The zinc chelator 1,10-phenanthroline enhances the stimulatory effects of protein kinase C activators and staurosporine, but not sphingosine and H₂O₂, on phospholipase D activity in NIH 3T3 fibroblasts

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Protein kinase C (PKC), an enzyme which is believed to mediate the stimulatory effects of the PKC activator phorbol 12-myristate 13-acetate (PMA) on phospholipase D (PLD) activity, has a zinc-dependent structure required for phorbol ester binding. Accordingly, zinc or zinc chelators would be expected to promote or inhibit, respectively, the stimulatory effects of PMA on PLD-mediated phospholipid hydrolysis. Instead, treatment of [¹⁴C]choline- and [¹⁴C]ethanolamine-labelled NIH 3T3 fibroblasts with the high-affinity zinc chelator 1,10-phenanthroline (0.2–1 mM) for 20–30 min was found to enhance the stimulatory effects of PMA on PLD-mediated hydrolysis of phosphatidylcholine and phosphatidylethanolamine. In [¹⁴C]palmitic acid-labelled fibroblasts, in the presence of ethanol, phenanthroline

also enhanced the stimulatory effect of PMA on the synthesis of phosphatidylethanol, a marker of PLD activity. Addition of zinc (250 μM) to phenanthroline-treated fibroblasts reversed the stimulatory effects of the chelator. The potentiating effects of phenanthroline were also partially reversed by cadmium, whereas iron, lead, copper, magnesium and calcium were without effects. Of the other activators of PLD tested, phenanthroline also enhanced the stimulatory effects of platelet-derived growth factor and staurosporine, but not that of sphingosine and H₂O₂, on the hydrolysis of both phospholipids. These results suggest that regulation of PLD by PKC activators and staurosporine involves a common intermediate step, which is inhibited by a chelatable cellular pool of zinc.

INTRODUCTION

Activators of protein kinase C (PKC), including phorbol 12-myristate 13-acetate (PMA) [1,2], 1,2-diacylglycerol-elevating hormones [3] and exogenous phospholipase C [4], commonly stimulate phospholipase D (PLD)-mediated hydrolysis of both phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEtn). Most recent evidence suggests that the effects of PKC activators on PLD activity are mediated by PKC, although a protein-phosphorylation step may not be involved [5].

Recent studies also established PKC as a zinc-binding protein [6–13]. Four atoms of zinc bind tightly to the regulatory domain of PKC, which appears to be a requirement for the binding of phorbol ester to this region [13]. As a consequence, the zinc status of PKC affects phorbol-ester-induced redistribution of this enzyme in cells [6,8,10]. In intact cells, phorbol-ester-induced membrane translocation of PKC was shown to be reversed by the high-affinity zinc-specific chelator 1,10-phenanthroline, indicating that membrane-bound PKC is regulated by a chelatable cellular zinc pool [11]. Accordingly, one would expect that 1,10-phenanthroline will inhibit phorbol ester stimulation of PLD. The present work was initiated to examine this possibility. I found that in NIH 3T3 fibroblasts 1,10-phenanthroline enhanced, rather than inhibited, the stimulatory effect of PMA and platelet-derived growth factor (PDGF) on PLD activity. In addition, phenanthroline also enhanced the stimulatory effect of staurosporine, which was shown to stimulate PLD activity by a PKC-independent mechanism [14]. These results indicate that in fibroblasts a chelatable pool of zinc negatively influences regulation of PLD activity by PKC activators and staurosporine.

EXPERIMENTAL

Materials

PMA, Dowex-50W (H⁺ form), 1,10-phenanthroline, sphingosine, H₂O₂, ZnCl₂, Pb(NO₃)₂, FeCl₃, CdCl₂, CaCl₂, MgCl₂, cupric acetate, choline chloride and ethanolamine were purchased from Sigma; staurosporine was from LC Services; PDGF-BB (human, recombinant) was from Boehringer Mannheim; [1-¹⁴C]palmitic acid (60 mCi/mmol), [2-¹⁴C]ethanolamine (50 μCi/mmol) and [methyl-¹⁴C]choline (55 μCi/mmol) were bought from Amersham Corp.; tissue culture reagents were bought from GIBCO; phosphatidylethanol (PtdEtOH) was purchased from Avanti Polar Lipids Inc.

