Bombesin and platelet-derived growth factor stimulate formation of inositol phosphates and Ca^{2+} mobilization in Swiss 3T3 cells by different mechanisms

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Highly purified platelet-derived growth factor (PDGF) or recombinant PDGF stimulate DNA synthesis in quiescent Swiss 3T3 cells. The dose–response curves for the natural and recombinant factors were similar, with half-maximal responses at 2–3 ng/ml and maximal responses at approx. 10 ng/ml. Over this dose range, both natural and recombinant PDGF stimulated a pronounced accumulation of [^{3}H]inositol phosphates in cells labelled for 72 h with [^{3}H]inositol. In addition, mitogenic concentrations of PDGF stimulated the release of ^{45}Ca^{2+} from cells prelabelled with the radioisotope. However, in comparison with the response to the peptide mitogens bombesin and vasopressin, a pronounced lag was evident in both the generation of inositol phosphates and the stimulation of ^{45}Ca^{2+} efflux in response to PDGF. Furthermore, although the bombesin-stimulated efflux of ^{45}Ca^{2+} was independent of extracellular Ca^{2+}, the PDGF-stimulated efflux was markedly inhibited by chelation of external Ca^{2+} by using EGTA. Neither the stimulation of formation of inositol phosphates nor the stimulation of ^{45}Ca^{2+} efflux in response to PDGF were affected by tumour-promoting phorbol esters such as 12-O-tetradecanoylphorbol 13-acetate (TPA). In contrast, TPA inhibited phosphoinositide hydrolysis and ^{45}Ca^{2+} efflux stimulated by either bombesin or vasopressin. Furthermore, whereas formation of inositol phosphates in response to both vasopressin and bombesin was increased in cells in which protein kinase C had been down-modulated by prolonged exposure to phorbol esters, the response to PDGF was decreased in these cells. These results suggest that, in Swiss 3T3 cells, PDGF receptors are coupled to phosphoinositidase activation by a mechanism that does not exhibit protein kinase C-mediated negative-feedback control and which appears to be fundamentally different from the coupling mechanism utilized by the receptors for bombesin and vasopressin.

INTRODUCTION

Platelet-derived growth factor (PDGF) is a powerful mitogen that stimulates DNA synthesis and proliferation in various cell types, including Swiss 3T3 cells (Ross & Vogel, 1978; Eide et al., 1986). In these cells (Isacke et al., 1986), as in other cell types, the binding of PDGF to its specific cell-surface receptors causes an increase in protein-tyrosine kinase activity which is integral to the receptor (reviewed by Cooper & Hunter, 1983; Hunter & Cooper, 1985). Like PDGF, the amphibian peptide bombesin (or its mammalian homologue gastrin-releasing peptide) and the peptide hormone vasopressin are also potent mitogens for Swiss 3T3 cells (Rozengurt et al., 1979; Rozengurt & Sinnett-Smith, 1983; Corps et al., 1985). Specific, high-affinity receptors for bombesin-related peptides (Zachary & Rozengurt, 1985; Brown & Laurie, 1986) as well as distinct high-affinity vasopressin receptors (Collins & Rozengurt, 1983) have been demonstrated on Swiss 3T3 cells. Whether the receptors for the peptides, like the PDGF receptor, possess integral tyrosine kinase activity is not yet unequivocally established, but Isacke et al. (1986) failed to find such activity associated with bombesin-receptor activation.

Other early events associated with the binding of PDGF, bombesin or vasopressin to Swiss 3T3 cells include stimulation of polyphosphoinositide hydrolysis (Habenicht et al., 1981; Berridge et al., 1984; Brown et al., 1984, 1987; Chu et al., 1985; Heslop et al., 1986; Besterman et al., 1986b; Takuwa et al., 1987), Ca^{2+} mobilization (Berridge et al., 1984; Mendoza et al., 1986b; Brown et al., 1987; Takuwa et al., 1987) and activation of protein kinase C (Isacke et al., 1986; Zachary et al., 1986). It remains to be established whether stimulated phosphoinositide hydrolysis is a necessary event for the stimulation of DNA synthesis and cell proliferation by these mitogens. Evidence in favour of a causal relationship has been reported by Matuoka et al. (1988), who found that micro-injection of a monoclonal antibody against phosphatidylinositol 4,5-bisphosphate (PIP$_2$) abolished [^{3}H]thymidine nuclear labelling induced by PDGF or bombesin (but not by fibroblast growth factor, epidermal growth factor, insulin or serum).

We have recently shown that bombesin-stimulated PIP$_2$ hydrolysis and Ca^{2+} mobilization are inhibited by tumour-promoting phorbol esters such as 12-O-tetradecanoylphorbol 13-acetate (TPA) (Brown et al., 1987). The TPA effect is apparently mediated via activation of protein kinase C, since the inhibitory action of the

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promotor is not observed in cells in which protein kinase C has been down-modulated (Brown *et al.*, 1987). Furthermore, cells in which protein kinase C has been down-modulated show an increase in bombesin-stimulated production of inositol phosphates, compared with bombesin-stimulated control cultures, suggesting that a diacylglycerol-activated feedback loop normally limits polyphosphoinositide turnover in bombesin-stimulated cells. We now report that, although a similar protein kinase C-mediated negative-feedback loop also operates in vasopressin-stimulated cells, no such mechanism is involved in regulating the formation of inositol phosphates in Swiss 3T3 cells stimulated by PDGF. In addition, we have shown that the time courses for the stimulation of both formation of inositol phosphates and $^{45}Ca^{2+}$ efflux are markedly different in response to PDGF as compared with bombesin.

MATERIALS AND METHODS

Cell-culture reagents were obtained from Flow Laboratories. [*methyl*-^{3}H]Thymidine, ^{125}I, $^{45}Ca^{2+}$ and *myo*-[2-^{3}H]inositol were from Amersham International. TPA was purchased from Sigma, and was dissolved in dimethyl sulphoxide at 1.6 mM and diluted immediately before use in Dulbecco's modified Eagle's medium (DMEM) containing 1 mg of bovine serum albumin/ml. Bombesin and [Arg^8]vasopressin were from Bachem U.K. Two preparations of pure PDGF were used in these experiments; recombinant PDGF$_{c\text{-}sis}$ was purchased from Amersham International, and human platelet PDGF was generously given by Carl Heldin (University of Uppsala, Sweden). The recombinant PDGF, estimated to be at least 95% pure, is prepared from a eukaryotic recombinant DNA host containing the human c-*sis* gene, which is the normal human gene for B-chain PDGF. The c-*sis* gene product has a structure equivalent to the PDGF B-chain homodimer (Johnsson *et al.*, 1984; Leal *et al.*, 1985). Similar results were obtained with either the natural or the recombinant factor.

