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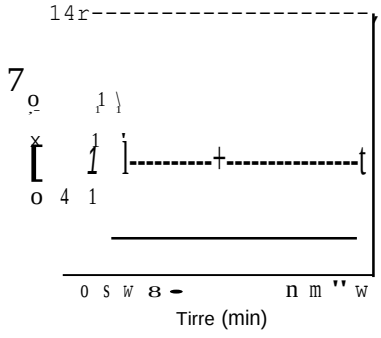
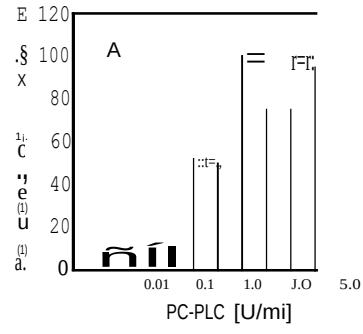


Figure 2. Time Course of PCho Ralease in Response to Addition of B. cereus PC-PLC

[Methyi-^{14}C]choline-labeled quiescent Swiss 3T3 fibroblasts were either untreated (Bl} or treated with 1 U/mi B. cereus PC-specific PLC (4), and reactions were terminated at different times. Intracellular PCho ralease was then determinad as described. Results are the mean ± SD of three independent experiments with incubations in



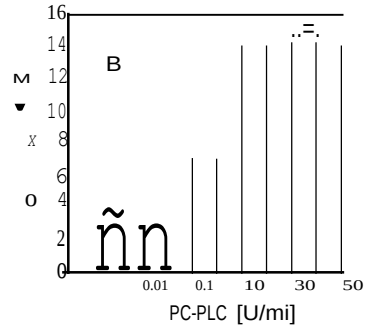


Figure 3. Dose Response of the Effect of B. cereus PC-PLC on DNA Synthesis and PCho Release

(A) Quiescent cells were stimulated with different concentrations of B. cereus PC-PLC, and DNA synthesis was determinad as described undar Experimental Procedures. Control value was 7100 ::t 500 dpmlwell and maximal stimulation was 92,000 ± 300 dpm/well.

(B) (Methyi-^{14}C]choline-labeled quiescent cells were treated with different concentrations of B. cereus PC-PLC for 5 min, alter which PCho ralease was determinad as described.

Results are the mean ± SO *ot* three independent experiments with incubations in duplicate.

DNA synthesis by itself. In the following experiments we examined whether the exogenous addition of PC-PLC was sufficient to elicit a mitogenic response in serum-starved Swiss 3T3 fibroblasts. For this purpose, we used a highly purified PC-hydrolyzing PLC from B. cereus that has been characterized extensively (Johansen et al., 1988; Little, 1988). Results shown in Figure 2 clearly indicate that the addition of 1 U/mi B. cereus PC-PLC to quiescent [^{14}C]choline-labeled fibroblast cultures activated the prompt hydrolysis of PC. This occurred without any detectable effect on inositol-containing phospholipids or sphingomyelin and with little or no change in the level of phosphatidylethanolamine or phosphatidylserine (Table 2). However, the hydrolysis of PC was concomitan!with a dramatic increase in DAG levels (data not shown). One unit of B. cereus PC-PLC activated PC turnover to an extent similar to that produced by a saturating concentration of PDGF (10 ng/ml; compare Figure 1A with Figure 2). Interestingly, this concentration of enzyme was able to promote DNA synthesis (Figure 3A) ata magnitude 60% of that induced by a maximal dose (10 ng/ml) of PDGF.

The mitogenic activity of exogenous B. cereus PC-PLC correlated excellently with its PC-hydrolyzing activity (Figures 3A and 3B). As a further proof of the specificity of this effect, the following experiment was carried out. B. cereus PC-PLC was added to quiescent cell cultures either alone or in the presence of a potent neutralizing affinity-purified anti-B. cereus PC-PLC antibody. Results shown in Table 3 indicate that PLC-catalyzed PCho release was completely inhibited in the presence of the neutralizing antibody. Concomitan!with the inhibition of the enzymatic activity by the antibody, a complete abolition of the ability of B. cereus PC-PLC to induce DNA synthesis was observad. These data support the specificity of the mitogenic effect of PC-PLC.

