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Inositol trisphosphate formation and calcium mobilization in Swiss 3T3 cells in response to platelet-derived growth factor

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Swiss 3T3 cells incubated for 60h with [^{3}H]inositol incorporated radioactivity into phosphatidylinositol (PI) and the two polyphosphoinositides phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2). On stimulation with platelet-derived growth factor (PDGF) there were significant increases in the levels of inositol 1-phosphate (IP_1), inositol 1,4-bisphosphate (IP_2) and inositol 1,4,5-trisphosphate (IP_3). The effect of PDGF and IP_3 on Ca^{2+} mobilization was studied in both intact cells and in 'leaky' cells that had been permeabilized with saponin. In intact cells, PDGF stimulated the efflux of $^{45}Ca^{2+}$, whereas IP_3 had no effect. Conversely, IP_3 stimulated $^{45}Ca^{2+}$ efflux from 'leaky' cells, which were insensitive to PDGF. 'Leaky' cells, which accumulated $^{45}Ca^{2+}$ to a steady state within 20min, were found to release approx. 40% of the label within 1min after addition of 10μM-IP_3. This stimulation of $^{45}Ca^{2+}$ release by IP_3 was reversible and was also dose-dependent, with a half-maximal effect at approx. 0.3μM. It seems likely that an important action of PDGF on Swiss 3T3 cells is to stimulate the hydrolysis of PIP_2 to form IP_3 and diacylglycerol, both of which may function as second messengers. Our results indicate that IP_3 mobilizes intracellular Ca^{2+}, and we propose that diacylglycerol may act through C-kinase to activate the Na^+/H^+ antiport. By generating two second messengers, PDGF can simultaneously elevate the intracellular level of Ca^{2+} and alkalinize the cytoplasm by lowering the level of H^+.

Swiss 3T3 cells in culture can be stimulated to proliferate by the addition of various growth factors such as epidermal growth factor, fibroblast growth factor, insulin-like growth factor and platelet-derived growth factor (PDGF). Interest in the last was heightened by the finding that the oncogene of the simian-sarcoma virus *v-sis* codes for a protein that is almost identical with PDGF (Doolittle *et al.*, 1983; Waterfield *et al.*, 1983). Doolittle *et al.* (1983) have suggested that one form of cellular transformation induced by oncogenes such as *v-sis* may depend on the 'constitutive expression' of a protein that resembles one of the growth factors that is released for a short period during the control of normal growth. This endogenous production of a growth factor that may feed back to activate the host cell is an example of autocrine secretion as originally proposed by Sporn & Todaro (1980). The possibility of a link between oncogenes and cell growth factors makes it all the more imperative that we understand the mechanisms by which mitogens stimulate DNA synthesis and cell proliferation.

Among the early responses stimulated by mitogens are changes in the levels of classical second messengers such as Ca^{2+} (Boynton *et al.*, 1974; Berridge, 1975; Whitfield *et al.*, 1976, 1981; Metcalfe *et al.*, 1980; Durham & Walton, 1982) and cyclic AMP (Rozengurt, 1983) that may contribute to the onset of DNA synthesis. The fact that one of these second messengers is Ca^{2+} suggests that these growth factors may act in a manner similar to other calcium-mobilizing agents

Abbreviations used: PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol 1,4,5-trisphosphate; IP_2, inositol 1,4-bisphosphate; IP_1, inositol 1-phosphate; PDGF, platelet-derived growth factor; DME medium, Dulbecco's modified Eagle's medium; Pipes, 1,4-piperazinediethanesulphonic acid.