Stock cultures of Swiss mouse 3T3 cells were maintained and passaged as described previously (Brown & Blakeley, 1983). For experimental use, cells were seeded into 35 mm-diam. culture dishes in DMEM containing 10% (v/v) newborn-calf serum, and used after 7 days' growth, by which time the cells were confluent and quiescent.

The extraction, measurement and identification of inositol phosphate fractions from mitogen-stimulated cells was performed as described previously (Heslop *et al.*, 1986; Brown *et al.*, 1987). Cells were pre-labelled for approx. 72 h by adding *myo*-[^{3}H]inositol (5 or 10 μCi/ml) directly to the growth medium 3–4 days after seeding. At the start of the experiment, the labelling medium was removed and the cells were washed and then incubated at 37 °C in phosphate-buffered saline (PBS, pH 7.4) containing 10 mM-LiCl and the test reagents at the concentrations indicated. [The composition of PBS is (mM): NaCl, 138; KCl, 2.8; Na$_2$HPO$_4$, 8; KH$_2$PO$_4$, 1.45; CaCl$_2$, 0.91; MgCl$_2$, 0.49.] The solution was removed by suction and replaced by 0.5 ml of 15% (w/v) trichloroacetic acid (4 °C), and the dishes were kept at 4 °C to extract cellular ^{3}H-labelled inositol phosphates, which were analysed by anion-exchange chromatography on small columns of Dowex 1-X8. The ether-extracted and neutralized cell extracts were applied to the columns,

which were then washed with 10 ml of water to remove [^{3}H]inositol and then with 5 ml of 60 mM-sodium formate to elute glycerophosphoinositol. [^{3}H]Inositol mono-, bis- and tris-phosphate fractions (IP$_1$, IP$_2$ and IP$_3$ fractions respectively) were eluted with sequential 5 ml batches of 0.1 M-formic acid/0.2 M-ammonium formate, 0.1 M-formic acid/0.5 M-ammonium formate and 0.1 M-formic acid/1.0 M-ammonium formate. Elution positions were confirmed by using tritiated standards. Although the 'IP$_3$ fraction' eluted with 0.1 M-formic acid/1.0 M-ammonium formate includes both 1,4,5-IP$_3$ and 1,3,4-IP$_3$ together with 1,3,4,5-IP$_4$ (Batty *et al.*, 1985; Heslop *et al.*, 1985), it is a valid measure of phospholipase C activity, because 1,4,5-IP$_3$ formed directly by PIP$_2$ hydrolysis appears to be the only precursor for the formation of 1,3,4,5-IP$_4$ and 1,3,4-IP$_3$ (see Taylor *et al.*, 1986). [In some experiments, the IP$_1$, IP$_2$ and IP$_3$ fractions were eluted together from the columns with 0.1 M-formic acid/1.0 M-ammonium formate immediately after the elution of glycerophosphoinositol.] The column eluates were collected directly into scintillation vials, to which were added 10 ml of scintillation fluid (ACS; Amersham). After cooling to 4 °C, the gelled samples were counted for ^{3}H.

The efflux of $^{45}Ca^{2+}$ from pre-labelled cells was measured as described previously (Berridge *et al.*, 1984; Brown *et al.*, 1987). The data are presented as a fractional efflux, i.e. the amount of radioactivity leaving the cells during a 2 min interval is expressed as a fraction of that present in the cells at the start of the interval.

Cellular protein kinase C was down-regulated by incubation with 300 nM-TPA for 48 h. Prolonged treatment with high concentrations of phorbol esters causes a dramatic decrease in amounts of kinase, as shown by decreased enzymic activity in cell extracts (Rodriguez-Pena & Rozengurt, 1984; Blackshear *et al.*, 1985), decreased binding of [^{3}H]phorbol dibutyrate (Blackshear *et al.*, 1985), and decreased amounts of immunoreactive protein kinase C (Ballester & Rosen, 1985; Stabel *et al.*, 1987).

RESULTS

DNA synthesis and formation of inositol phosphates in Swiss 3T3 cells in response to highly purified or recombinant PDGF

The recent production of recombinant PDGF has greatly increased the availability of this growth factor for investigation of its mechanisms of action. However, since previous studies have been carried out using partially or highly purified PDGF from human or pig platelets, it was important to compare the cell-growth-promoting activity of the natural and recombinant factors. In serum-free medium, in the absence of any other mitogens, both recombinant and highly purified natural PDGF stimulated DNA synthesis in Swiss 3T3 cells with a half-maximal response at 2–3 ng/ml and a maximal response at approx. 10 ng/ml (Fig. 1*a*). These values are similar to those reported for stimulation of Swiss 3T3-cell DNA synthesis by highly purified PDGF from human platelets (Eide *et al.*, 1986), and significantly lower than the values obtained by Lopez-Rivas *et al.* (1987) using highly purified PDGF from pig platelets. In the presence of insulin (1 μg/ml), the dose–response curve for the stimulation of DNA synthesis by the natural or recombinant factors was shifted substantially to the left (half-maximal
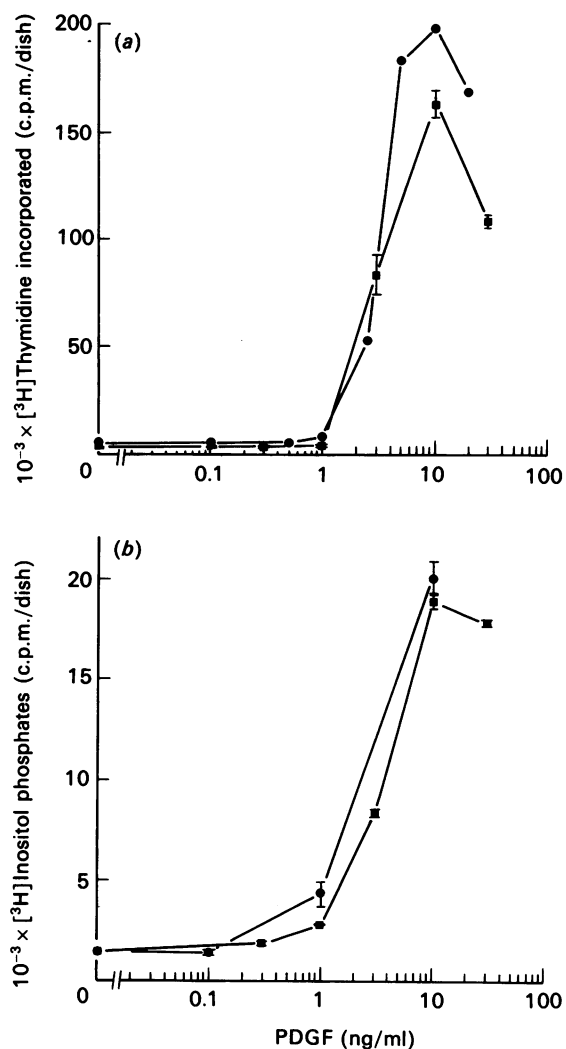
Fig. 1. Stimulation of DNA synthesis (a) and production of inositol phosphates (b) by highly purified or recombinant PDGF