Table 2. Specificity of B. cereus PC-PLC

	Phospholipid Levels (Percent of Control)				
Addition	PC	PE	PS	SM	PIPS
None	100	100	100	100	100
B. cereus PC-PLC (1 U/mi)	75 + 3	93 + 2	98 + 2	100 + 3	100 + 5

Swíss 3T3 fibroblasts were labeled with difieren!precursors as described under Experimental Procedures. Cells were treated with 1 U/mi B.cereus PC-PLC for 30 min, reactions were stopped, and lípids were extractad and fractionated as descríbed. Results are expressed as the percent of control values and are the mean ± SD of three independent experiments performed in duplicate. Control values for different phOspholipids were as follows: PC, 549,000 dpm/well; SM (sphingomyelin), 38,000 dpm/well; PE (phosphatidylethanolamine), 445,000 dpm/well; PS (phosphatidylserine), 83,000 dpmlwell; PIPs (polyphosphoinositides), 150,000 dpm/well.

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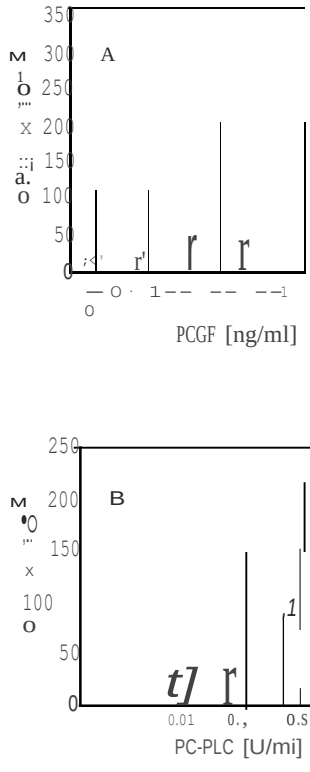

Figure 5. Synergistic and Nonadditive Eflects ol B. cereus PC-PLC, PDGF, and Insulin on DNA Synthesis

(A) Quiescent cells were treated with difieren!concentrations olPDGF in the absence (open bars) or presence (stippled bars) of 1 U/mi B. cereus PC-PLC or were treated with 10% serum (black bar) and incubated with 2 ttCi/ml [^{3}H)thymidine. Alter 24 hr of incubation, reactions were stopped and DNA synthesis was determinad.

(B) Ouiescent cells were treated with difieren!concentrations of B. cereus PC-PLC in either the absence (open bars) or presence (stippled bars) olinsulin (10 ttQ/ml). DNA synthesis under these conditions was determinad as described above.

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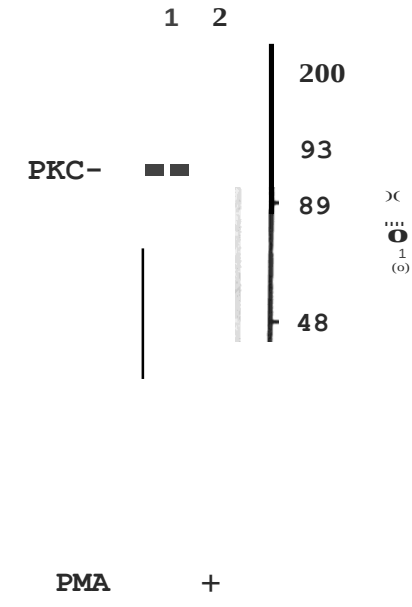

Figure 6. Effect of PMA Treatment on PKC Levels

Lysates containing 100 tt9 of total cellular protein were resolved by electroblotting as described in Experimental Procedures. PKC was visualizad using a monoclonal anti-PKC antibody. Lane 1, control; lane 2, treated with PMA (500 ng/ml) for 24 hr. Molecular weight markers are shown on the right.

prediction is confirmad by the results in Figure 58. Thus, although insulin alone induces little or no changa of the proliferative state of quiescent Swiss 3T3 fibroblasts, it greatly augments the mitogenic response elicited by the addition of different concentrations of B. cereus PC-PLC.