Cell culture

NIH 3T3 clone-7 fibroblasts were continuously cultured in Dulbecco's modified Eagle's medium, supplemented with 10% (v/v) fetal-calf serum, penicillin (50 units/ml)/streptomycin (50 μg/ml) and glutamine (2 mM). Fibroblasts were seeded in 150 mm-diameter plastic dishes (Costar), and growing (70–80% confluent) cell populations were harvested (3–6 dishes) after 2 days in culture.

Measurement of formation of PtdEtOH in fibroblasts prelabelled with [¹⁴C]palmitic acid

Fibroblasts were grown in 6-well tissue-culture dishes for 48 h up to about 90% confluency, and labelled with [1-¹⁴C]palmitic acid (0.35 μCi/ml) for the last 24 h. Fibroblasts were washed, in-

cubated in fresh Dulbecco's medium for an additional 2 h (to minimize the amount of unesterified labelled palmitic acid), and then treated with PMA and/or phenanthroline in the presence of 200 mM ethanol for 20 min. Incubations were performed in an incubator at 37 °C and were terminated by scraping the fibroblasts into 2 ml of ice-cold methanol, followed by rapid transfer of the methanol extract to 2 ml of chloroform. PtdEtOH was separated from other phospholipids on potassium oxalate (1%)-impregnated silica gel H plates (Analtech) by using the solvent system chloroform/methanol/acetone/acetic acid/water (50:10:15:10:2, by vol.).

Measurement of PLD-mediated hydrolysis of PtdCho and PtdEtn in NIH 3T3 fibroblasts

Fibroblasts, grown in 150 mm-diameter dishes, were incubated with either [*methyl*- ^{14}C]choline (0.3 $\mu\text{Ci}/\text{ml}$) or [^{14}C]ethanolamine (0.25 $\mu\text{Ci}/\text{ml}$) for 48 h, washed, incubated in fresh medium for 3 h (to decrease the level of water-soluble ^{14}C -labelled phospholipid precursors), then harvested by scraping from four to six dishes. As discussed in detail elsewhere [3,15], the major advantage of using suspended fibroblasts is that they contain much lower background levels of [^{14}C]ethanolamine (500–700 d.p.m./ 10^6 cells) than do attached cells, which permits accurate determination of [^{14}C]PtdEtn hydrolysis. Because scraping transiently enhances cellular 1,2-diacylglycerol levels in NIH 3T3 fibroblasts (discussed in ref. [6]), an additional 20 min incubation period between scraping and centrifugation (10 min at 500 g) was inserted. A 30 min period is needed for the return of cellular 1,2-diacylglycerol to the control level [16]. At this step, about 95% of cells were viable, determined by the Trypan Blue dye exclusion assay. Scraped cells retained sensitivity to activators of PLD, including PMA [1,2], 1,2-diacylglycerol-elevating hormones [3], sphingosine [17,18] and staurosporine [18]. Portions (0.2 ml) of cell suspension [$(1.6\text{--}2.0) \times 10^6$ cells/ml] were incubated (final volume 0.25 ml) in the presence of unlabelled choline (20 mM) or ethanolamine (2 mM) (to prevent further metabolism of newly formed ^{14}C -labelled bases [1,15]). Fractionation of choline and ethanolamine metabolites was performed on Dowex-50W (H^+)-packed columns (Bio-Rad Econo-columns; 1 ml bed volume) as described by Cook and Wakelam [19] for the choline derivatives with some modifications. The initial flow-through (4.5 ml) along with a following 3.5 ml or 5 ml water wash contained glycerophosphoethanolamine or glycerophosphocholine, respectively. Ethanolamine phosphate and choline phosphate were eluted by 15 ml and 20 ml of water, respectively. Finally, ethanolamine and choline were eluted by 12 ml and 20 ml of 1 M HCl, respectively. The metabolites of [^{14}C]ethanolamine and [^{14}C]choline were further identified by t.l.c. [1]. In previous studies we determined that, under these conditions, formation of [^{14}C]choline and [^{14}C]ethanolamine is due to PLD activity [1]. Phospholipids were separated as described previously [20].