such as acetylcholine, noradrenaline (norepinephrine), vasopressin and thrombin. This similarity becomes all the more striking in view of the fact that, like these calcium-mobilizing agents, growth factors can stimulate changes in the metabolism of the phosphoinositides (Fisher & Mueller, 1968; Ristow *et al.*, 1973; Diringer & Friis, 1977; Habenicht *et al.*, 1981; Michell, 1982), which appears to function as an integral part of a receptor-transduction mechanism that generates Ca^{2+} signals (Michell, 1975; Berridge, 1981). Recent studies on these Ca^{2+}-mobilizing agents have established that their primary action is to stimulate the hydrolysis of PIP_2 to form diacylglycerol and IP_3 (Michell *et al.*, 1981; Agranoff *et al.*, 1983; Berridge, 1983; Berridge *et al.*, 1983; Creba *et al.*, 1983; Downes & Wusteman, 1983; Martin, 1983; Rebecchi & Gerschengorn, 1983). The latter may then act as a second messenger to mobilize intracellular Ca^{2+} (Berridge, 1983; Streb *et al.*, 1983; Berridge & Irvine, 1984; Burgess *et al.*, 1984; Joseph *et al.*, 1984). In the light of these new developments on the mode of action of Ca^{2+}-mobilizing receptors, it seemed important to re-evaluate the effect of PDGF on PI metabolism in Swiss 3T3 cells. In the present paper we show that PDGF acts on Swiss 3T3 cells to stimulate the breakdown of PIP_2 with the formation of IP_3. We have also demonstrated, by using permeabilized 3T3 cells, that this IP_3 may function as a second messenger to mobilize intracellular Ca^{2+}.

Materials and methods

Cell cultures

Stock cultures of Swiss-mouse 3T3 cells were grown on 9 cm-diameter plastic culture dishes (Nunc) in DME medium containing 10% (v/v) newborn-calf serum, 100 units of penicillin/ml and 100 μg of streptomycin/ml. Dishes were incubated at 37°C in a humidified atmosphere of CO_2/air (1:9). For experimental use, 5×10^4 cells were seeded in 2 ml of medium into 33 mm-diameter dishes (Nunc) and used after 6–7 days, by which time the cells were confluent and quiescent.

Extraction and determination of inositol lipids and inositol phosphates

For the phosphoinositide experiments, the cells were prelabelled for 60 h with *myo*-[2-^{3}H]inositol (20 μCi/dish) added directly to the growth medium. The labelled medium was removed, the cells were rinsed twice and incubated for 1 h in phosphate-buffered saline, pH 7.4, to remove as much as possible of the free [^{3}H]inositol. The phosphate-buffered saline had the following composition (mmol/litre): NaCl, 138; KCl, 2.8; NaH_2PO_4, 8; KH_2PO_4, 1.45; $CaCl_2$, 0.91; $MgCl_2$, 0.49. After a further rinse the cells were incubated for various times in 1 ml of phosphate-buffered saline with or without PDGF. At 6 s before the due time the medium was removed by aspiration and reactions were terminated by adding 1 ml of 15% (w/v) trichloroacetic acid. The dishes were left on ice for 30 min to extract the water-soluble inositol phosphates. The extract was transferred to a test tube and the cells were washed twice with 1 ml of distilled water. The washings were combined with the original acid extract and the trichloroacetic acid was removed with diethyl ether (four extractions). The final extract was neutralized and applied to anion-exchange columns to separate the water-soluble inositol phosphates as described previously (Berridge *et al.*, 1983).

The inositol lipids that remained in the dishes after removing the inositol phosphates were extracted three times with 1 ml of chloroform/methanol/conc. HCl (200:100:1, by vol.). The deacylation procedure for converting these lipids into their corresponding glycerophosphoderivatives, which were then separated on anion-exchange columns, was identical with that described by Berridge (1983).

Preparation of permeabilized cells

The medium used to permeabilize cells had the following composition (mmol/l): KCl, 140; NaCl, 20; $MgCl_2$, 2; ATP, 2; Pipes, 20; EGTA, 1 (at pH 6.8), with a free Ca^{2+} concentration of 0.13 μM. Cells were washed for 30 s in this high-K^+ medium before adding saponin (50 μg/ml) for a period of 4 min. The saponin was removed by washing cells four times with high-K^+ medium. These saponin-treated cells will be referred to as 'leaky' cells.

Ca^{2+} studies on intact and 'leaky' cells

In the experiments performed on intact cells, the cultures were incubated for 24 h in DME medium containing $^{45}Ca^{2+}$ (4.4 μCi/ml). The radioactive medium was removed and the cells were washed five times with 2 ml aliquots of phosphate-buffered saline. $^{45}Ca^{2+}$ efflux was measured by incubating cells in 1 ml of phosphate-buffered saline that was replaced at 3 min intervals with fresh solution. At certain intervals, control portions of phosphate-buffered saline were replaced with a similar solution containing either PDGF or IP_3. Each efflux sample was added to 9 ml of scintillant (NE260) and counted for $^{45}Ca^{2+}$ radioactivity. The radioactivity remaining in the cells at the end of the experiment was determined after extraction (room temperature for 30 min) into 500 μl of 0.1 M-HCl (see below for details). The efflux data are represented as a fractional efflux, i.e. the amount of radioactivity leaving the cells each 3 min is

expressed as a percentage of that present in the cells at the beginning of each period.

