Phosphoinositides and Inositol Phosphates

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Inositol lipids and phosphates in growing, stimulated and differentiating cells

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Introduction

Receptor-stimulated phosphatidylinositol (PtdIns) metabolism was discovered by Hokin & Hokin in 1953 and substantially characterized by the same workers during the following few years (see Hawthorne, 1960). However, the function of this response remained obscure until Michell (1975) realized that it might in some way be responsible for the receptor-stimulated elevation in the cytoplasmic concentration of Ca**{2+}** that is a major intracellular trigger to the physiological responses of target cells, from rapid contraction or secretion through to the initiation of cell proliferation. In 1979, Nishizuka and co-workers discovered that 1,2-diacylglycerol released during receptor-activated inositol lipid hydrolysis could serve as a second messenger activating protein kinase C, an observation that transformed inositol lipid hydrolysis into a signalling reaction responsible for the generation of at least two intracellular signals (Nishizuka, 1984). Finally, during the period from 1981 to 1984, it was recognized that phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] is the major or only lipid hydrolysed as a direct result of receptor stimulation (Michell et al., 1981) and that the inositol 1,4,5-trisphosphate [Ins_{1,4,5}P_3] liberated by this reaction causes the release of Ca**{2+}** from an intracellular pool into the cytosol (Berridge & Irvine, 1984). Thus, in 1984, we briefly had the luxury of the satisfying and tidy conclusion that many receptors cause the activation of phosphoinositidase-C-catalysed PtdIns(4,5)P_2 hydrolysis, directly yielding two cellular messengers [1, 2-diacylglycerol and Ins_{1,4,5}P_3] and indirectly causing an increase in the cytosolic concentration of a third (the Ca**{2+}** ion) (reviewed in Downes & Michell, 1985; Nishizuka, 1986; Berridge, 1987; Berridge & Michell, 1988).

Several roles for inositol lipids in cell regulation

This ‘simple’ picture of a branching inositol-lipid-mediated pathway leading to several intracellular signals was obviously much more complex than the previously characterized and essentially linear sequence leading from other receptors through adenylyl cyclase to the protein substrates of cyclic-AMP-dependent protein kinase. However, at least three lines of subsequent work have shown that this 1984 synthesis was only the beginning of a much more complex story of the roles of inositol lipids in cellular control processes.

First, we now recognize that cells of many types contain interconvert a very large number of inositol polyphosphates, some of which are present at remarkably high intracellular concentrations (see below). Enzymic pathways have been discovered that lead from PtdIns(4,5)P_2, via Ins_{1,4,5}P_3, to many of these, notably Ins_{1,4}P_2, Ins_{1,3,4,5}P_4, Ins_{1,3,4,6}P_4, Ins_{1,3,4}P_3, Ins_{1,4,5}P_2, while the complete pathways to others, particularly Ins_{3,4,5,6}P_6, Ins_{1,3,4,5,6}P_6, and Ins_{1,2,3,4,5,6}P_6, are still unknown (see Irvine et al., 1988; Carpenter et al., 1989).

Secondly, inositol lipids have some as yet undefined role in the control of cell behaviour by cell surface receptors and/or (proto)oncogene-encoded proteins that possess intrinsic protein tyrosine kinase activity. Cantley and co-workers have discovered that, when activated, these plasma membrane tyrosine kinases become tightly associated with an 80–85 kDa tyrosine-phosphorylated protein that is almost certainly the so-called ‘Type I’ PtdIns kinase (Morrison et al., 1988), and an observation that has been confirmed by Kypka et al. (1988). Phosphatidylinositol 4-phosphate (PtdIns4P), the precursor of PtdIns(4,5)P_2, had previously been thought to be the only isomer of phosphatidylinositol monophosphate (PtdInsP) present in cells, but Whitman et al. (1988) showed that the this ‘Type I’ PtdIns kinase phosphorylates the 3-position of PtdIns and thus synthesizes the novel isomer phosphatidylinositol 3-phosphate (PtdIns3P). The further metabolism of PtdIns3P, possibly to additional lipids and inositol polyphosphates containing a 3-phosphate group, is yet to be explored, as are the functions of PtdIns3P and its metabolites in the growth-stimulating actions of protein tyrosine kinase receptors and (proto)oncogenes.