RESULTS

Effects of 1,10-phenanthroline and zinc on PMA-stimulated PLD activity

Formation of metabolically stable PtdEtOH from phospholipids and ethanol is a well-accepted diagnostic tool to determine the activity of PLD. In unstimulated NIH 3T3 fibroblasts the rate of PtdEtOH synthesis was very low, and it was not significantly affected by 150 or 300 μM concentrations of zinc (employed in the form of ZnCl_2) or by 1 mM 1,10-phenanthroline, a high-

affinity zinc chelator, during a 20 min incubation period (Figure 1a). PMA concentrations of 5 and 100 nM stimulated PtdEtOH synthesis about 5.6- and 14.5-fold respectively. Although zinc had the tendency to enhance PMA-induced PtdEtOH synthesis slightly, these effects were usually within the margin of experimental error. In contrast, 1 mM phenanthroline enhanced the effects of 5 and 100 nM concentrations of PMA about 1.7- and 1.45-fold respectively (Figure 1a).

Previous work from our laboratory established that in NIH 3T3 fibroblasts PMA-activated PLD hydrolyses both PtdCho and PtdEtn [1,2]. Thus it was decided to determine whether phenanthroline had similar or different effects on the hydrolysis of these phospholipids. For reasons discussed in the Experimental section, suspended fibroblasts were used for these experiments. It should be emphasized that, by using an optimally effective concentration (100 nM) of PMA, when higher background levels of [^{14}C]ethanolamine have less effect on the determination of PMA effect on phospholipid hydrolysis (because phospholipid hydrolysis is so extensive), we found that scraping causes only about 15% decrease in PMA-induced PtdEtn hydrolysis. Addition of zinc to [^{14}C]ethanolamine-labelled suspended fibroblasts failed to modify the hydrolysis of [^{14}C]PtdEtn in the absence or presence of PMA (Figure 1b). Addition of 1 mM 1,10-phenanthroline alone to these cells also was without effect on [^{14}C]PtdEtn hydrolysis. However, this chelator sig-

Figure 1 Effects of zinc and 1,10-phenanthroline on the synthesis of PtdEtOH and on the hydrolysis of PtdCho and PtdEtn in NIH 3T3 fibroblasts

NIH 3T3 fibroblasts, prelabelled with [^{14}C]palmitic acid (a), [^{14}C]ethanolamine (b) or [^{14}C]choline (c) as described in the Experimental section, were treated with PMA for 20 min as indicated in the absence (□) or presence of 150 μM ZnCl_2 (▨), 300 μM ZnCl_2 (▩) or 1 mM 1,10-phenanthroline (■). Data are means $\pm$ S.E.M. of three experiments each performed in triplicate: *significantly ($P < 0.05$) different from PMA-treated samples (Student's t test).

Figure 2 Inhibition of PMA-induced hydrolysis of PtdEtn and PtdCho by zinc in NIH 3T3 fibroblasts pretreated with 1,10-phenanthroline

NIH 3T3 fibroblasts were labelled with [^{14}C]ethanolamine (a) or [^{14}C]choline (b) for 48 h, washed, then incubated for an additional 3 h period in the absence (I) or presence of 1 mM 1,10-phenanthroline (II) added for the last 30 min of incubation. Attached fibroblasts were washed, collected by scraping from dishes, and then suspended fibroblasts were incubated for 20 min in the absence ($\square$) or presence of 250 μM ZnCl_2 ($\square$), 100 nM PMA ($\boxtimes$), or PMA plus ZnCl_2 ($\blacksquare$). Data are means $\pm$ S.E.M. of three experiments each performed in triplicate: *, **, significantly ($P < 0.01$) different from samples treated with PMA in the absence of 1,10-phenanthroline, or from the corresponding PMA-treated samples, respectively.

nificantly enhanced the stimulatory effect of both lower (5 nM) and higher (100 nM) concentrations of PMA (Figure 1b). In [^{14}C]choline-labelled fibroblasts, zinc occasionally had small, but not reproducible, stimulatory effects on [^{14}C]PtdCho hydrolysis in the presence of PMA (Figure 1c). In contrast, 1,10-phenanthroline significantly enhanced the stimulatory effects of 5 and 100 nM PMA on [^{14}C]PtdCho hydrolysis (Figure 1c). On the whole, the potentiating effects of 1,10-phenanthroline on PMA-induced hydrolysis of PtdEtn and PtdCho were comparable with those on PMA-induced PtdEtOH formation.

