Inositol lipids and cell proliferation

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Control of cell proliferation by growth factors depends upon an orderly transfer of information from the cell surface into the nucleus. Although these intracellular signal pathways responsible for relaying information into the nucleus are very much a mystery, clues are beginning to emerge that the inositol lipids may play a crucial role in mediating the action of growth factors (Michell, 1982). The first indication of a relationship between inositol lipids and cell proliferation became apparent in studies on lymphocytes where phymo-gagglutinin was found to stimulate the turnover of PtdIns (Fisher & Mueller, 1968, 1971). Stimulation of PtdIns turnover by growth-promoting agents has since been observed with embryonic rat cells (Ristow et al., 1973), rat fibroblasts (Hoffmann et al., 1974), avian fibroblasts (Diringer & Friis, 1977), A-431 cells (Sawyer & Cohen, 1981) and Swiss 3T3 cells (Habenicht et al., 1981). The studies on Swiss 3T3 cells were particularly important because they revealed that, during the action of PDGF, there was an increase in the formation of DG, suggesting that the inositol lipid was being hydrolysed by the same mechanism as used by many external stimuli (e.g. hormones and neurotransmitters) which act by hydrolysing PtdIns(4,5)P₂ to give DG and Ins(1,4,5)P₃. (Michell et al., 1981, Downes & Michell, 1982, Berridge, 1983, 1984a; Berridge et al., 1984a). The formation of these two products represents a bifurcation in the signal pathway in that both DG and Ins(1,4,5)P₃ are now recognized to function as second messengers. DG stimulates C-kinase (Nishizuka, 1984) whereas Ins(1,4,5)P₃ functions as a second messenger to mobilize Ca²⁺ (Berridge, 1983, 1984a; Streb et al., 1983; Burgess et al., 1984a,b; Joseph et al., 1984). The aim of this paper is to present a hypothesis that this bifurcating signalling system based on DG and Ins(1,4,5)P₃ plays a major role in relaying information from the cell periphery into the nucleus. The appeal of this hypothesis is that it brings together much of the information in the literature, including the recent work on the function of specific oncogenes. Indeed, the hypothesis makes specific predictions as to the sites of action of many of the oncogenes whose functions are still unknown (Berridge, 1984b).

Bifurcating signal hypothesis of cell growth

There appear to be two major ionic signals responsible for initiating cell growth (Fig. 1). An increase in the intracellular level of Ca²⁺ has been recognized for some time to be part of the mitogenic signal for many different cell types (Boynton et al., 1974; Berridge, 1975; Metcalfe et al., 1980; Whitfield et al., 1981). More recently, a change in pH has also been identified as another intracellular mitogenic signal (Epel, 1978; Moolenaar et al., 1981; Pouyssegur et al., 1982; Cassel et al., 1983; Moolenaar et al., 1983). The first clue that pH was important came from studies on fertilization of sea-urchin eggs, which can be triggered simply by increasing cytosolic pH with NH₄⁺ ions. Subsequent studies showed that immediately after fertilization the intracellular pH increased markedly as acid was extruded from the egg (Lopo & Vacquier, 1977; Shen & Steinhardt, 1978). Such increases in intracellular pH appear to be a ubiquitous feature when cells are stimulated to grow, since it has been described in lymphocytes (Rothenburg et al., 1983) and in fibroblasts (Moolenaar et al., 1981; Cassel et al., 1983; Moolenaar et al., 1983). The bifurcating signal hypothesis attempts to explain how these two major ionic events are regulated independently of each other. It is proposed that growth factors stimulate the hydrolysis of PtdIns(4,5)P₂ to give the two second messengers, DG and Ins(1,4,5)P₃, which then regulate the two major ionic pathways responsible for cell proliferation. Ins(1,4,5)P₃ mobilizes Ca²⁺, whereas DG is thought to control the change in pH. For a more detailed description of the hypothesis, it is convenient to separate the control system into three separate sections (Fig. 1). Firstly, there are the metabolic pathways responsible for supplying the PtdIns(4,5)P₂ required by the receptor. Secondly, there is the transduction step itself whereby growth factors stimulate the hydrolysis of PtdIns(4,5)P₂ to give the two second messengers. Thirdly, the two second messengers function to stimulate the two ionic pathways responsible for initiating the proliferative response.