The Mltogenic Response Actlvated by PC-PLC Is Independent of PKC

DAG, which is a potent activator of PKC, is produced as a consequence of the PLC-mediated hydrolysis of PC. Therefore, PKC may be importan!for the mitogenic properties of PC-PLC. To address this possibility, PKC was down-regulated in Swiss 3T3 fibroblasts by treating cell cultures with phorbol myristate acetate (PMA) (500 ng/ml) for 24 hr. This treatment completely removed PKC from Swiss 3T3 fibroblasts, as determinad by Western blot analysis with a specific monoclonal anti-PKC antibody (Figure 6). The antibody used recognizes the a subtype of PKC, which is the sole PKC isotype present in Swiss 3T3 fibroblasts (Rose-John et al., 1988). Interestingly, results shown in Table 5 indicate that the mitogenic response produced by different concentrations of B. cereus PC-PLC is not inhibited in cells with down-regulated PKC. This strongly suggests that PKC activation is not a required step in the mitogenic cascada triggered by PC-PLC. The fact that PMA was unable to promote DNA synthesis in down-regulated cells confirms the lack of any residual PKC in depleted cells (Tabla 5). Furthermore, when the phosphorylation of p80-a very well established substrate for PKC in Swiss 3T3 fibroblasts (Biackshear et al., 1985; Rodríguez-Peña and Rozengurt, 1986)-was determinad in response to PMA in PKC down-regulated cells, no response was found, although a potent activation of that parameter was observed in control cells (data not shown).

Table 5. Effect of PKC Down-Regulation on B. cereus PC-PLC-Induced DNA Synthesis

Additions	PMA	DNA Synthesis (DPM x 10"3)
None		7 ± 2
	+	6 ± 1
PC-PLC (0.1 U/mi)		38 ± 3
	+	31 ± 5
PC-PLC (1.0 U/mi)		82 ± 8
	+	76 ± 7
PMA (100 ng/ml)		48 ± 6
	+	6 ± 2

Ouiescent Swiss 3T3 fibroblasts were either untreated or treated with PMA (500 ng/ml) for 24 hr. Afterward, cells were stimulated with B. cereus PC-PLC or PMA, and DNA synthesis was determinad. Results are the mean ± SO of three independent experiments with incubations in duplicate.

Discussion

A number of recent studies have unveiled the existence of a novel source of DAG in mammalian cells activated by different agonists (Besterman et al., 1986; lrving and Exton, 1987; Rosoff et al., 1988; Slivka et al., 1988; Diaz-Meco et al., 1989). This pathway is mediated by an as yet poorly characterized PC-hydrolyzing PLC activity. Since we and others have shown that the product of the *ras* oncogene, *ras* p21, potently activates this novel phospholipid degradative mechanism (Lacal et al., 1987; Moscat et al., 1989a; Price et al., 1989), this enzymatic activity could be important in the control of cell growth and transformation. Further support for this notion comes from studies showing the activation of the phosphodiesterase degradation of PC in response to severa! growth factors (Besterman et al., 1986; Muir and Murray, 1987; Pessin and Raben, 1989).

The results reportad here indicate that following the addition of human recombinant B-homodimer PDGF to Swiss 3T3 fibroblasts, a delayed and dramatic elevation in intracellular PCho levels is observad prior to any detectable increase in DNA synthesis. In contrast to the results of Besterman et al. (1986) with 3T3 L-1 cells stimulated by PDGF from an unspecified source, we did not detect any increase in intracellular PCho levels until 4 hr after PDGF challenge. The recent discovery of more than one PDGF receptor (Matsui et al., 1989) together with the fact that different cell lines were used in both studies could account for this discrepancy. On the other hand, as far as we know, the results shown here are the first report of long-term effects of a growth factor on PLC-mediated hydrolysis of PC.

