650 ± 520	6280 ± 310	1750 ± 170	4890 ± 130
PDGF	8410 ± 450	6510 ± 160	4410 ± 210	5180 ± 330
PMA	38 550 ± 1370	7980 ± 450	25 230 ± 160	4630 ± 400
Serum	9120 ± 240	9940 ± 410	7620 ± 430	4910 ± 470
Serum + PDGF	11 540 ± 510	10 710 ± 280	8980 ± 370	5330 ± 390

Fig. 3. Long-term effects of serum, PDGF-BB and PMA on the hydrolysis of PtdEtn in NIH 3T3 cells

Cells were prelabelled with [¹⁴C]Etn for 48 h, followed by incubation of suspended cells as described in the Materials and methods section. (a) Incubations were performed in the absence (●) or presence of 100 nM-PMA (▲), 10 % serum (■) or PMA plus serum (◆). (b) Incubations were performed in the absence (●) or presence of 100 nM-PMA (▲), 50 ng of PDGF-BB/ml (■) or PMA + PDGF-BB (◆). Each point represents the mean ± S.E.M. of four determinations. Similar results were obtained in another experiment.

maximal effects on [¹⁴C]Etn formation during the first 15 min of the incubation period, whereas after 1 h its effect was barely distinguishable from the control value. On the other hand, serum had little influence on the effect of PMA during the first 15 min, but it clearly potentiated PMA-induced PtdEtn hydrolysis after 1 h of incubation. In contrast to serum, PDGF failed to enhance the effect of PMA on PtdEtn hydrolysis even after 1 h of incubation (Fig. 3b).

In previous studies [17,22], the role of phospholipase D in the mediation of alcohol and sphingosine effects on PtdEtn hydrolysis was verified by using [³²P]PtdEtn-prelabelled cells. Using a similar approach, I found that treatment of [³²P]PtdEtn-prelabelled (suspended) cells with PDGF (50 ng/ml) in the presence of 100 μM-propranolol (an agent commonly used to inhibit phosphatidic acid phosphohydrolase; see, for example, [25–27]) resulted in about a 2-fold increase in the formation of [³²P]phosphatidic acid (Table 2). The ³²P content of PtdCho

Table 2. Stimulation by PDGF-BB of [³²P]phosphatidic acid formation from [³²P]PtdEtn in NIH 3T3 cells

Suspended cells (3.6 × 10⁶/ml), prelabelled with [³²P]PtdEtn as described in the Materials and methods section, were incubated in standard culture medium containing 100 μM-propranolol for 10 min; 50 ng of PDGF-BB/ml was added where indicated. Data are the means ± S.E.M. of four determinations. Qualitatively similar results were obtained in two other experiments. The ³²P content of cellular PtdEtn and PtdCho was 14470 and 860 d.p.m./10⁶ cells respectively. * *P* < 0.05 compared with control, according to Student's *t* test.

Addition	Incubation time (min)	[³² P]Phosphatidic acid (d.p.m./10 ⁶ cells)
None	0	142 ± 11
None	10	120 ± 17
PDGF-BB	10	238 ± 22*

Table 3. Effect of bombesin on PtdEtn hydrolysis in NIH 3T3 fibroblasts

Cells were prelabelled with [¹⁴C]Etn for 48 h as described in the Materials and methods section. Labelled cells were further incubated for 20 min in the absence or presence of bombesin (50 nM), PDGF-BB (50 ng/ml), PMA (100 nM) or fetal bovine serum (10 %, v/v). Data are the means ± S.E.M. of four determinations. Similar results were obtained in two other experiments. * Significantly different from values obtained in the absence of bombesin (*P* < 0.05).

Addition	Formation of [¹⁴ C]Etn (d.p.m. of [¹⁴ C]product/20 min per 10 ⁶ d.p.m. of [¹⁴ C]PtdEtn)	
	None	Bombesin
None	7560 ± 350	11 180 ± 120
PMA	49 650 ± 1790	40 980 ± 850*
PDGF	17 090 ± 440	14 430 ± 520*
Serum	19 320 ± 430	18 950 ± 340

(860 d.p.m./10⁶ cells) was clearly too low, compared with the ³²P content of PtdEtn (14470 d.p.m./10⁶ cells), to serve as a precursor for [³²P]phosphatidic acid. I should add here that, because of a probably uneven distribution of [³²P]PtdEtn in cells, these data can prove the role of phospholipase D in PtdEtn hydrolysis, but they do not reliably indicate changes in the mass of phosphatidic acid.

In fibroblasts, bombesin, similar to PDGF, stimulates the

hydrolysis of both PtdIns(4,5) P_2 [28,29] and PtdCho [7,10–12]. Thus, if it were a general rule that increased PtdIns(4,5) P_2 hydrolysis is coupled to increased hydrolysis of PtdEtn, then bombesin would be expected to have PDGF-like effects on the turnover of PtdEtn. In contrast to this expectation, exposure of prelabelled NIH 3T3 cells to an optimal (50 nM) concentration of bombesin for 5 min (results not shown) or 20 min (Table 3) caused a less than 50%, and somewhat variable, increase in the formation of [14 C]Etn. In addition, bombesin, unlike the other agents, inhibited the stimulatory effects of both PMA and PDGF, but not that of serum, on PtdEtn hydrolysis (Table 3). Addition of FGF (0.2–100 ng/ml) or insulin (1–100 nM) to NIH 3T3 cells for 5–20 min failed to elicit any increase in PtdEtn hydrolysis. It should be noted that these latter agents are not known to elicit enhanced hydrolysis of PtdIns(4,5) P_2 in fibroblasts.