Both $^{45}Ca^{2+}$-efflux and $^{45}Ca^{2+}$-uptake studies were carried out on 'leaky' cells. Saponin-treated cells were incubated with $^{45}Ca^{2+}$ (10 μCi/ml) in the high-K^+ medium for 30 min. The radioactive solution was removed and the cells were washed five times with 2 ml aliquots of the high-K^+ medium before beginning the efflux measurements as described above for the intact cells. The technique used for measuring $^{45}Ca^{2+}$ uptake was adapted from that described by Burgess *et al.* (1983). 'Leaky' cells were incubated with $^{45}Ca^{2+}$ (10 μCi/ml) for various times, after which the label was removed and the cells washed five times with 1 ml aliquots of high-K^+ medium over a period of 1 min. After the last wash the $^{45}Ca^{2+}$ remaining in the cells was extracted into 500 μl of 0.1 M-HCl. A 100 μl portion of the HCl extract was removed, added to 5 ml of scintillant and counted for radioactivity. In order to study the effect of inositol phosphates, the cells were first allowed to accumulate $^{45}Ca^{2+}$ until a steady state was achieved (20 min). Inositol phosphates were then added for a further period before measuring the $^{45}Ca^{2+}$ content of the cells. The inositol phosphates were added in the same $^{45}Ca^{2+}$-containing buffer as that used to achieve a steady state, so that no dilution of isotope occurred.

Chemicals

IP_3 and IP_2 were prepared by strong alkaline hydrolysis of the corresponding ox brain phosphoinositides followed by Dowex-column fractionation and paper chromatography (Irvine, *et al.*, 1984). IP_1 and inositol 1,2-cyclic phosphate were prepared as described previously (Irvine *et al.*, 1980; Streb *et al.*, 1983). *myo*-Inositol, inositol 2-phosphate and fructose 1,6-bisphosphate were purchased from Sigma. [^{32}P]IP_3 was prepared from red blood cells as described by Downes *et al.* (1982).

PDGF was partially purified from clinically outdated platelet concentrates by heparin–agarose and carboxymethyl-agarose chromatography as described previously (Brown *et al.*, 1983). The PDGF-containing fractions from the cation-exchange column were combined; the protein concentration of the pool was found to be 670 μg/ml.

Results

Effects of PDGF on inositol lipids

Most of the [^{3}H]inositol incorporated into the inositol lipids was found in PI (90.1%), with smaller amounts in PIP (6.4%) and PIP_2 (3.5%) (Table 1). After stimulation with PDGF the proportion of label in PI decreased, with a corresponding increase in the polyphosphoinositides, particularly PIP. A similar increase was noted for PIP_2 at the low concentration of PDGF, but at the higher concentration the proportion as PIP_2 fell, suggesting that this might be associated with the changes in IP_3 described below.

PDGF stimulates the production of inositol phosphates in Swiss 3T3 cells

Stimulation of 3T3 cells with PDGF results in significant increases in the levels of IP_1, IP_2 and IP_3 (Table 2). At the lowest dose tested, there was a significant increase in the level of IP_3, but no change in IP_1 or IP_2. This dose of PDGF was capable of inducing a maximal stimulation of DNA synthesis (Table 2). At the higher doses of PDGF there was no further increase in DNA synthesis, but there were significant increases in all three inositol phosphates.

Access of IP_3 to 'leaky' cells

Before studying the effect of IP_3 on $^{45}Ca^{2+}$ fluxes, we attempted to establish that the 'leaky' cells were accessible to IP_3. Therefore normal or 'leaky' cells were incubated with [^{32}P]IP_3 in the presence of 5 μM unlabelled IP_3 for various times

Table 1. *Effect of PDGF on the distribution of [^{3}H]inositol in the three inositol lipids in 3T3 cells*

Swiss 3T3 cells were prelabelled for 60 h with [^{3}H]inositol. Dishes of cells were treated with partially purified PDGF at various concentrations for 5 min at 21°C. The radioactivity in the three inositol lipids is represented as a percentage of the total lipid inositol (±S.E.M.). The average incorporation into PI in the control cells was 34800±2800 c.p.m./dish. The corresponding water-soluble inositol phosphates from this experiment are shown in Table 2. Differences from control: $^*P<0.01$.