Severa! authors have demonstrated that besides PLC, a PLD specific for PC can also be responsible for the activation of PC turnover in cells stimulated by severa! agonists (Bocckino et al., 1987; Agwu et al., 1989; Billah et al., 1989a, 1989b; Cook and Wakelam, 1989). Although from our results the action of a PLD cannot be completely ruled out in PDGF-stimulated Swiss 3T3 fibroblasts, such a possibility seems unlikely. Thus, the fact that addition of B-homodimer PDGF to Swiss 3T3 fibroblasts activates the release of PCho and DAG without detectable change in choline or PA levels strongly supports the notion that PC-PLC is the main route activated by B-homodimer PDGF in Swiss 3T3 fibroblasts. One attractive hypothesis could be that different agonists actívate distinct mechanisms leading to PC turnover. Further work is necessary to evaluate a possible role for PC-PLD in mitogenic pathways.

To examine the possible importance of PC-PLC activation in the mitogenic signaling cascada triggered by PDGF, we carried out a series of experiments aimed to investigate the potential mitogenic activity of a highly purified PC-hydrolyzing PLC from B. cereus. This enzyme has been characterized extensively (Johansen et al., 1988; Little, 1988), and we show that its addition to fibroblast cultures leads to the specific hydrolysis of PC. Our results also clearly demonstrate that the simple exogenous addition of this enzyme is able to induce a potent mitogenic response in quiescent Swiss 3T3 fibroblasts. Preliminary data obtained by an independent experimental strategy confirm the mitogenic effect of B. cereus PC-PLC: overexpression of this enzyme in Swiss 3T3 cells stably transfected within a plasmid containing the PC-PLC gene from B. cereus, under the control of a dexamethasone-inducible enhancer/promoter, leads to a potent mitogenic response associated with the generation of PCho (T. J., M. T. D.-M., and J. M., unpublished data).

The magnitude of the mitogenic effect elicited by a maximal dose of B. cereus PC-PLC is 60% of that produced by a saturating concentration (10 ng/ml) of PDGF. Smith et al. (1989) have recently demonstrated that microinjection of a PI-specific PLC into NIH 3T3 fibroblasts produces a mitogenic response similar to that described here by B. cereus PC-PLC. The fact that both PLCs cannot account for the full mitogenic potential of PDGF suggests that this growth factor may also be activating PLC-independent pathways that may be important for an optimal proliferative response.

On the other hand, the time course of the effect of B. cereus PC-PLC-activated DNA synthesis differs significantly from that produced by PI-PLC (Smith et al., 1989). Thus, whereas the peak of maximal mitogenic activity elicited by PI-PLC coincides with that produced by serum, the maximal DNA synthesis in response to B. cereus PC-PLC is detectad rv9 hr befare the maximal response triggered by PDGF, which is considerad to be the main mitogen in serum. Interestingly, the 9 hr interval between both maxima is very similar to the lag sean between the maximum of PDGF-induced PCho ralease and the activation, by this growth factor, of maximal DNA synthesis (compare Figure 4 with Figures 1A and 1C). Therefore, the fact that B. cereus PC-PLC mimics the mitogenic activity of PDGF in a time course that is in agreement with the kinetics of induction of PCho and DNA by this growth factor is consistent with a modal whereby PC-PLC is important in the control of cell proliferation. Yet whereas PI-PLC activation is an early step in the mitogenic signaling cascade, PC-PLC activation is a late event in this pathway.