Substrate formation

The previous studies on inositol lipids and proliferation discussed earlier concentrated on PtdIns, which is the major inositol lipid in cells. Recent studies, however, indicate that the immediate substrate used by receptors is a quantitatively minor inositol lipid, PtdIns(4,5)P₂, which is derived by phosphorylation of PtdIns (Fig. 1). A PtdIns kinase converts PtdIns to PtdIns4P, which is phosphorylated to PtdIns(4,5)P₂ by a PtdIns(4)P kinase. It has been proposed that the PtdIns(4,5)P₂ used by the receptor is immediately replaced by phosphorylation of PtdIns(4,5)P₂ is crucial for the continued operation of the receptor mechanism. It is of some interest, therefore, to find that the src and ras oncogenes appear to code for proteins which have PtdIns and PtdIns4P kinase activities (Fig. 1) (Macara et al., 1984; Sugimoto et al., 1984).

These kinases which convert PtdIns to PtdIns(4,5)P₂ are counteracted by phosphomonoestersases which sequentially remove the phosphates from the 5- and 4-positions. The significance of having this futile cycle operating between PtdIns and PtdIns(4,5)P₂ is not clear but it may be part of an adaptive mechanism which enables the system to
Fig. 1. The bifurcating signal hypothesis of cell growth

A variety of growth factors, all acting on separate receptors, stimulate the hydrolysis of PtdIns(4,5)P₂ to produce DG and Ins(1,4,5)P₃. The latter is proposed to act on a specific receptor (R) on the endoplasmic reticulum (ER) to mobilize Ca²⁺. The other second messenger, DG, stimulates C-kinase, which is thought to activate on Na⁺/H⁺ exchange carrier to increase intracellular pH.

Second messenger formation

The key step in the signal transduction pathway is the agonist-dependent hydrolysis of PtdIns(4,5)P₂ to give DG and Ins(1,4,5)P₃ (Fig. 1). The cellular level of these two second messengers is determined by the balance which exists between their rate of formation from PtdIns(4,5)P₂ and their rate of removal as they are processed back to PtdIns. DG is either phosphorylated to give phosphatidic acid, or it is hydrolysed to monoacylglycerol with the release of arachidonic acid, while the Ins(1,4,5)P₃ is dephosphorylated to Ins(1,4)P₂ (Fig. 1). Growth factors appear to act by stimulating the rate at which these two second messengers are formed. Stimulation of Swiss 3T3 cells with PDGF results in a rapid formation of DG, some of which may be converted to monoacylglycerol with the release of arachidonic acid (Habenicht et al., 1981). The other product of PtdIns(4,5)P₂ hydrolysis is Ins(1,4,5)P₃ which is also increased in Swiss 3T3 cells after stimulation with PDGF (Berridge et al., 1984). In addition to PDGF, increases in Ins(1,4,5)P₃ in Swiss 3T3 cells have also been recorded in response to vasopressin, bombesin (Brown et al., 1984) and EGF but not to insulin (Table 1). The responsiveness of cells to EGF was somewhat capricious: a significant effect was seen in some experiments (as shown in Table 1), but not in others. The accumulation of Ins(1,4,5)P₃ after stimulation with PDGF can be amplified if the cells are incubated with 10mM-Li⁺ (Table 1). The finding that Li⁺ inhibits the inositol 1-phosphomonoesterase (Hallcher & Sherman, 1980) has been introduced as a method for amplifying the agonist-induced formation of inositol phosphates (Berridge et al., 1982). The ability of 10mM-Li⁺ to increase the formation of Ins(1,4,5)P₃ and Ins(1,4)P₂ has also been described in liver (Charest et al., 1984; Thomas et al., 1984) and in GH₂ pituitary cells (Drummond et al., 1984). The finding that 10mM-Li⁺ enhances the formation of Ins(1,4,5)P₃ induced by PDGF (Table 1) is particularly interesting because this concentration of Li⁺ is mitogenic in lymphoid cells (Hart, 1979), lymphocytes (Gelfand et al., 1979), mammary epithelium (Hori & Oka, 1979; Ptashne et al., 1980; Tomooka et al., 1983), kidney epithelial cells (Toback, 1980) and Balb/c3T3 fibroblasts (Ryback & Stockdale, 1981). In general, Li⁺ had no effect when added alone but appeared to act either additively or synergistically
with various growth factors. In discussing this mitogenic action of Li+, Hart (1979) suggested that growth factors may induce a distinct mitogenic signal that is retarded by some rate-limiting enzyme whose activity is altered by Li+. In the light of the data in Table 1, it is tempting to speculate that the mitogenic signal is Ins(1,4,5)P₃ and that the rate-limiting enzyme which is inhibited by Li+ is the inositol trisphosphatase that degrades Ins(1,4,5)P₃ to Ins(1,4)P₂ (Fig. 1).