[PDGF] (μg of protein/ml)	Inositol lipid ...	Radioactivity (%) PI	PIP	PIP_2	No. of replicates
Control		90.1±0.30	6.4±0.16	3.5±0.13	6
3.3		88.9±0.41*	7.3±0.26*	3.8±0.16*	5
11.0		88.4±0.72*	8.3±0.53*	3.3±0.21	6
32.9		88.7±0.32*	8.2±0.25*	3.1±0.13*	6

up to 30min. The medium surrounding the cells was removed and applied to anion-exchange columns. After a 30min incubation with *intact* cells there was virtually no detectable $[^{32}P]P_i$ in the bathing medium, indicating that there had been very little breakdown of IP_3. In contrast, the medium that had bathed the *'leaky'* cells contained appreciable quantities of $[^{32}P]P_i$ (9.3% of the $[^{32}P]IP_3$ originally applied to the cells).

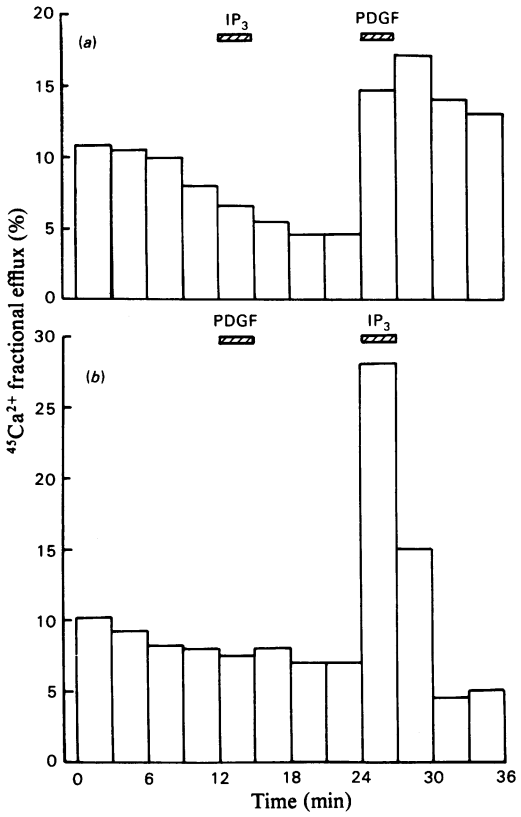

Fig. 1. *Effect of PDGF and IP_3 on the efflux of $^{45}Ca^{2+}$ from intact (a) and 'leaky' (b) 3T3 cells*

(*a*) Intact cells were incubated for 24h in DME medium containing $^{45}Ca^{2+}$ (4.4μCi/ml). (*b*) 'Leaky' cells were incubated with $^{45}Ca^{2+}$ (10μCi/ml) in the high-K^+ medium for 30 min. After this labelling period, both groups were treated in the same way. The radioactive medium was removed, the cells were washed five times and then incubated in 1ml of phosphate-buffered saline (*a*) or high-K^+ medium (*b*), which was replaced at 3min intervals. The amount of $^{45}Ca^{2+}$ leaving the cells each 3min is expressed as a percentage of that present in the cells at the beginning of each period. The hatched bars mark the intervals during which the cells were treated either with PDGF (11μg/ml) or with IP_3 (10μM).

Effect of PDGF and IP_3 on the efflux of Ca^{2+} from normal and 'leaky' 3T3 cells

The efflux of $^{45}Ca^{2+}$ from intact 3T3 cells that had been prelabelled with $^{45}Ca^{2+}$ for 24h is shown in Fig. 1(*a*). The addition of 10μM-IP_3 had no effect on the fractional $^{45}Ca^{2+}$ efflux, which varied between 5 and 10%. The addition of PDGF to the bathing solution caused a marked increase in the rate of $^{45}Ca^{2+}$ efflux from these cells. In contrast, the $^{45}Ca^{2+}$ efflux from 'leaky' cells was not increased by PDGF, but there was a massive release of $^{45}Ca^{2+}$ in response to 10μM-IP_3, a release that over the first 3min of stimulation amounted to a large proportion of the available pool (Fig. 1).