(a) Confluent monolayers of cells were rinsed with serum-free DMEM and 2 ml of this medium containing [^{3}H]-thymidine (1 μCi/ml; 1 μM) was added per dish. Samples of purified ($\bullet$) or recombinant ($\blacksquare$) PDGF were added to give the indicated final concentration of the factor. Cultures were incubated for 40 h at 37 °C, washed and extracted, and incorporated radioactivity was measured. Each point represents the mean value for two or three separate determinations and, where shown, error bars indicate $\pm$ S.E.M. The purified and recombinant factors were tested in separate experiments in which [^{3}H]thymidine incorporation in response to the addition of 10 % (v/v) calf serum was 296 × 10^3 and 241 × 10^3 c.p.m./dish respectively. (b) Cells were grown for 7 days and labelled with [^{3}H]-inositol for the final 72 h by adding it directly to the growth medium. The medium was removed, and the cells were washed with PBS (pH 7.4) for 30 min to remove as much as possible of the unincorporated radioactivity. The solutions were removed and replaced by 1 ml of PBS (pH 7.4) containing 10 mM-LiCl and the indicated concentration of either purified ($\bullet$) or recombinant ($\blacksquare$) PDGF, and the dishes were incubated for 30 min at 37 °C. The cells were extracted into cold trichloroacetic acid, and the [^{3}H]inositol phosphates were measured. Each point represents the mean value ($\pm$ S.E.M.) of three or four determinations of [^{3}H]inositol phosphates from separate dishes of cells.

response at approx. 0.5 ng/ml) and an enhanced maximal response (95 % of the response to 10 % calf serum) was obtained (results not shown).

We have previously observed (Berridge et al., 1984) that partially purified PDGF from human platelets stimulates phosphoinositide hydrolysis and the production of inositol phosphates in Swiss 3T3 cells. Highly purified natural and recombinant PDGF also stimulated a marked increase in inositol phosphates in these cells, with dose–response curves (Fig. 1b) very similar to those observed for the stimulation of DNA synthesis by the PDGF preparations (Fig. 1a). The results shown in Fig. 1(b) represent total accumulation of inositol phosphates as measured by elution from ion-exchange columns with 0.1 M-formic acid/1.0 M-ammonium formate. When the IP$_1$, IP$_2$ and IP$_3$ fractions were collected separately by stepwise elution from the anion-exchange columns (see the Materials and methods section), the dose–response curves for the stimulation of the production of each fraction by recombinant PDGF were very similar (results not shown).

Different time courses for PDGF- or bombesin-stimulated formation of inositol phosphates

We have compared the time courses of the production of inositol phosphates in Swiss 3T3 cells stimulated by either bombesin or recombinant PDGF. Bombesin stimulated a biphasic response, which was most clearly seen in the IP$_3$ and IP$_2$ fractions, although a similar trend was also discernible in the IP$_1$ fraction (Fig. 2). The stimulation of the IP$_3$ fraction reached a peak value within 1 min after bombesin addition, and then decreased somewhat (to 5 min) before rising slowly, but continuously, to the end of the experiment at 30 min. The stimulation of the IP$_2$ fraction followed a very similar time course (Fig. 2). In contrast, stimulation of the cells with PDGF produced a markedly different pattern of production of inositol phosphates (Fig. 2). There was a clear lag of approx. 2 min before there was a significant increase in the size of any of the inositol phosphate fractions. Thereafter, the size of the IP$_1$, IP$_2$ and IP$_3$ fractions increased linearly to the end of the experiment. Similar results were obtained in three other experiments in which the effects of bombesin and PDGF were compared.

In order to test whether the length of the lag period preceding accumulation of inositol phosphates was dependent on PDGF concentration, the time course of stimulation in response to increasing concentrations of recombinant PDGF was measured. In the 3–10 ng/ml concentration range, the length of the lag period was not greatly influenced by the concentration of PDGF used (results not shown). However, it was difficult to obtain useful results on this question for lower concentrations of PDGF (e.g. 1 ng/ml). Although these doses were sufficient to give a significant accumulation of inositol phosphates after a 30 or 60 min stimulation (see, e.g., Fig. 1), the proportional changes that might be expected at early time points are very small, and it was difficult to determine whether such changes occurred or whether there was indeed an increased lag time in response to low doses of the factor.