Data in Fig. 1 implied that removal of a chelatable cellular pool of zinc by 1,10-phenanthroline results in enhanced activation of PLD activity by PMA. If so, re-addition of zinc (or another heavy metal chelated by 1,10-phenanthroline) to phenanthroline-treated fibroblasts should decrease the effect of PMA on phospholipid hydrolysis. To test if this was the case, attached fibroblasts, prelabelled with either [^{14}C]ethanolamine or [^{14}C]choline, were treated with 1 mM 1,10-phenanthroline for 30 min, followed by the incubation of suspended (washed) fibroblasts in the absence or presence of zinc. In untreated fibroblasts, 250 μM zinc had little or no effect on PMA-induced hydrolysis of PtdEtn (Figure 2a) or PtdCho (Figure 2b). Treatment of fibroblasts with phenanthroline enhanced the stimulatory effect of 100 nM PMA on the hydrolysis of [^{14}C]PtdEtn (Figure 2a) and [^{14}C]PtdCho (Figure 2b) about 1.35- and 1.6-fold respectively. Importantly,

Figure 3 Concentration-dependent combined effects of PMA and 1,10-phenanthroline on the hydrolysis of PtdEtn and PtdCho in NIH 3T3 fibroblasts

Attached fibroblasts were labelled with [^{14}C]ethanolamine (a) or [^{14}C]choline (b) for 48 h, followed by treatments of suspended fibroblasts with different concentrations (0–1 mM) of 1,10-phenanthroline for 60 min in the absence ($\bullet$) or presence of 5 nM PMA ($\blacktriangle$), 20 nM PMA ($\blacksquare$) or 100 nM PMA ($\blacklozenge$). Each point represents the mean $\pm$ S.E.M. of three incubations. Similar results were obtained in three other experiments.

Figure 4 Effect of zinc on PMA-stimulated PtdEtn hydrolysis in NIH 3T3 fibroblasts

[^{14}C]Ethanolamine-labelled suspended fibroblasts were treated with various concentrations (3–100 nM) of PMA in the absence ($\bullet$) or presence ($\blacktriangle$) of 250 μM ZnCl_2 for 60 min. Each point represents the mean $\pm$ S.E.M. of two experiments each performed in triplicate: *significantly ($P < 0.01$) different from the corresponding value.

addition of 250 μM zinc to phenanthroline-treated fibroblasts reversed the potentiating effects of chelator on PMA-induced hydrolysis of PtdEtn and PtdCho (Figure 2). Increasing the concentration of ZnCl_2 up to 1 mM failed to result in greater inhibition of PMA effects (results not shown). Of the other metals examined, 250 μM cadmium partially (about 60%) reversed the potentiating effects of phenanthroline, whereas similar

Table 1 Effects of 1,10-phenanthroline on PLD-mediated hydrolysis of PtdEtn and PtdCho induced by PDGF, sphingosine or H₂O₂

Fibroblasts were labelled with [¹⁴C]ethanolamine or [¹⁴C]choline for 48 h, followed by incubation of suspended fibroblasts for 20 min in the absence or presence of 1 mM phenanthroline and/or PDGF, sphingosine and H₂O₂ at concentrations as indicated. Data are means $\pm$ S.E.M. of three incubations. Similar results were obtained in two other experiments.