The observation that a number of growth factors stimulate an increase in the formation of Ins(1,4,5)P₃ provides clear evidence that PtdIns(4,5)P₂ is the substrate of the mitogenic signal pathway responsible for regulating the two major ionic events associated with cellular proliferation.

Second messenger action

One of the effects of growth factors is to stimulate an increase in the intracellular level of Ca²⁺. Lopez-Rivas & Rozengurt (1983, 1984) found that fresh serum or vasoressin caused a rapid increase in Ca²⁺ efflux from Swiss 3T3 cells associated with a significant fall in intracellular Ca²⁺ content. This agonist-dependent mobilization of intracellular Ca²⁺ is a characteristic feature of many other hormones and neurotransmitters. Direct measurements in human fibroblasts with quin 2 have revealed that PDGF, and to a lesser extent EGF, can stimulate an increase in intracellular Ca²⁺ (Moolenaar et al., 1984). The possibility that Ins(1,4,5)P₃ might mediate the Ca²⁺-mobilizing action of growth factors has been investigated by using Swiss 3T3 cells permeabilized with saponin (Berridge, 1983, 1984). The effect of Ins(1,4,5)P₃ on Ca²⁺ mobilization was also investigated in permeabilized Swiss 3T3 cells (Berridge, 1983; Berridge & Irvine, 1984). Some evidence to support this hypothesis has come from studies showing that Ins(1,4,5)P₃, but not Ins(1,4)P₂, will release Ca²⁺ from intracellular stores in permeabilized pancreatic cells (Strub et al., 1983), liver cells (Burgess et al., 1984a; Joseph et al., 1984), porcine artery cells (Suzumatsu et al., 1984) and insulinoma cells (Bidén et al., 1984). The Ins(1,4,5)P₃-sensitive store appears to be a non-mitochondrial ATP-dependent membrane store, which is most likely to be the endoplasmic reticulum. This conclusion is supported by the observation that Ins(1,4,5)P₃ will stimulate the release of Ca²⁺ from microsomes but not from mitochondria (Dawson & Irvine, 1984; Prentki et al., 1984). There is thus growing evidence for the idea that Ins(1,4,5)P₃ functions as a second messenger to mobilize intracellular Ca²⁺.

Table 1. Effect of growth factors on inositol phosphate levels in Swiss 3T3 cells

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<tr>
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<th>Radioactivity (c.p.m./10⁶ cells)</th>
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<tr>
<td></td>
<td>Ins(1,4,5)P₃</td>
</tr>
<tr>
<td>Expt 1</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>Insulin (1 μg/ml)</td>
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<tr>
<td></td>
<td>EGF (30 μg/ml)</td>
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<tr>
<td>Expt 2</td>
<td>PDGF</td>
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<td>PDGF + 10 mM Li⁺</td>
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*P < 0.01.