Protein kinase C-mediated feedback control of formation of inositol phosphates

The stimulation of PIP$_2$ hydrolysis by various agonists in several cell types is inhibited by phorbol esters such as
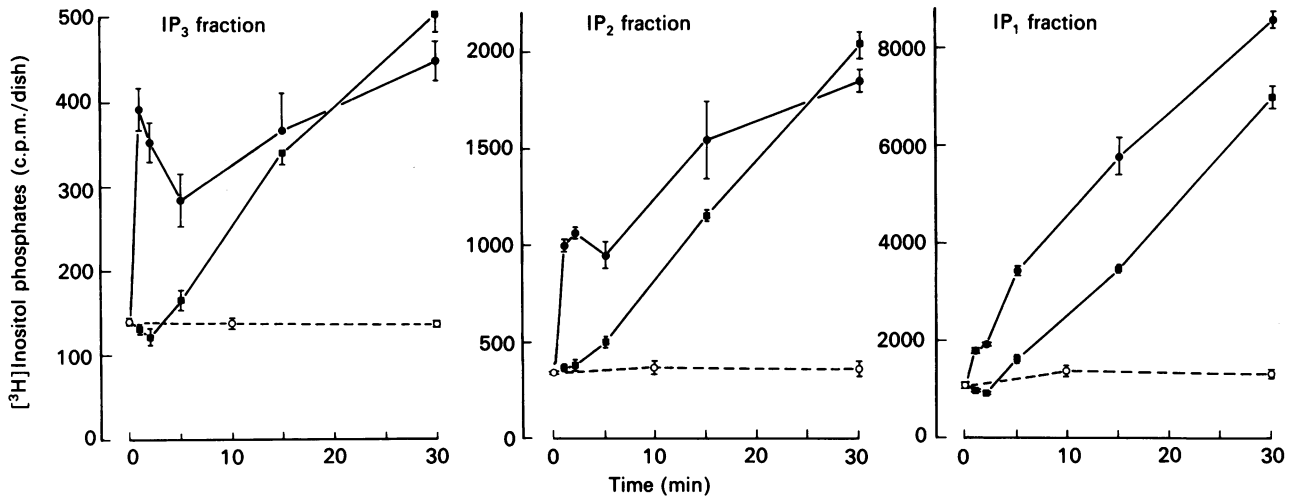
Fig. 2. Time courses of PDGF- and bombesin-stimulated production of inositol phosphates in Swiss 3T3 cells

Cells were grown for 7 days and labelled for 72 h by adding [^{3}H]inositol directly to the growth medium. The medium was removed, and the cells were washed with PBS (pH 7.4) for 30 min to remove as much as possible of the unincorporated radioactivity. LiCl (final concn. 10 mM) was added for the final 10 min of this incubation. The solutions were removed and replaced by 1 ml of PBS (pH 7.4) containing 10 mM-LiCl and no addition (○), 10 nM-bombesin (●) or 10 ng of recombinant PDGF/ml (■). The dishes were incubated for the indicated times at 37 °C. The cells were extracted into cold trichloroacetic acid, and the [^{3}H]inositol phosphates were separated and measured. Each point represents the mean value (±S.E.M.) for three determinations of [^{3}H]inositol phosphates from separate dishes of cells.

phorbol dibutyrate or TPA (see, e.g., Brock *et al.*, 1985; Orellana *et al.*, 1985; Griendling *et al.*, 1986; Monaco & Mufson, 1986). In Swiss 3T3 cells, we have found that TPA markedly inhibits bombesin-stimulated PIP$_2$ hydrolysis through a protein kinase C-dependent mechanism (Brown *et al.*, 1987). In contrast, Sturani *et al.* (1986) have reported that formation of inositol phosphates in response to PDGF is not affected by TPA. We have therefore compared the effects of the tumour promotor on the production of inositol phosphates in cells stimulated by pure PDGF, vasopressin or bombesin. The results show that, although TPA inhibits formation of inositol phosphates in response to either bombesin or vasopressin, the response to PDGF was not affected by TPA (Table 1). The magnitude of the inhibitory effects of TPA on accumulation of inositol phosphates (30 min) in response to high doses (3–10 nM) of bombesin has varied from approx. 30 to 50 % in several experiments. The response to a lower dose of bombesin (0.1 nM) was inhibited by over 90 % (results not shown). In contrast, we have never observed inhibitory effects of TPA on PDGF-stimulated production of inositol phosphates. Indeed, TPA was observed to cause a significant increase in the response to low doses of PDGF. For example, total inositol phosphates accumulated in response to PDGF (1 ng/ml for 30 min) in the absence or presence of TPA were 254±6 % and 351±7 % of control unstimulated values respectively.

The effect of TPA on the time course of the production of IP$_3$ in bombesin-treated cells is shown in Fig. 3. The biphasic response to bombesin was again evident, and the maximal inhibitory action of TPA was reproducibly in the first 1 min of the stimulation (Fig. 3, and results not shown). Similarly, the production of the IP$_2$ fraction also showed maximal inhibition during the first 1–2 min of stimulation (results not shown). These results suggest that the early phase of bombesin-stimulated PIP$_2$ hydro-

lysis is most sensitive to protein kinase C-mediated inhibition. However, the substantial inhibitory effect of TPA on all three [^{3}H]inositol phosphate fractions at later

Table 1. Effect of TPA on mitogen-stimulated production of inositol phosphates

Cells labelled with [^{3}H]inositol were washed with PBS (pH 7.4) and incubated for 45 min at 37 °C in PBS to remove as much as possible of the unincorporated radioactivity. TPA (30 nM) was added to some dishes of cells for the final 15 min of this incubation. The solutions were removed and replaced by 1 ml of PBS containing 10 mM-LiCl and TPA (30 nM), PDGF (10 ng/ml), bombesin (3 nM) or vasopressin (100 nM) as indicated. The cells were incubated at 37 °C for 30 min and extracted into cold trichloroacetic acid. Extracted ^{3}H-labelled inositol phosphates were separated by anion-exchange chromatography. Each point represents the mean (±S.E.M.) of three determinations of inositol phosphates from separate dishes of cells.

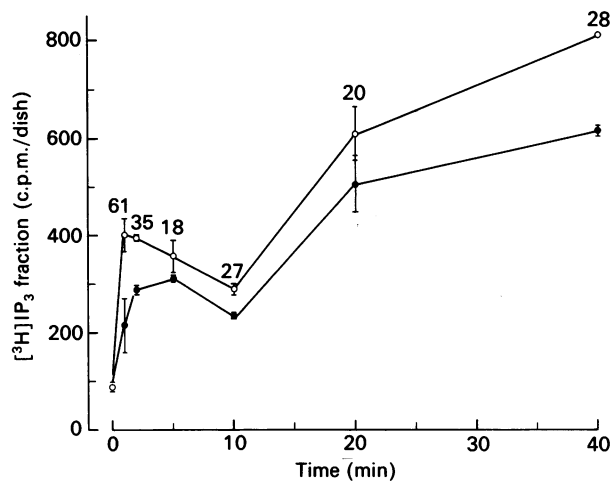| Addition	[^{3}H]Inositol phosphate (c.p.m./dish)		
	IP$_1$ fraction	IP$_2$ fraction	IP$_3$ fraction
None	1061±77	349±30	167±14
TPA	1215±144	466±75	195±18
PDGF	15875±559	2717±288	607±42
PDGF+TPA	16648±665	2388±87	564±29
Bombesin	12776±518	1624±193	380±60
Bombesin+TPA	6870±277	958±67	244±9
Vasopressin	5041±77	948±100	233±20
Vasopressin+TPA	1627±89	581±28	172±6