Addition	Formation (d.p.m./20 min per 10 ⁶ d.p.m. of [¹⁴ C]phospholipid) of:			
	[¹⁴ C]ethanolamine		[¹⁴ C]choline	
	None	Phenanthroline	None	Phenanthroline
None	1840 $\pm$ 230	1460 $\pm$ 140	2230 $\pm$ 370	2010 $\pm$ 260
PDGF (50 nM)	3780 $\pm$ 170	6540 $\pm$ 190	5050 $\pm$ 420	7420 $\pm$ 480
Sphingosine (12.5 μ M)	4930 $\pm$ 150	4810 $\pm$ 290	4810 $\pm$ 380	4360 $\pm$ 310
Sphingosine (25 μ M)	12110 $\pm$ 270	11370 $\pm$ 330	9150 $\pm$ 620	8340 $\pm$ 440
Sphingosine (40 μ M)	18550 $\pm$ 360	17820 $\pm$ 680	12990 $\pm$ 840	11190 $\pm$ 630
H ₂ O ₂ (0.5 mM)	3060 $\pm$ 290	3120 $\pm$ 330	3990 $\pm$ 210	4060 $\pm$ 370
H ₂ O ₂ (2 mM)	5450 $\pm$ 140	5110 $\pm$ 110	5840 $\pm$ 670	6500 $\pm$ 540

Table 2 Potentiating effects of 1,10-phenanthroline on staurosporine-induced hydrolysis of PtdEtn and PtdCho

NIH 3T3 fibroblasts were labelled with [¹⁴C]ethanolamine or [¹⁴C]choline for 48 h, followed by incubation of suspended fibroblasts for 40 min in the absence or presence of 1 mM phenanthroline and/or staurosporine as indicated. Data are means $\pm$ S.E.M. of three incubations. Similar results were obtained in two other experiments.

Addition	Formation (d.p.m./40 min per 10 ⁶ d.p.m. of [¹⁴ C]phospholipid) of:			
	[¹⁴ C]ethanolamine		[¹⁴ C]choline	
	None	Phenanthroline	None	Phenanthroline
None	4280 $\pm$ 670	4410 $\pm$ 340	6000 $\pm$ 150	5780 $\pm$ 340
Staurosporine (2 μ M)	8810 $\pm$ 340	18730 $\pm$ 350	13570 $\pm$ 170	24980 $\pm$ 1360
Staurosporine (5 μ M)	12780 $\pm$ 470	19670 $\pm$ 370	17620 $\pm$ 750	26420 $\pm$ 830

concentrations of iron (Fe³⁺ ions), lead, copper, magnesium and calcium had no such effects (results not shown).

Since dissociation of zinc from zinc-binding proteins is likely to be slow [13], significant depletion of the total cellular (including protein-bound) zinc pool may require longer treatments with 1,10-phenanthroline. Thus, in principle, increasing the time of treatment may result in greater potentiating effects of 1,10-phenanthroline. To examine if this was the case, in the next experiments [¹⁴C]ethanolamine- and [¹⁴C]choline-labelled suspended fibroblasts were treated with different concentrations (0.2–1 mM) of 1,10-phenanthroline in the absence or presence of various concentrations (5, 20 and 100 nM) of PMA for 60 min (after longer than 60 min treatment, 1 mM phenanthroline was slightly toxic to these cells, judged from their decreased re-attachment to substratum). Surprisingly, although increasing concentrations of 1,10-phenanthroline proportionally enhanced the effect of 5 nM PMA on [¹⁴C]PtdEtn hydrolysis, the effects of 20 nM and 100 nM PMA were not significantly enhanced by any concentrations of 1,10-phenanthroline (Figure 3a). In contrast, 1,10-phenanthroline enhanced, although with somewhat different concentration-dependency, the effects of both lower (5 nM) and higher (20 and 100 nM) concentrations of PMA on [¹⁴C]PtdCho hydrolysis (Figure 3b). These differences in the effects of zinc chelator on the hydrolysis of PtdEtn and PtdCho were highly reproducible (four experiments were performed).

It has been shown previously [6] that treatment of rabbit thymocytes with 20 nM PMA for 90 min resulted in intracellular redistribution of zinc. A similarly slow PMA-induced redistribution of zinc from the site of PtdEtn hydrolysis to other intracellular sites might explain why 1,10-phenanthroline failed

to enhance the effects of 20–100 nM PMA on PtdEtn hydrolysis after 60 min treatment. However, if such a mechanism indeed operates in fibroblasts, then restoration of zinc in 'PMA-sensitive' intracellular pool(s) by externally added zinc should lead to an inhibition of PtdEtn hydrolysis induced by higher concentrations of PMA. To test if this might be the case, the effects of 250 μ M zinc on PtdEtn hydrolysis, induced by various concentrations of PMA for 60 min, were next examined. As shown in Figure 4, zinc had no significant effect on PtdEtn hydrolysis in the presence of low concentrations (3–10 nM) of PMA, whereas it significantly inhibited the stimulatory effects of higher concentrations (20–100 nM) of PMA. In similar experiments performed with [¹⁴C]choline-labelled fibroblasts, zinc had no effect on [¹⁴C]PtdCho hydrolysis stimulated by 5, 20 or 100 nM PMA (results not shown).