Thirdly, Saltiel and his colleagues have discovered that mammalian cells contain very small quantities of PtdIns glycan(s) of as yet undetermined structure(s) which are hydrolysed in insulin-stimulated cells. Furthermore, the water-soluble inositol phosphate glycans that are liberated by this reaction, presumably within the cell, have some of the actions expected of the long-sought intracellular messenger(s) of insulin action (see Low & Saltiel, 1988). Closest related to this set of observations is the earlier discovery that similar PtdIns glycans (for structures, see Homans et al., 1988) serve as the lipophilic anchors for an
appreciable proportion of the diverse proteins that are exposed on the surfaces of eukaryote cells: the metabolic relationship between the (presumably) intracellular events of Ptdlns glycan metabolism in response to insulin and the origin and fate of Ptdlns glycan-anchored cell surface proteins is a topic of intense investigation at present (see Low & Saltiel, 1988).

Some of the questions that have recently engaged my laboratory relate to control of the supply of substrate for inositol lipid signalling and to the intracellular levels and metabolism of the various inositol polyphosphates in relation to cell state.

**Metabolic pooling of inositol lipids in cells**

PtdIns(4,5)P₂ for signalling is synthesized from Ptdlns, probably at the plasma membrane, by the sequential action of Ptdlns 4-kinase and PtdIns4P 5-kinase. Previous work has suggested that cellular inositol lipid is metabolically heterogeneous in its relationship with the polyphosphoinositides involved in signalling (reviewed by Michell et al., 1988a). First, only a minority of the PtdIns4P and PtdIns(4,5)P₂ of human erythrocyte plasma membranes is metabolically active over a timescale of a few hours (Müller et al., 1986; King et al., 1987): this active fraction has been estimated to be one-quarter to one-third of the total complement of each lipid, when measured either by equilibrium labelling or kinetic methods (King et al., 1989; E. King & R. H. Michell, unpublished work). Secondly, some workers have obtained apparently clear evidence that the inositol lipid that is employed for PtdIns(4,5)P₂-based signalling comes from a metabolically distinct pool that is only a minority of the total cellular inositol lipids. However, other past evidence appears incompatible with this view (reviewed by Michell et al., 1988a). This question has been re-investigated in WRK1 cells stimulated with vasopressin and in 3T3 cells stimulated with prostaglandin F₂α. To do this, a new technique has been devised for determining whether the PtdIns(4,5)P₂ that is the immediate substrate for receptor-activated phosphoinositidase C action is derived from a metabolically atypical pool of Ptdlns or from random from all of the cellular Ptdlns (see Michell et al., 1988a). This involves equilibrium labelling of cells with [3H]inositol, by incubating with [3H]inositol, stimulation, and finally determination of the 14C/3H ratios of the inositol phosphate products harvested from the stimulated cells. To date, all of the evidence obtained by using this method has been consistent with the idea that the inositol lipid that is hydrolysed in response to stimulation comes from a metabolically average sample of cellular lipid (Michell et al., 1988a; Stephens et al., 1988), and it seems possible that the putative Ins(3,4,5)P₃ of WRK1 cells is also a metabolite on these lipid-independent pathways. In general, the other compounds we have observed are those expected as intermediates on recently defined metabolic pathways, and their changes on stimulation are in accord with the behaviour expected of the appropriate compounds traversing those pathways.

**Concentrations of inositol phosphates in growing and differentiating HL60 cells**

HL60 promyeloid cells were labelled to equilibrium with [3H]inositol while growing either in serum-containing medium at 35 mg inositol/l or serum-free at 1 mg/l, and were differentiated towards neutrophils using dimethyl sulphoxide. Intracellular concentrations of individual inositol phosphates were calculated by reference to the specific radioactivities of the inositol precursor and the measured volumes of the cells (Michell et al., 1988a; French et al., 1988).

The growing cells contained inositol phosphates at a total concentration of about 120 μM, bearing a total of approximately 450 μM-phosphate groups. More than half consisted of InsP₃ (50 μM) and InsP₂ (22 μM) of high metabolic stability. During differentiation, the concentration of InsP₂ fell, and the latter rose about 3-fold (French et al., 1988). At least six different inositol monophosphates and bisphosphates were also present at substantial concentrations, with InsP₁ and/or InsP₃ showing a particularly striking accumulation and InsP₂ a large decline during differentiation towards neutrophils (see French et al., 1988, for details).