Fig. 3. Effect of TPA on the time course of bombesin-stimulated IP$_3$ production

Cells were grown for 7 days and labelled for 72 h by adding [^{3}H]inositol directly to the growth medium. The medium was removed, and the cells were washed with PBS (pH 7.4) for 30 min to remove as much as possible of the unincorporated radioactivity. LiCl (10 mM) and, where indicated, TPA (30 nM) were added for the final 15 min of this incubation. The solutions were removed and replaced by 1 ml of PBS (pH 7.4) containing 10 mM-LiCl and bombesin (10 nM) in the absence (○) or presence (●) of TPA (30 nM). The dishes were incubated for the indicated times at 37 °C. The cells were extracted into cold trichloro-acetic acid, and the [^{3}H]inositol phosphates were separated and measured. Each point represents the mean value (±S.E.M.) for three determinations of the [^{3}H]IP$_3$ fraction from separate dishes of cells. The numbers above the symbols show the percentage inhibition of [^{3}H]IP$_3$ production in the TPA-treated cells compared with the control cultures.

Fig. 4. Effect of protein kinase C down-regulation on mitogen-stimulated production of inositol phosphates

Cells were grown for 7 days and labelled with [^{3}H]inositol for the final 72 h. Some of the dishes (▨) received TPA (300 nM) for the final 48 h of the growth period, while other dishes (□) remained untreated. The cells were washed with PBS (pH 7.4) and incubated for 45 min at 37 °C in PBS to remove as much as possible of the un-incorporated radioactivity. The solutions were removed and replaced by 1 ml of PBS containing 10 mM-LiCl and TPA (30 nM), bombesin (10 nM), vasopressin (100 nM) or recombinant PDGF (10 ng/ml) as indicated. The cells were extracted after a 30 min incubation at 37 °C. The combined IP$_1$, IP$_2$ and IP$_3$ fraction eluted with 0.1 M-formic acid/1.0 M-ammonium formate is shown. Each value represents the mean value (±S.E.M.) for three determinations of the inositol phosphates from separate dishes of cells.

times (Fig. 3 and Table 1) suggests that both phases of the bombesin response are subject to inhibition via the kinase.

The inhibitory effect of TPA on bombesin-stimulated phosphoinositide hydrolysis is apparently mediated via protein kinase C, since the inhibition is not seen in cells in which the kinase has been down-regulated by pro-longed exposure to high concentrations of TPA (Brown *et al.*, 1987). Such cells exhibit a marked increase in inositol phosphates produced in response to bombesin stimulation, possibly owing to relief of endogenous feedback inhibition operating through diacylglycerol activation of protein kinase C (Brown *et al.*, 1987). On the basis of this interpretation, the finding (Table 1) that production of inositol phosphates in response to vaso-pressin is also inhibited by TPA predicts that the vaso-pressin response should also be increased in cells down-modulated for protein kinase C. However, since TPA does not inhibit PDGF-stimulated production of inositol phosphates (Table 1), one might predict that an endo-genous feedback loop acting via protein kinase C would not operate for this agonist, and consequently the PDGF response would not be increased in cells in which protein kinase C had been down-modulated. The results (Fig. 4) show that the response to vasopressin is indeed greatly increased in TPA-pretreated cells. In contrast, the re-sponse to PDGF was not potentiated in TPA-pretreated
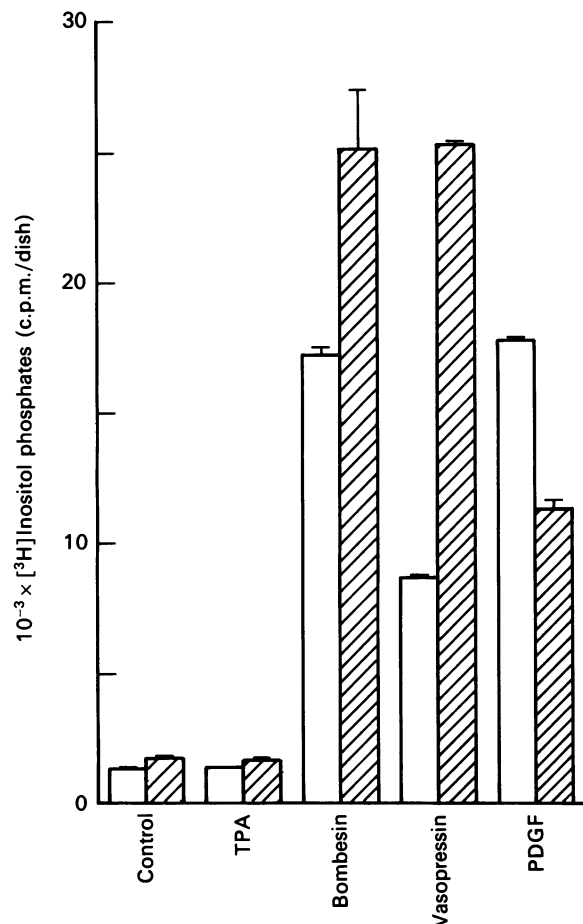
cells; in fact, a substantial decrease in the response to PDGF was evident in these cultures (Fig. 4).

Stimulation of Ca^{2+} efflux by PDGF

Partially purified PDGF stimulated a pronounced increase in the release of ^{45}Ca^{2+} from Swiss 3T3 cells which had been pre-labelled with the radioisotope (Ber-ridge *et al.*, 1984). Similarly, highly purified PDGF, at a concentration of 5 ng/ml, also stimulated a marked increase in ^{45}Ca^{2+} release from the cells (Fig. 5). This release was not altered by adding TPA to the efflux medium (Fig. 5), which contrasts with previous findings for bombesin or vasopressin, whose stimulation of ^{45}Ca^{2+} efflux was markedly attenuated by TPA (Mendoza *et al.*, 1986*a*; Brown *et al.*, 1987).
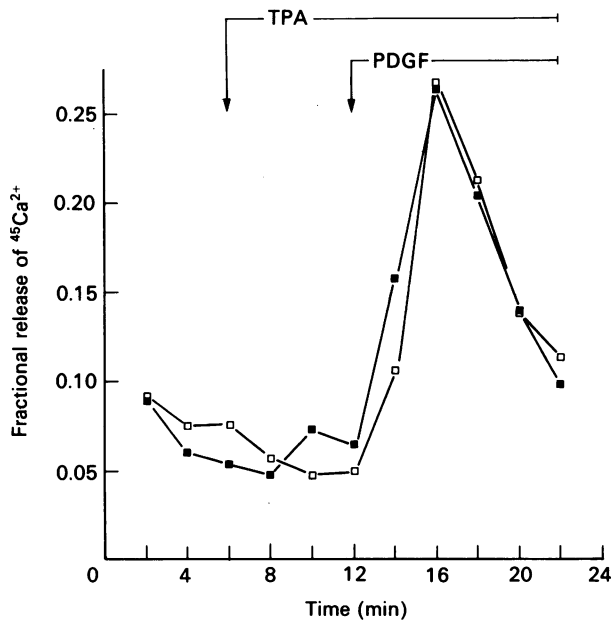
Fig. 5. TPA does not inhibit PDGF-stimulated $^{45}Ca^{2+}$efflux from Swiss 3T3 cells