Effect of 1,10-phenanthroline on phospholipid hydrolysis in the presence of other stimulators of PLD

In NIH 3T3 fibroblasts, hormonal activators of PKC, such as PDGF and bombesin, also increase the rate of hydrolysis of both PtdCho and PtdEtn [3]. In addition to PKC activators, PLD-mediated hydrolysis of these phospholipids is also commonly elicited by sphingosine [17,18], H₂O₂ [21] and staurosporine [14,18]. To define better the site of action of 1,10-phenanthroline, I next examined the combined effects of this chelator and the above PLD stimulators. As shown in Table 1, 1,10-phenanthroline significantly enhanced the stimulatory effects of PDGF on the hydrolysis of both PtdCho and PtdEtn, whereas it failed to modify the stimulatory effects of both sub-optimal and

optimal concentrations of sphingosine and H_2O_2 . It should be emphasized here that the length of incubation time (20 min) was optimal for the effects of these PLD stimulators.

In contrast with the above stimulators of PLD, the optimal incubation time for the stimulatory effect of staurosporine on phospholipid hydrolysis was about 40 min, as determined in preliminary time-course experiments (results not shown). During this incubation time, 1 mM 1,10-phenanthroline significantly (1.5–2.1-fold) enhanced the stimulatory effects of both sub-optimal (2 μ M) and optimal (5 μ M) concentrations of staurosporine on PLD-mediated hydrolysis of PtdCho and PtdEtn (Table 2). Addition of 250 μ M zinc to fibroblasts pretreated with 1 mM phenanthroline for 30 min also reversed the potentiating effect (1.6–1.8-fold) of chelator on staurosporine (5 μ M)-induced phospholipid hydrolysis (results not shown).

DISCUSSION

The major finding of this work is that 1,10-phenanthroline, a high-affinity zinc chelator, potentiated, rather than inhibited, the stimulatory effects of PKC activators PMA and PDGF on PLD activity. The potentiating effects of 1,10-phenanthroline were reversed by zinc and to a lesser extent by cadmium, but not by other metals. At the present, I cannot exclude the possibility that some of these metals (in particular, iron, lead and copper) were ineffective because of their poor cellular uptake. The inhibitory effect of zinc was unexpected because zinc has a positive influence on PKC activity [6–13], and because PKC appears to mediate the stimulatory effect of PMA on PLD activity [5]. There are three possible mechanisms which may account for the observed potentiating effects of 1,10-phenanthroline and for the inhibitory effects of zinc.

The first possibility is that a chelatable cellular pool of zinc directly inhibits PLD activity, whereas PKC is regulated by tightly bound (non-chelatable) zinc. In this case, 1,10-phenanthroline would act at the level of PLD, and would be expected to inhibit the effect of any PLD activator. However, 1,10-phenanthroline clearly failed to enhance the stimulatory effects of both sub-optimal and optimal concentrations of sphingosine and H_2O_2 . For this reason, direct inhibition of PLD (which, in fact, has very low activity in unstimulated cells) by zinc is unlikely.

The second possibility is that the effect of PMA on PLD activity is mediated by a PKC-independent protein which (in contrast with PKC) is inhibited by a chelatable cellular pool of zinc. Although it may be argued that more evidence is required to establish firmly the role of PKC in the regulation of PLD, no evidence has yet been presented against such role of PKC. Thus, although not totally impossible, presently available experimental evidence [5] disfavours the role of a zinc-inhibitable non-PKC protein in the mediation of PMA effect on PLD activity.