Effect of IP_3 on Ca^{2+} accumulation by 'leaky' 3T3 cells

The uptake of $^{45}Ca^{2+}$ into 'leaky' cells incubated in a high-K^+ medium is rapid and reaches an equilibrium within 20min (Fig. 2). Once equilibrium has been reached, the addition of 2μM-IP_3 caused a rapid release of approx. 40% of the stored Ca^{2+}. Most of this $^{45}Ca^{2+}$ was lost within the first minute of stimulation with IP_3 (Fig. 2). This ability of IP_3 to release ^{45}Ca from the intracellular stores

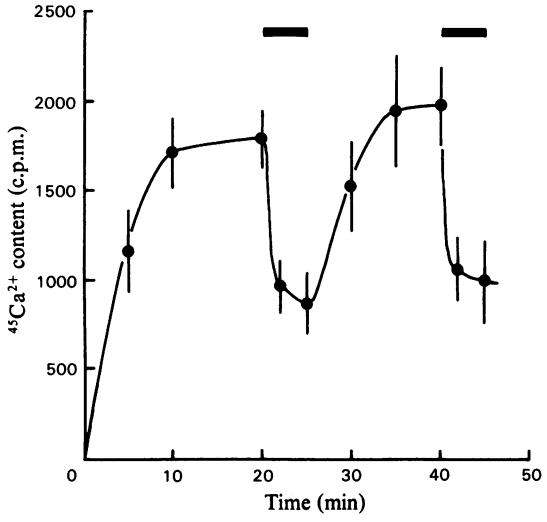

Fig. 2. *Reversibility of the Ca^{2+}-mobilizing action of IP_3 on 'leaky' 3T3 cells*

'Leaky' 3T3 cells were incubated with $^{45}Ca^{2+}$ (10μCi/ml) for various times. The solid bars represent periods when cells were stimulated with 2μM-IP_3. At each time point, label was removed, the cells were washed five times with 1ml aliquots of high-K^+ medium and the $^{45}Ca^{2+}$ remaining in the cells was counted after extraction with 0.1M-HCl. Results are means $\pm$S.E.M. for four separate dishes.

Table 2. *Effect of PDGF concentration on inositol phosphate accumulation and DNA synthesis*
Separate experiments were carried out on closely comparable cultures of Swiss 3T3 cells. For the inositol phosphate measurements, cells that had been prelabelled with [^{3}H]inositol (20 μCi/dish for 60 h) were stimulated for 5 min with three different concentrations of PDGF. Data are means ± S.E.M. obtained from six dishes. For the DNA measurements, radioactive incorporation after 36 h incubation with [^{3}H]thymidine was measured as described by Brown & Blakeley (1983). *P versus control incubations <0.01.

[PDGF] (μg of protein/ml)	Inositol phosphate ... Radioactivity (c.p.m./10^6 cells) IP_1	IP_2	IP_3	$10^{-3} \times$ [^{3}H]Thymidine incorporation (c.p.m./dish)
Control	5229 ± 240	1421 ± 50	493 ± 60	5
3.3	4900 ± 100	1436 ± 100	691 ± 60*	351
6.7	—	—	—	380
11.0	5904 ± 210*	1912 ± 50*	835 ± 120*	—
16.75	—	—	—	327
32.9	7614 ± 350*	2824 ± 160*	746 ± 20*	—
33.5	—	—	—	172

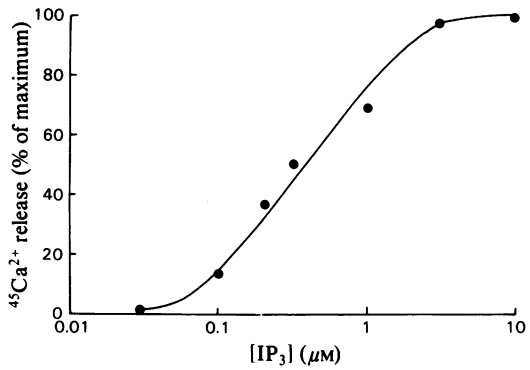

Fig. 3. *Effect of IP_3 concentration on $^{45}Ca^{2+}$ accumulation by 'leaky' 3T3 cells.*
Groups of three dishes were incubated with $^{45}Ca^{2+}$ (10 μCi/ml) in a high-K^+ medium. After 20 min, the incubation medium was replaced with medium containing different concentrations of IP_3 for a further 5 min. The $^{45}Ca^{2+}$ content was measured as described in the legend to Fig. 2. Results are expressed as a percentage of the amount of $^{45}Ca^{2+}$ released by a maximal dose of IP_3.