The possibility that Ins(1,4,5)P₃ might mediate the Ca²⁺-mobilizing action of growth factors has been investigated by using Swiss 3T3 cells permeabilized with saponin (Berridge et al., 1984). A preliminary experiment showed that Ins(1,4,5)P₃ had no effect on Ca²⁺ efflux when applied to intact cells, whereas PDGF induced a rapid efflux similar to that described previously (Lopez-Rivas & Rozengurt, 1983). Conversely, PDGF had no effect on permeabilized cells, which responded to Ins(1,4,5)P₃ with a prompt increase in the efflux of Ca²⁺ (Berridge et al., 1984). The effect of Ins(1,4,5)P₃ on Ca²⁺ mobilization was also investigated in cells which had been labelled to equilibrium with ⁴⁰Ca²⁺ (Fig. 2). If permeabilized Swiss 3T3 cells are incubated with ⁴⁰Ca²⁺ in the presence of ATP, they rapidly accumulate tracer to an equilibrium level within about 20 min. Once this...
equilibrium has been achieved, the addition of Ins(1,4,5)P$_3$ results in a rapid release of approx. 40% of the accumulated 45Ca$^{2+}$ (Berridge et al., 1984). Testing different concentrations revealed that Ca$^{2+}$ release was half-maximal at 0.3 µM-Ins(1,4,5)P$_3$ (Berridge et al., 1984; Irvine et al., 1984). There was no release of Ca$^{2+}$ upon addition of inositol, inositol 2-phosphate, inositol 1,2-cyclic phosphate, inositol 1,4-bisphosphate or phytic acid. Structure-activity studies on the four inositol phosphates which can release Ca$^{2+}$ have been carried out in 3T3 cells (Irvine et al., 1984) and in liver (Burgess et al., 1984b). There is a small reduction in activity when glycerol is esterified to the phosphate on the 1-position or if this 1-phosphate is shifted to the 2-position. Removing this 1-phosphate completely caused a much greater reduction in potency, but the resulting inositol 4,5-bisphosphate can still induce a maximal release of Ca$^{2+}$. These structure-activity studies reveal that the vicinal phosphates on the 4- and 5-positions of the inositol ring are essential for the Ca$^{2+}$-mobilizing effect of Ins(1,4,5)P$_3$. In addition, the affinity of the molecule for its receptors is greatly enhanced by having a phosphate on the opposite side of the molecule, with a preference for the 1-position. The ability of Ins(1,4,5)P$_3$ to mobilize intracellular Ca$^{2+}$ would seem to depend upon the existence of a unique receptor which binds to the naturally occurring Ins(1,4,5)P$_3$ with a high degree of specificity.

The other limb of the bifurcating signal pathway is controlled through DG, which functions as a second messenger operating in the plane of the membrane to stimulate C-kinase (Fig. 1) (Nishizuka, 1984). At present, the identity and function of the protein substrates which are phosphorylated by C-kinase are unknown. However, there is sufficient evidence to propose that one of these substrates might be a Na$^+$/H$^+$ exchange carrier. When cells are stimulated with growth factors there is a marked increase in DG (Irvine et al., 1984), with a high degree of specificity.

The hydrolysis of PtdIns(4,5)P$_2$ may thus be a key event in the action of mitogenic agents because it initiates a bifurcating signal cascade resulting in a rise in the intracellular level of Ca$^{2+}$ and a fall in the level of protons. The fact that these two ionic events are controlled by two second messengers suggests that Ins(1,4,5)P$_3$ will induce a maximal release of Ca$^{2+}$ and a fall in the level of protons. These two ionic events are controlled by two DG, which functions as a second messenger in the liver (Irvine et al., 1984). The observation that the phorbol esters, which are precursors of DG, stimulate proliferation in cells (Irvine et al., 1984) suggests that DG may be responsible for activating this protein kinase system (Fig. 1).