The third, and the most likely, possibility is that regulation of PLD activity by activated PKC occurs by a multiple-step process, and one of the intermediate stimulatory steps is inhibited by zinc. Work from several laboratories established synergistic interaction between the activators of PKC and G-proteins on the stimulation of PLD activity [22–25]. In addition, the action of staurosporine on PLD also appears to involve a G-protein [14]. Thus it is quite possible that, in NIH 3T3 fibroblasts as well, a G-protein is involved in the mediation, or at least in the potentiation, of phorbol ester effects on PLD activity. In fact, we have previously shown [2] that in membranes from Ha-*ras*- or v-*raf*-transformed NIH 3T3 fibroblasts PMA can significantly stimulate PLD activity only in the concomitant presence of ATP and a hydrolysis-resistant analogue of GTP. Based on this evidence,

we have started investigating the possibilities that zinc inhibits either GTP activation of PLD, or the interaction between the PKC and GTP-binding proteins involved in the regulation of PLD. Preliminary results indicate that in isolated membranes [17] stimulation of PLD by stable GTP analogues is not significantly enhanced by 1,10-phenanthroline. Thus it seems more likely either that zinc inhibits interaction between PKC and a regulatory G-protein, or that the action of phorbol ester on PLD depends, in addition to PKC, on a presently unknown factor. Clearly, understanding of the exact mechanism of phorbol ester action on PLD is an important prerequisite for a precise determination of the site of inhibitory zinc action.

In fibroblasts, nearly all activators of PLD examined so far, including PMA [1,2], PKC-activating hormones [3], nucleotides [17], sphingosine [17,18] and staurosporine (the present work), stimulate the hydrolysis of both PtdCho and PtdEtn. The only exceptions are alcohols, which stimulate only PtdEtn hydrolysis, and do so in a strictly PKC-dependent manner [26]. In the present work, at a longer incubation time (60 min), phenanthroline enhanced the hydrolysis of only PtdCho, but not PtdEtn, at optimal (100 nM) or near-optimal (20 nM) concentrations of PMA. Conversely, at this incubation time (60 min) added zinc inhibited only the hydrolysis of PtdEtn, but not PtdCho, in the presence of higher concentrations (20–100 nM) of PMA. The exact reason(s) for the observed differences between the effects of zinc on PtdEtn and PtdCho hydrolysis is not known. However, it has been reported previously [6] that PMA can induce slow intracellular redistribution of zinc. Thus it is possible that in fibroblasts PMA can deplete a chelatable zinc pool which specifically regulates PtdEtn hydrolysis. Such process would (i) result in a maximal stimulation of PtdEtn hydrolysis by PMA, (ii) diminish the potentiating effect of 1,10-phenanthroline, and (iii) sensitize the PtdEtn-hydrolysing system to the inhibitory effect of added zinc. Although our results are in agreement with these predictions, further experiments are required to determine that PMA indeed induces redistribution of zinc in fibroblasts and that this specifically correlates with altered regulation of PtdEtn hydrolysis by PMA.

Regardless of the exact mechanism(s) by which 1,10-phenanthroline and zinc acted, these observations indicate that the PtdCho- and PtdEtn-hydrolysing systems are somewhat differentially regulated by zinc. This may reflect the existence of separate PLD enzyme systems, acting on PtdCho and PtdEtn with some selectivity, which may be differentially sensitive to the inhibitory effect of zinc. In addition, these phospholipases may catalyse the hydrolysis of PtdCho and PtdEtn in different subcellular compartments which contain different levels of chelatable zinc. Recent data, indicating that most cell types express two different PLD molecules hydrolysing either PtdEtn and/or PtdCho [27], could provide a basis for the observed differential effects of zinc on phospholipid hydrolysis. However, further experiments are required to prove that fibroblasts indeed express different, PtdCho- and PtdEtn-specific, PLD activities.

In summary, I showed that, despite the positive effect of zinc on the PKC system, a chelatable cellular pool of zinc inhibits, rather than potentiates, the stimulatory effects of PMA, PDGF and staurosporine on PLD-mediated hydrolysis of PtdCho and PtdEtn. The results suggest that the inhibitory effects of zinc are not due to its direct interaction with PLD or PKC; thus they probably occur at an intermediate level. This implies that in fibroblasts regulation of PLD by activated PKC occurs by a multi-step process.

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