Table 3. *Effect of free inositol and various phosphorylated derivatives on ^{45}Ca uptake by 'leaky' 3T3 cells.*
Saponin-treated cells were incubated with $^{45}Ca^{2+}$ (10 μCi/ml) in the high-K^+ medium for 20 min. The different compounds (all at 10 μM) were then added for a further 5 min period before measuring the $^{45}Ca^{2+}$ content of the cells.

Compounds	$^{45}Ca^{2+}$ content (c.p.m./dish)
No additions	2435 ± 309
Fructose 1,6-bisphosphate	2226 ± 365
myo-Inositol	2416 ± 342
Inositol 2-monophosphate	2154 ± 115
Inositol 1,2-cyclic monophosphate	2107 ± 185
IP_2	2419 ± 383
IP_3	981 ± 105

was readily reversible. When IP_3 was removed, the cells rapidly reaccumulated ^{45}Ca. Re-addition of IP_3 produced a further release of $^{45}Ca^{2+}$ similar in magnitude to that induced by the initial stimulation (Fig. 2). The stimulation of $^{45}Ca^{2+}$ release by IP_3 was dose-dependent, with a half-maximal effect at approx. 0.3 μM (Fig. 3). Furthermore, the effect of IP_3 appears to be specific in that there was no stimulation of $^{45}Ca^{2+}$ loss from 'leaky' cells treated with either free inositol or a number of closely related compounds (Table 3).

Discussion

Inositol lipids exist in cells in three interconvertible forms. The major species is PI, which can be phosphorylated to form PIP, which can, in turn, be further phosphorylated to form PIP_2. Phosphomonoesterase enzymes are able to convert these two phosphorylated derivatives (the polyphosphoinositides) back into PI (Irvine, 1982). Recent studies have shown that it is not PI, but the relatively minor constituent PIP_2 (perhaps also PIP), that is the primary substrate cleaved in response to receptor activation (Kirk *et al.*, 1981; Michell *et al.*, 1981; Berridge, 1983; Berridge *et al.*, 1983; Downes & Wusteman, 1983; Martin, 1983; Rebecchi & Gershengorn, 1983). PI participates indirectly as a reservoir of lipid that can be rapidly called on to replace the PIP_2 consumed by the receptor mechanism (Michell *et al.*, 1981).

An important action of PDGF on Swiss 3T3 cells is to stimulate the hydrolysis of PIP_2, resulting in the formation of IP_3 and diacylglycerol. This growth factor thus has a mode of action that is remarkably similar to that of many hormones which use the inositol lipids as part of a transduction mechanism for generating second messengers. After stimulation with PDGF, there was an increase in the proportion of label present in the polyphosphoinositides relative to PI. The cells

apparently have a feedback mechanism whereby they increase the proportion of PI that is phosphorylated, so as to enlarge the pool of PIP_2 that is used by the receptor mechanism to generate diacylglycerol and IP_3. This utilization of PI to replace the polyphosphoinositides being hydrolysed by the receptor would explain the decline in labelled PI that was described previously in PDGF-treated 3T3 cells (Habenicht *et al.*, 1981). Habenicht *et al.* (1981) also reported that PDGF induced a large and rapid increase in the formation of diacylglycerol, which is consistent with the cleavage of one of the inositol lipids through a phospholipase C-type reaction. The finding that PDGF stimulates a significant increase in the level of IP_3 suggests that the receptor mechanism in 3T3 cells is using PIP_2 as a substrate. The DNA-synthesis results show that the concentrations of PDGF used were adequate to initiate cell growth if continued for a sufficient time. The hydrolysis of PIP_2 may thus be of fundamental importance to the proliferative action of PDGF in that the two products, diacylglycerol and IP_3, may function as second messengers to switch on some of the early events thought to be responsible for cell growth.