Further support of this notion comes from our results concerning the additivity and synergism between B. cereus PC-PLC, PDGF, and insulin in the mitogenic response. Thus, the fact that B. cereus PC-PLC mimics the ability of PDGF-induced DNA synthesis to be synergized by insulin indicates that PC·PLC is actually activating the same route as PDGF. Furthermore, the lack of additivity of the presence of a maximal dose of B. cereus PC-PLC on the mitogenic response elicited by a saturating concentration of PDGF strongly indicates that PLC-mediated PC hydrolysis is actually an important step in the PDGF mitogenic signaling cascade.

The mechanism whereby B. cereus PC-PLC activates the release of intracellular PCho and induces DNA synthesis in Swiss 3T3 fibroblasts remains to be clarified. The use of the specific antibody described here, in immunofluorescence experiments underway in our laboratory, will help explain how the exogenous addition of this enzyme is able to mimic the increase in intracellular PCho levels triggered by PDGF. On the other hand, since DAG is a metabolic product of PC-PLC action, a feasible hypothesis to explain the mechanisms used by this enzyme to activate DNA synthesis should consider PKC activation as a

possible link in the chain of events triggered by that stimulus. Our results, however, show that in celis in which PKC is completely down-regulated, B. cereus PC-PLC elicits a mitogenic response identical to that produced in fibroblasts with normal PKC levels. Therefore, although DAG is generated after the addition of B. cereus PC-PLC, PKC is not required for the mitogenic activity of this PLC. Of note is that recent reports have found that the generation of biological signals associated with DAG production is not always mediated by PKC (van Corven et al., 1989; Hockberger et al., 1989). Whatever the mechanism, the results presentad here clearly demonstrate the importance of PDGF-activated PLC-mediated hydrolysis of PC in cell proliferation.

Experimental Procedures

Cell Cultures

Swiss 3T3 fibroblasts (passage 123) were purchased from Flow Laboratories and culturad in Dulbeccds modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 100 U/mi penicillin, 100 J!Q/ml streptomycin, and 2 mM L-glutamine. Cells were grown in standard tissue culture flasks in a humidified air:CO_2 (19:1) incubator al 37"C. Cells were made quiescent by incubation for 24 hr in !he presence of serum-free medium supplemented with 5 J!Q/ml transferrin and 1 J.tM Na_2SeO_3. The recombinant PDGF B-chain homodimer was from Amersham lnternational and bovine insulin was from Collaborative Research (Waltham, MA).

Isolation of PC-PLC from B. cereus and Preparation of Affinity-Purified Antibody

PC-PLC was isolated from cultures of B. cereus SE-1 essentially as described by Myrnes and Little (1980). Following the Johansen et al. (1988) protocol, the enzyme preparation was purified lo complete homogeneity as confirmad by SDS-PAGE followed by silver staining. The specifíc activíty of the purifíed enzyme was 1.5 U/J!Q (see Little, 1981). A rabbit antiserum was raised against this B. cereus PC-PLC by multiple intradermal injections with 75 1!9 of this enzyme. Serum was diluted 1:3 in phosphate-buffered saline (PBS) and applied to an Affigel10 (BioRad) column containing immobilized B. cereus PC-PLC. The column was washed with PBS, with PBS with increasing sal! (up lo 1 M NaCl), and with PBS containing 3 M urea befare elution with 4 M urea, 0.5 M NaCI adjusted lo pH 3.0 with acetic acid. The affinity-purified antibody was eluted directly into 1 M glycine-NaOH (pH 10.5] and dialyzed extensively against a suitable buffer. The sole presence of heavy and light antibody chains in the final preparation was confirmad by SDS-PAGE followed by silver staining.

Analysls of Products of PC Hydrolysls

Cells were labeled for 48 hr with 2 J.tCi of [methyi-^{14}C)choline (Amersham lnternational; spec. radioactivity 50--60 mCi/mmol)/dish. The last 24 hr of labeling was performed in serum-free medium supplemented as described above. Afterward, cells were treated with the corresponding agonists for difieren!times. Reactions were stopped by removing the supernatants and adding ice-cold methanol to cells. Methanolic cell extracts were fractionated into chloroform and aqueous phases as previously described (Biigh and Dyer, 1959). The presence and levels of intra- and extracellular water-soluble choline metabolites were evaluated in the aqueous phases and extracellular medium, respectively, by thin-layer chromatography (Diaz-Meco et al., 1989) followed by autoradiography of plates in which standards corresponding to the different water-soluble choline metabolites were included.