DISCUSSION

An important difference between [14 C]Cho- and [14 C]Etn-labelled attached fibroblasts is that the latter cells contain unacceptably high background radioactivity in Etn and its water-soluble derivatives. For example, similar to my observations, Wright *et al.* [30] found that after labelling of serum-starved fibroblast cultures with [14 C]Etn or [14 C]Cho for 48 h, cells contained about 50 times more [14 C]Etn than [14 C]Cho (502836 versus 8123 d.p.m./well). Addition of epidermal growth factor to [14 C]Cho-labelled cells enhanced the formation of [14 C]Cho by about 25%, a change which is still detectable [30]. However, it is clearly impossible to detect a similar magnitude of change in the cellular content of [14 C]Etn. During the course of this work, I observed the same high background [14 C]Etn levels in attached NIH 3T3 fibroblasts. Therefore, in order to properly investigate possible hormonal effects on PtdEtn hydrolysis, the intracellular level of [14 C]Etn derivatives had to be decreased about two orders of magnitude. As described earlier [13,21], this can be achieved by a four-step procedure: (i) incubation of labelled cells in a radioactive-precursor-free medium for several hours, (ii) separation of [14 C]Etn from other products, (iii) gentle scraping and washing of suspended cells, and (iv) adding a sufficient amount of unlabelled Etn to the medium to prevent the metabolism of newly formed [14 C]Etn. In the present study, the above strategy ensured that at the beginning of incubation the cells uniformly contained only low amounts of [14 C]Etn (background levels were 800–1000 d.p.m./ 10^6 cells), which made accurate determination of newly formed [14 C]Etn feasible even in control cells.

At the same time, the above approach may not allow optimal detection of hormonal effects on PtdEtn hydrolysis. For example, it is possible that during the preincubation period (in the absence of [14 C]Etn), a substantial portion of the hormone-sensitive pool of PtdEtn had already been hydrolysed before the hormones were added. Another possibility is that scraping might have decreased the response of cells to hormones. However, despite these possible drawbacks, suspended fibroblasts provided important information which could not be obtained by using attached cells.

It has been proposed that in fibroblasts the hydrolysis of PtdCho by phospholipase D is a common response to mitogens which stimulate PtdCho hydrolysis [11]. Although both PDGF [1–4] and bombesin [7,10–12] have been shown to stimulate the hydrolysis of PtdIns(4,5) P_2 in fibroblasts, these agents had different effects on PtdEtn hydrolysis. In particular, bombesin had only a relatively small stimulatory effect on PtdEtn hydrolysis, and, in contrast to PDGF, it inhibited the stimulatory effect of PMA. While the mechanism and significance of this inhibitory bombesin effect remain to be determined, these data

clearly indicate that one cannot anticipate the effects of hormones on PtdEtn hydrolysis on the basis of their ability to stimulate PtdIns(4,5) P_2 turnover.

The effects of PDGF on the hydrolysis of both PtdEtn and PtdCho appear to involve protein kinase C. However, it should be stressed that this assumption is based solely on the inhibition of the PDGF effect by prolonged treatment of cells with PMA. In addition to down-regulation of protein kinase C [23,24], prolonged treatment of cells with PMA may also cause changes both in the level of receptors and in the ability of receptors to bind PDGF. Accordingly, further experiments are needed to clarify the role of protein kinase C in the mediation of the effects of PDGF.

Similar to PDGF and bombesin, serum was shown to stimulate the hydrolysis of PtdIns(4,5) P_2 in fibroblasts [31–33]. Here I have shown that serum, like PDGF, also stimulates the hydrolysis of both PtdEtn and PtdCho. However, whereas pretreatment of cells with PMA for 30 h completely abolished the effect of serum on PtdCho hydrolysis and that of PDGF on the hydrolysis of both phospholipids, PMA pretreatment only slightly affected serum-induced PtdEtn hydrolysis. Thus serum clearly differentially regulates phospholipase D-mediated hydrolysis of PtdEtn and PtdCho. Even though PMA pretreatment failed to abolish the effect of serum on PtdEtn hydrolysis, possible mediation of the serum effect by a protein kinase C isoform still cannot be excluded. In fact, the potentiating effect of serum on PMA-induced PtdEtn hydrolysis would support this possibility. In this context, it is worth mentioning that, in our hands, prolonged treatment of NIH 3T3 fibroblasts with PMA causes nearly complete (< 90%) down-regulation of only the α -isoform, but not the ζ -isoform, of protein kinase C (Z. Kiss & N. Gavamszegi, unpublished work). It remains to be seen whether hydrolysis of PtdCho and PtdEtn, which might be mediated by different isoforms of phospholipase D [20], are regulated by different isoforms of protein kinase C.

In conclusion, I report for the first time that PDGF and serum, and to a lesser extent bombesin, stimulate the phospholipase D-mediated hydrolysis of PtdEtn. While the effects of PDGF on the hydrolysis of PtdCho and PtdEtn may be mediated by a common mechanism involving protein kinase C, serum clearly regulates the hydrolysis of these phospholipids by distinct mechanisms, which may or may not involve protein kinase C. In addition, despite similar effects of PDGF and bombesin on PtdIns(4,5) P_2 hydrolysis, these growth factors have distinct effects on PtdEtn hydrolysis.

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