Ca^{2+} has long been implicated as a second messenger in the control of cell growth (Boynton *et al.*, 1974; Berridge, 1975; Whitfield *et al.*, 1976, 1981; Metcalfe *et al.*, 1980; Durham & Walton, 1982). An interesting aspect of the Ca^{2+}-signalling system in 3T3 cells is that growth factors stimulate a rapid mobilization of intracellular Ca^{2+} (Lopez-Rivas & Rozengurt, 1983). By using the fluorescent indicator quin 2, Moolenaar *et al.* (1984) have shown that PDGF induces an increase in the intracellular level of Ca^{2+} even when cells are bathed in a Ca^{2+}-free medium. The large increase in $^{45}Ca^{2+}$ efflux recorded from intact cells in response to PDGF (Fig. 1*a*) is certainly consistent with the notion that this growth factor can mobilize internal Ca^{2+}. As in many other cell types, however, the link between surface receptors and the internal Ca^{2+} reservoirs has been missing. Recently, it has been proposed that IP_3 might function as a second messenger for Ca^{2+} mobilization (Berridge, 1983; Streb *et al.*, 1983; Berridge & Irvine, 1984; Burgess *et al.*, 1984; Joseph *et al.*, 1984). Such an idea might be applicable to the action of mitogens such as PDGF. The present results indicate that IP_3 may be involved in the PDGF-mediated mobilization of cellular Ca^{2+}, since IP_3 stimulated the release of large quantities of $^{45}Ca^{2+}$ from 'leaky' 3T3 cells (Figs. 1*b* and 2). The effect appears to be specific for IP_3, because there was no stimulation of $^{45}Ca^{2+}$ release after the addition of related phosphates. The dose–response relationship for IP_3 shows that a half-maximal release is achieved at approx. 0.3 μM, which is similar to the values of 0.5 μM and 0.22 μM reported for permeabilized pancreatic (Streb *et al.*, 1983) and liver cells (Burgess *et al.*, 1984; Joseph *et al.*, 1984) respectively. One important action of PDGF, therefore, appears to be a stimulation of the hydrolysis of PIP_2 to release IP_3, which then acts as a second messenger to mobilize Ca^{2+} from intracellular stores.

Diacylglycerol, the other product of PIP_2 hydrolysis, may also function as a second messenger, because it is known to activate a protein kinase (C-kinase) that phosphorylates specific proteins (Takai *et al.*, 1979; Nishizuka, 1983). Diacylglycerol may function in 3T3 cells to switch on a Na^+/H^+ antiport. Growth factors are known to activate such a Na^+/H^+ antiport, resulting in a pronounced alkalinization of the cytoplasm (Schuldiner & Rozengurt, 1982; Moolenaar *et al.*, 1981, 1982, 1983). The significance of this ionic mechanism is apparent from the fact that the proliferative action of fresh serum can be blocked by amiloride, which is a potent inhibitor of this carrier (Rozengurt, 1981). The idea that diacylglycerol might provide the link between receptors and the stimulation of Na^+/H^+ exchange stems from the observation that the effect of growth factors on Na^+ entry can be mimicked by phorbol esters (Moroney *et al.*, 1978; Dicker & Rozengurt, 1981; Burns & Rozengurt, 1983). Since these phorbol esters are thought to act by stimulating the diacylglycerol-sensitive C-kinase (Castagna *et al.*, 1983), it is reasonable to speculate that diacylglycerol may function as an intramembraneous second messenger responsible for regulating the activity of the Na^+/H^+ carrier.

The ability of PDGF to stimulate the hydrolysis of PIP_2 to yield IP_3 and diacylglycerol thus represents a bifurcating control mechanism responsible for controlling at least two of the early ionic events that occur when cells are stimulated to grow. IP_3 acts to mobilize intracellular Ca^{2+}, whereas diacylglycerol could function to switch on the Na^+/H^+ antiport. Studies on blood platelets have revealed that these two separate signal pathways appear to act synergistically with each other to control various physiological processes (Kaibuchi *et al.*, 1982). Such synergistic interactions between the two pathways may also occur in the control of cell proliferation. Boynton *et al.* (1976) have shown that cells blocked in a low-Ca^{2+} medium can be induced to begin DNA synthesis if they are stimulated by a phorbol ester, and likewise the Ca^{2+}-ionophore A23187 can act synergistically with a phorbol ester to promote lymphocyte activation (Mastro & Smith, 1983). It remains to be seen whether these two intracellular signal pathways will underly the synergistic interactions that exist between the phorbol esters and a wide variety of growth factors in both fibroblasts (Frantz *et al.*,

1979; Dicker & Rozengurt, 1979; Brown *et al.*, 1979) and lymphocytes (Mastro & Mueller, 1974).

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