Analysis of DAG and PA Release, and Levels of Other Phosphollpids

For determination of DAG production, cells were labeled with 10 J.tCi of [U-'4C)glycerol (spec. radioactivity 141 mCi/mmol)/dish as described above. For determination of the levels of different phospholipids, cells were labeled as follows: for PC and sphingomyelin, with 10 J!Ci of [methyi-^{14}C)choline (spec. radioactivity 55 mCi/mmol)/well; for phosphatidylethanolamine, with 10 J.tCi of (2-^{14}C]ethan-1-olamine (spec. radioactivity 55 mCi/mmol)/ml; for phosphatidylserine, with 10 J.tCi of L-[U-^{14}C)serine (spec. radioactivity 55 mCilmmol)lwell; for polyphosphoinositides, with 10 J.tCi of myo-[2-^{3}H]inositol (spec. radioactivity 16.3 Ci/mmol)lwell. Labeled compounds were obtained from Amersham International. Afterward, cells were treated with the corresponding agonists, according to the different experiments, and reactions were stopped by removing the supernatants and adding ice-cold methanol to cells. Methanol extracts were fractionated into chloroform and aqueous phases as descríbed above. Organic phases were dried down under N_2, and lipids were fractionated by thin-layer chromatography using the following solvent systems. For the separation of DAG: hexane:diethylether:acetic acid (60:40:1) (vol/vol/vol) was usad. For the fractionation of different phospholipids: chloroform:methanol:ammonia (65:25:4) (vol/vol/vol) was used in the first dimension and chloroform:acetone:methanol:acetic acid:water (30:40:10:10:5) (vol/vol/vol/vol/vol) was used in the second dimension.

For determination of PA production, the following protocol was carried out. Quiescent cells were incubated overnight with phosphate and serum-free culture medium supplemented with 10 J.tCi of [^{32}P)orthophosphate (Du Pont-New England Nuclear; 9000 Ci/mmol). Afterward, cells were stimulated and phospholipids were extractad as above. PA levels were determinad alter separation by thin-layer chromatography with the upper phase of the following solvent system: ethyl acetate:trimethylpentane:acetic acid (90:50:20) (vol/vol/vol). Different lipids were visualizad alter autoradiography of plates where the corresponding standards were included.

[^{3}H]Thymidine Incorporatlon Assays

Quiescent cells were incubated with the corresponding stimulants in the presence of [^{3}H]thymidine (2 J.tCi/ml) either for different times or for 24 hr according to the experiments. Afterward, de novo DNA synthesis was determinad as previously described (Leal et al., 1985).

IdentlfIcation of PKC by Immunoblottlng

Cell extracts containing 100 119 of total cell protein were obtained from cultures treated as described in Table 5. Following denaturation in SOS sample buffer, proteins were resolved in 10% SDS-PAGE and then transferred electrophoretically onto a polyvinylidene difluoride membrana (lmmobilon, Millipore Water Systems, Bedford, MA). To visualiza PKC, the membrana was incubated as previously described (Moscat et al., 1989b) using a monoclonal anti-PKC antibody (clone MC5, Amersham lnternational). This antibody recognizes the a form of PKC,which is the sola subtype present in Swiss 3T3 fibroblasts (Rose-John et al., 1988).

Estlmation of PKC Activatlon by Analysls of Endogenous 80 kd Proteln Phosphorylatlon

Phosphorylation of endogenous proteíns in response to PMA either in untreated cells or in cells chronically exposed to PMA was performed by two-dimensional gel electrophoresis following a slight modification of the method of Rodríguez-Peña and Rozengurt (1986) as described previously (Moscat et al., 1989b).

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