For the first time, therefore, it is clear that the transition of HL60 cells from a proliferating to a neutrophil-like differentiated state is accompanied by large and characteristic changes in the intracellular concentrations of a variety of inositol polyphosphates. Preliminary studies suggest that a quite different set of changes occurs when the same cells are differentiated to a macrophage-like condition following exposure to tetradecanoil phorbol acetate (P. J. French, C. M. Bunce, J. M. Lord & G. Brown, unpublished work), suggesting that particular patterns of inositol phosphates may characterize particular differentiated cell states. As yet, these striking changes in inositol polyphosphate profiles have no known function, and our next experiments will aim to determine whether the changes are involved in the process of cell
commitment to differentiation or are reflections of the new
differentiated states.

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The metabolism and functions of inositol pentakisphosphate and inositol hexakisphosphate

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Over the last few years, the investigation of inositol lipid metabolism has elucidated a fundamental mechanism by which cells respond to a variety of external stimuli (Berridge & Irvine, 1984; Downes & Michell, 1985). Activation of appropriate cell-surface receptors stimulates the hydrolysis of a membrane lipid, phosphatidylinositol 4,5-bisphosphate, to form two informational products, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate [Ins(1,4,5)P3]. DAG and Ins(1,4,5)P3 are now generally recognized as authentic ‘second-messengers’ in intracellular communication, controlling the activity of protein kinase C and the elevation of cytosolic Ca2+ respectively (Nishizuka, 1984; Streb et al., 1983). These findings have provided the impetus for more detailed investigations of inositol metabolism in cells, and these have recently revealed that cells contain a hitherto unsuspected and quite startling diversity of inositol phosphates (see for example; Batty et al., 1985; Heslop et al., 1985; Hawkins et al., 1986; Hansen et al., 1986; Balla et al., 1987; Shears et al., 1987; Inhorn et al., 1987; Stephens et al., 1988a; Dean & Moyer, 1988). Some of these compounds are produced via the receptor-stimulated formation and subsequent metabolism of the second-messenger Ins(1,4,5)P3, but it is becoming increasingly clear that others are normal constituents of quiescent cells with no clear connection with hormone-stimulated events. Two compounds which appear to fit into this latter category are inositol pentakisphosphate (Ins(5)P5 and inositol hexakisphosphate (Ins(6)P6).

Preliminary evidence first suggested that the levels of InsP2 and InsP3 went up (Morgan et al., 1987), down (Tilly et al., 1987) or even oscillated (Heslop et al., 1985) in response to cellular stimulation by agonists coupled to inositol lipid hydrolysis. However, we have measured the levels of [3H]InsP2 and/or [3H]InsP3 in a variety of cell types labelled with [3H]inositol and stimulated with the appropriate agonists, and can find no evidence for rapid changes in the levels of these two compounds. The systems we have studied are: bradykinin-stimulated NG115-401L neuroblastoma-glioma hybrid cells, carbachol-stimulated 132 1N myoblasts (see Fig. 1 and data not shown).

If InsP2 and InsP3 are not directly involved in the inositol lipid signal-transduction pathway, what do they do? These compounds have only recently been observed in mammalian cells, but they have been known as major constituents of plant seeds (as phytic acid) and avian erythrocytes for a number of years (Posternak, 1903; Jackson et al., 1982; Dyer, 1940; Johnson & Tate, 1969; Bartlett, 1980). Are there any clues in this older literature as to their possible functions in mammalian cells? Unfortunately, the answer appears to be no. It seems that InsP2 has a rather specialized function as a modulator of haemoglobin oxygen affinity in avian erythrocytes (Bartlett, 1980) and InsP3 is generally assigned the role of a phosphorus and/or energy store in avian erythrocytes (Bartlett, 1980) and InsP6 is generally lacking. Preliminary evidence suggests that InsP2 and InsP3 can accumulate in mammalian tissues to levels of the order 10–100 μM (Szwergold et al., 1987 and our own unpublished work). This poses a problem, in that one might expect these concentrations to be insoluble in the presence of the intracellular concentration of free Mg2+ of the order of 1 mM