Cells were grown for 7 days and prelabelled with $^{45}Ca^{2+}$ by an overnight incubation with the radioisotope (4.4 μCi/ml). The cells were washed, and $^{45}Ca^{2+}$ efflux was measured during successive 2 min intervals as described in the Materials and methods section. Additions to the efflux solutions were: □, 5 ng of PDGF/ml from 12 min; ■, 20 nM-TPA from 6 min and 5 ng of PDGF/ml from 12 min. The amount of $^{45}Ca^{2+}$ leaving the cells during each 2 min interval is expressed as a fraction of that present at the beginning of the interval. The results are typical of those obtained in three separate experiments.

An additional interesting difference between the effects of PDGF and bombesin (or vasopressin) on $^{45}Ca^{2+}$ release concerns the time course of the response. In cells stimulated with the peptide mitogens, the maximal release of radioisotope is invariably seen during the first 2 min collection interval after the addition of the agonist even when the dose of agonist is substantially sub-maximal (Fig. 6a). In contrast, in PDGF-stimulated cells, the maximal release of $^{45}Ca^{2+}$ is not observed until the second or even the third (depending on PDGF concentration) 2 min collection period after adding agonist (Figs. 5 and 6b).

Thus, in comparison with the peptide mitogens, a distinct lag is evident for the stimulation of either phosphoinositide hydrolysis or Ca^{2+} release from Swiss 3T3 cells in response to pure PDGF. It would be tempting, therefore, to suppose that cellular Ca^{2+} mobilization in response to PDGF is mediated by 1,4,5-IP_3 and that the delay in $^{45}Ca^{2+}$ efflux reflects the observed delay in phosphoinositide hydrolysis. That this attractively simple hypothesis may not be correct is indicated by two findings. Firstly, the PDGF-stimulated release of $^{45}Ca^{2+}$from the cells apparently precedes generation of $[^3H]IP_3$ when high doses of the factor are used (compare Fig. 2 with Figs. 5 and 6). Secondly, PDGF-stimulated $^{45}Ca^{2+}$ release is markedly inhibited by chelation of extracellular Ca^{2+} with EGTA (Fig. 7a). In contrast, $^{45}Ca^{2+}$ efflux in response to bombesin is totally unaffected
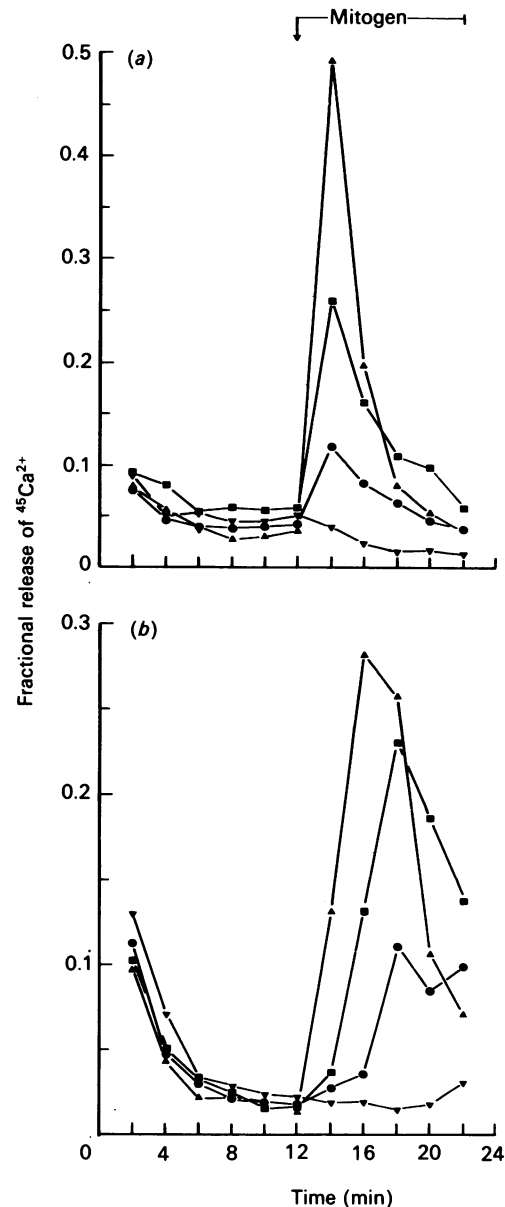

Fig. 6. Different time courses for bombesin- and PDGF-mediated Ca^{2+} release from Swiss 3T3 cells

Cells were grown for 7 days and prelabelled with $^{45}Ca^{2+}$ by an overnight incubation with the radioisotope (4.4 μCi/ml). The cells were washed and $^{45}Ca^{2+}$ efflux was measured during successive 2 min intervals. Additions to the efflux solution from 12 min were: (a) no addition (▼), 0.3 (●), 1 (■) or 10 (▲) nM-bombesin; (b) 0.3 (▼), 1 (●), 3 (■) or 10 (▲) ng of recombinant PDGF/ml. The amount of $^{45}Ca^{2+}$ leaving the cells during each 2 min interval is expressed as a fraction of that present at the beginning of the interval.

by the presence of EGTA (Fig. 7b). Although there are several possible explanations for this effect of EGTA (see the Discussion section), the results support the possibility that a mechanism other than 1,4,5-IP_3-mediated Ca^{2+} mobilization may underlie the primary effect of PDGF on $^{45}Ca^{2+}$ efflux. However, it should be noted that there is reproducibly a small residual PDGF-stimulated efflux of $^{45}Ca^{2+}$ even in the presence of EGTA (Fig. 7a).
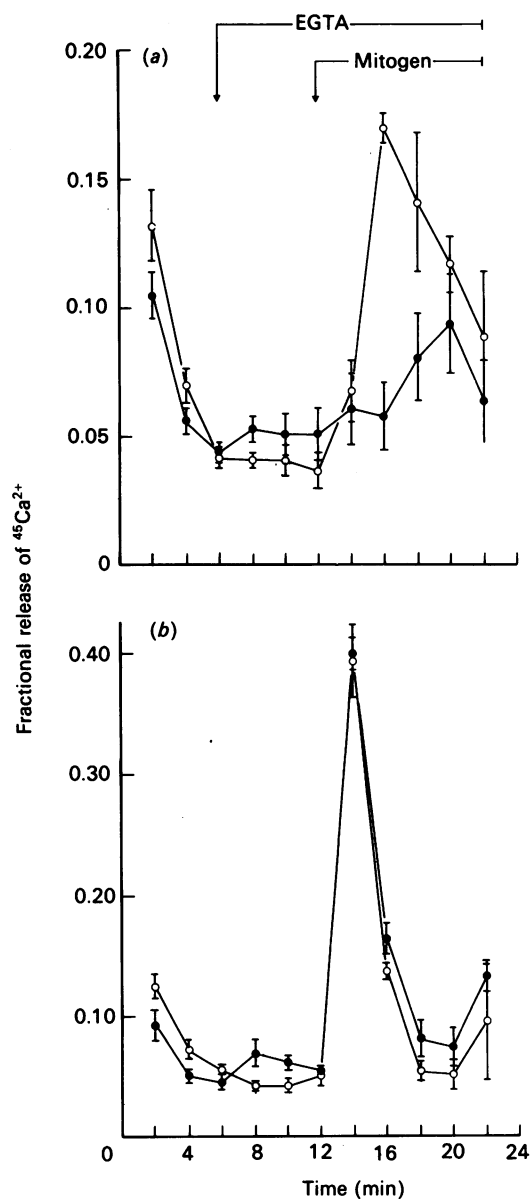
Fig. 7. EGTA inhibits PDGF-stimulated $^{45}Ca^{2+}$ efflux

Cells were grown for 7 days and prelabelled with $^{45}Ca^{2+}$ by an overnight incubation with the radioisotope (4.4 μCi/ml). The cells were washed, and $^{45}Ca^{2+}$ efflux was measured during successive 2 min intervals. Additions to the efflux solutions were: (a) ○, 2.5 ng of PDGF/ml from 12 min; ●, 1 mM-EGTA from 6 min and 2.5 ng of PDGF/ml from 12 min; (b) ○, 3 nM-bombesin from 12 min; ●, 1 mM-EGTA from 6 min and 3 nM-bombesin from 12 min. The amount of $^{45}Ca^{2+}$ leaving the cells during each 2 min interval is expressed as a fraction of that present at the beginning of the interval. The mean values for six (a) or four (b) determinations from separate dishes of cells are shown.

DISCUSSION

It has been reported (Lopez-Rivas et al., 1987) that highly purified PDGF from pig platelets, even at saturating concentrations, caused little if any increase in IP$_3$ production compared with the pronounced response seen with bombesin. Those authors point out that

previous studies (Habenicht et al., 1981; Berridge et al., 1984; Hasegawa-Sasaki, 1985) have generally used partially purified PDGF, and imply that this may explain the difference in results. The results presented here indicate that this is not the explanation, since both highly purified human PDGF and pure recombinant PDGF are both very effective stimulators of production of inositol phosphates. An alternative and intriguing possibility is that the PIP$_2$-hydrolysing activity of human PDGF requires the presence of the PDGF A-chain. [Human PDGF contains both A and B chains, although it is unclear whether it exists as a mixture of A and B homodimers or as A-B heterodimers (see Nister et al., 1988). In contrast, pig PDGF is a B-B-chain homodimer (Stroobant & Waterfield, 1984).] That this is not the case is indicated by the full activity of the recombinant PDGF, which is a B-B-chain homodimer (see the Materials and methods section). The results presented in Fig. 2, showing a lag of at least 2 min before the increase in inositol phosphates in response to PDGF, indicate that the failure to detect either 1,4,5-IP$_3$ or 1,3,4-IP$_3$ after stimulation with pig PDGF (Lopez-Rivas et al., 1987) was probably due to the short (1 min) stimulation used in their experiments.

It is possible that the delayed response to PDGF observed here simply reflects a slow binding of the factor to its cellular receptors. However, this seems most unlikely, since increased phosphorylation of PDGF-receptor substrates is seen within 15 s of PDGF addition at 37 °C (Frackleton et al., 1984; Kaplan et al., 1987). In addition, in 3T3 cells labelled with [^{3}H]choline, PDGF (10 ng/ml) markedly stimulates the formation of phosphocholine (presumably through hydrolysis of phosphatidylcholine), with a maximal response between 30 and 60 s (Besterman et al., 1986a). Furthermore, using fluorescent Ca^{2+} indicators, several groups have detected a rise in intracellular Ca^{2+} concentrations within 1 min of PDGF addition to Swiss 3T3 cells or human fibroblasts (Moolenaar et al., 1984; McNeil et al., 1985; Tsuda et al., 1985, 1986), although the response to PDGF was observed to be slower than the very rapid responses to either bradykinin (Ives & Daniel, 1987; Berk et al., 1987) or bombesin (Ives & Daniel, 1987; Lopez-Rivas et al., 1987). Our own studies, using digital video fluorescence microscopy (A. N. Corps, T. Cheek & K. D. Brown, unpublished work), reveal substantial heterogeneity between individual cells in the time course of the rise in Ca^{2+} concentrations in response to PDGF (10 ng/ml). However, over 60 % of Swiss 3T3 cells have substantially increased Ca^{2+} concentrations within 90 s of PDGF addition. Finally, it should be noted that, in response to high (10 ng/ml) doses of PDGF, significant $^{45}Ca^{2+}$ release was reproducibly observed during the first 2 min after addition of the mitogen (Figs. 5 and 6). Taken together, these results indicate that the lag of at least 2 min before inositol phosphates accumulate in response to PDGF is not caused by slow binding of the agonist to its receptors.

Although the efflux of $^{45}Ca^{2+}$ in response to PDGF is always slow by comparison with the response to bombesin, at high doses of PDGF (10 ng/ml) the stimulation of $^{45}Ca^{2+}$ release appears to precede the generation of [^{3}H]IP$_3$, supporting the suggestion (Lopez-Rivas et al., 1987) that Ca^{2+} mobilization in response to PDGF is primarily due to a process other than IP$_3$-mediated release from intracellular stores. Furthermore, in contrast with the response to bombesin, the PDGF-stimulated

$^{45}Ca^{2+}$ efflux appears to be dependent on extracellular Ca^{2+}, since it is markedly inhibited by EGTA. There are several possible explanations for this effect. Chelation of Ca^{2+} could decrease (or eliminate) PDGF binding to its cellular receptors, although this is unlikely, since binding of ^{125}I-PDGF to Swiss 3T3 cells was unaffected by omission of extracellular Ca^{2+} from the binding medium (Bowen-Pope & Ross, 1982). A second possibility is that the PDGF-stimulated production of inositol phosphates is blocked by EGTA at a step distal to PDGF binding. A third possibility is that PDGF stimulates an inflow of extracellular Ca^{2+}, leading to a Ca^{2+}-induced Ca^{2+} release of the type described by Fabiato & Fabiato (1975) and recently reviewed by Berridge et al. (1988). Further experiments will be required to distinguish between these mechanisms.

We have previously shown that bombesin-stimulated PIP_2 hydrolysis is markedly inhibited by phorbol esters through a mechanism which requires cellular protein kinase C but does not involve a decrease in bombesin binding (Brown et al., 1987). We have now extended these findings to show that PIP_2 hydrolysis in response to vasopressin is also inhibited by the phorbol ester. Furthermore, we have observed a biphasic stimulation of accumulation of inositol phosphates in response to either peptide, and we have found that production of inositol phosphates in the first minutes of stimulation is most subject to inhibition by phorbol esters. In contrast with these results, Sturani et al. (1986) have reported that PDGF-stimulated production of inositol phosphates is not inhibited by TPA. We have confirmed this result and have found that the time course of accumulation of inositol phosphates in response to PDGF is considerably different from that observed for bombesin or vasopressin. In particular, PDGF failed to generate the very rapid increase in inositol polyphosphates which is a characteristic feature of the response to the peptide mitogens. However, it should also be stressed that we do not know whether a common mechanism underlies the delayed PDGF response and the second phase of the bombesin response. Whatever the mechanisms involved, these results provide strong evidence for a fundamental difference in the way that PDGF receptors and bombesin (or vasopressin) receptors are coupled to the activation of phosphoinositidase(s). Rebecchi & Rosen (1987) reached a similar conclusion on the basis of their finding that, although PDGF stimulated PIP_2 hydrolysis in intact human fibroblasts, the factor had no effect on production of inositol phosphates by an isolated membrane fraction which retained demonstrable PDGF-receptor tyrosine kinase activity. In contrast, thrombin stimulated formation of inositol phosphates from both cells and membranes.

It is tempting to speculate that these different mechanisms may be related to the presence of an integral tyrosine kinase activity in the PDGF receptor and its apparent absence from the bombesin receptor (Isacke et al., 1986; but see also Cirillo et al., 1986). Consequently, it is interesting to consider what is known about accumulation of inositol phosphates in response to activation of other receptors with tyrosine kinase activity. Insulin and insulin-like growth factor 1 do not stimulate phosphoinositide hydrolysis (L'Allemain & Pouysségur, 1986; Besterman et al., 1986b; Heslop et al., 1986), although insulin-mediated potentiation of bombesin-stimulated accumulation of inositol phosphates has been

observed (Heslop et al., 1986). Epidermal growth factor (EGF) does not stimulate accumulation of inositol phosphates in 3T3 cells (Macphee et al., 1984; Besterman et al., 1986b), or in hamster lung fibroblasts (L'Allemain & Pouysségur, 1986), but the factor is effective at stimulating phosphoinositide hydrolysis in A431 cells (Hepler et al., 1987; Pike & Eakes, 1987; Wahl et al., 1987). The reasons for this difference are not known, but it is likely that the exceptionally high number of EGF receptors on A431 cells is involved, since it has been shown that EGF also stimulates inositol phosphates formation in other squamous carcinoma cell lines which over-express the EGF receptor (Wahl et al., 1987). In addition, there is evidence for a time course of EGF-stimulated formation of inositol phosphates which is very similar to that which we have seen for PDGF activation of Swiss 3T3 cells (Wahl et al., 1987; Tilley et al., 1988; but see also Hepler et al., 1987).

Nister et al. (1988) have raised the possibility that the different functional responses to PDGF may be mediated by different receptor populations. However, Escobedo et al. (1988) have transfected cloned PDGF-receptor cDNA (Yarden et al., 1986) into CHO cells, which normally lack PDGF receptors. Stable transfectants, expressing immunoreactive receptor protein, exhibited PDGF-stimulated tyrosine phosphorylation, phosphoinositide hydrolysis, increase in intracellular Ca^{2+} and [^3H]thymidine incorporation, indicating that a single receptor cDNA sequence confers PDGF-sensitivity to these various responses. Similar studies using mutated receptors should provide additional evidence on whether the tyrosine kinase and phosphoinositide responses are dissociable, or whether the latter is a secondary response to activation of the former. If this is the case, it will be important to determine the mechanisms by which receptor tyrosine kinase activation leads to stimulated phosphoinositide hydrolysis. Recently, a minor phosphatidylinositol kinase (designated type I) has been identified in mouse fibroblasts. The kinase stimulates the production of a novel phospholipid, phosphatidylinositol 3-phosphate, and this lipid has been tentatively identified as a minor component of total PIP in intact cells (Whitman et al., 1988). Type I kinase activity was rapidly (< 1 min) increased in PDGF-stimulated cells and, by using antibodies directed against phosphotyrosine, activity was co-immunoprecipitated with the PDGF receptor and an 85 kDa phosphoprotein whose presence correlated with phosphatidylinositol kinase activity through several purification steps (Kaplan et al., 1987). These results suggest that the 85 kDa protein is a phosphatidylinositol kinase which is a substrate for the PGF-receptor tyrosine kinase. It will be important to consider a possible involvement of this novel kinase in phosphoinositide metabolism in Swiss 3T3 cells in response to PDGF.

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