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Inositol lipids: receptor-stimulated hydrolysis and cellular lipid pools

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Our current knowledge of the process by which receptors stimulate the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) has its origin in the discovery by Hokin & Hokin (*J. biol. Chem.* **263**, 967 (1953)) that some pancreatic secretagogues not only elicit exocrine secretion but also stimulate the metabolism of membrane phospholipids. Despite the recent elucidation of many aspects of this widespread signalling system, there is still little information on the control of the supply of its substrate, PtdIns(4,5)P_2. In particular, some studies have suggested that inositol-lipid-mediated signalling involves much or all of the inositol lipid complement of the stimulated cells, whereas other observations have equally clearly implicated the receptor-activated hydrolysis of an inositol phospholipid pool that comprises only a small fraction of the total cellular complement of these lipids. These studies, which have largely employed radiochemical analyses using single isotopes, are briefly reviewed. In addition, we report the first information obtained by a new procedure for analysing the metabolic characteristics of the inositol lipids that are broken down during stimulation. This technique employs cells that are doubly labelled in the inositol moiety of their lipids (to isotopic equilibrium with ^{14}C and only briefly with ^{3}H) to search for functional metabolic heterogeneity among the inositol lipids of stimulated cells. Using this method, we have found that the inositol phosphates liberated in stimulated cells during brief stimulation of V_{1a}-vasopressin receptors or prostaglandin $F_{2\alpha}$ receptors come from phospholipid that has a turnover rate typical of the bulk of the cellular inositol lipids.

The history of inositol-lipid-mediated signalling

The observation by Hokin & Hokin (1953) that some secretagogues stimulate phospholipid metabolism marked the opening of a research field that has recently served to unite biochemistry, pharmacology, physiology, immunology and cell biology in a common search for the mechanisms by which extracellular agents control intracellular responses. However, the development of this research area has been a relatively slow process: the double helical structure of DNA was discovered at exactly the same time as hormone-stimulated phospholipid metabolism, but the widespread importance of the latter process took about 20 years longer to become widely appreciated.

Once Hokin & Hokin had discovered stimulated phospholipid metabolism, they soon went on to show that this response is largely restricted to two quantitatively minor membrane phospholipids, namely phosphatidylinositol(PtdIns) and phosphatidate (PtdOH): the latter is a biosynthetic precursor of PtdIns. In 1964, they suggested that the initiating reaction in the stimulation of PtdIns metabolism by acetylcholine in the nasal salt-secreting glands of seabirds was the hydrolysis of PtdIns by an inositol-lipid-specific phospholipase (now often known as phosphoinositidase C, or PIC), yielding as products 1,2-diacylglycerol and an inositol phosphate (Hokin & Hokin 1964). However, it was many more years before it was widely

agreed that activation of PIC is, indeed, the reaction at the heart of stimulated inositol phospholipid metabolism (Durell *et al.* 1969; Lapetina & Michell 1973; Michell 1975). Durell *et al.* (1969) pointed out that the major substrate of the activated PIC might be either phosphatidylinositol or phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2): the latter lipid is less abundant than PtdIns but is concentrated in the plasma membrane (see Downes & Michell 1982). However, it was not until the early 1980s that PtdIns(4,5)P_2 was shown to the major, and possibly the only, inositol lipid hydrolysed by hormone-activated PIC (Michell *et al.* 1981; Kirk *et al.* 1981; Weiss *et al.* 1982; Berridge 1983; reviewed in Berridge 1984; Downes & Michell 1985). In all eukaryotic cells investigated until the present, at least some of the PtdIns(4,5)P_2 of the cell is in rapid metabolic equilibrium with some or all of the cellular PtdIns through two kinase plus phosphatase cycles (see, for example, Downes & Michell 1982; Palmer *et al.* 1986; King *et al.* 1987).

Contradictory evidence for and against the hydrolysis and resynthesis of a metabolically unique pool of hormone-responsive inositol lipid

In their 1964 study of ^{32}P-labelled salt glands, Hokin & Hokin showed that a labelled pool of PtdIns was replaced by labelled PtdOH during stimulation, and that this lipid was resynthesized to PtdIns on removal of the stimulus. Concomitant with this marked shift of radioactivity out of and back into the cellular PtdIns pool, there was little or no change in the chemical concentration of PtdIns in the tissue. Thus it seemed that stimulation provoked the turnover of an inositol lipid pool that was only a minor fraction of the total quantity of PtdIns present. However, the function of this pool in the response of the salt gland to stimulation was unknown, and there was even a possibility that the quantitatively minor hormone-responsive pool of PtdIns was present in some minor cellular elements of the glands rather than in the salt-secreting cells.

During the following 10 years, the idea that stimulated PtdIns turnover might be in some way implicated either in the complex membrane events of exocytotic secretion or in the transmission of receptor signals into the cell provoked several groups to analyse the intracellular distribution of the labelled PtdIns accumulated in response to stimulation. These studies used techniques of autoradiography or subcellular fractionation, and the tissues investigated included sympathetic ganglia, exocrine pancreas, cerebral cortex and lymphocytes (for reviews see Lapetina & Michell 1973; Michell 1975). In general, these studies gave relatively concordant results, in that none of them suggested any unique intracellular locus for the PtdIns that became newly labelled in response to stimulation. In particular, there was no convincing evidence for localization either in secretory vesicles or at the plasma membrane. This information provided one of the major arguments for the predominant view, before 1975, that stimulated inositol lipid metabolism could play no central role in the transmembrane signalling mechanisms controlled by cell-surface receptors.

Recognition in the mid-1970s that PIC-catalysed hydrolysis of an inositol lipid is the reaction stimulated by receptor activation (see Michell 1975) to a large degree relegated the receptor-stimulated labelling of inositol lipids to a secondary position, where it was regarded as a 'compensatory resynthesis' of new inositol lipid to replace that degraded in the primary response to receptor activation. (Even now, more than 30 years after its discovery, we have very little idea of how either the normal biosynthesis of PtdIns or this 'compensatory resynthesis'

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is regulated within cells.) By that time, cytosolic phospholipid exchange proteins of high activity that could potentially shuttle lipid molecules between different intracellular membranes had been identified. It was therefore proposed that receptors activated PIC at the plasma membrane, and that the liberated 1,2-diacylglycerol was then phosphorylated to PtdOH and shuttled to the endoplasmic reticulum (the known site of PtdIns biosynthetic enzymes) for resynthesis into labelled PtdIns and subsequent redistribution around the cell (Lapetina & Michell 1973; Michell 1975). This model readily reconciled two apparently contradictory demands, that receptors could be directly linked to PIC-catalysed inositol lipid hydrolysis at the plasma membrane and that the newly labelled PtdIns that is synthesized in stimulated cells should be widely distributed through the cell. An additional experimental observation that was readily accommodated by this model was that hormonal stimulation can cause the hydrolysis of up to half of the inositol lipid of some cells (Jones & Michell 1974; Hokin-Neaverson 1974), a result that seems incompatible with any suggestion that hormone-sensitive inositol lipid hydrolysis can only draw substrate from the lipid complement of some quantitatively minor cell organelle such as the plasma membrane. Also implicit in this model was the assumption, based on early studies by Hokin & Hokin, that the PtdOH synthesized from inositol lipid-derived 1,2-diacylglycerol somehow becomes specifically committed to the resynthesis of PtdIns (see Michell 1975).

Once it had been recognized that receptors activate PIC, several studies attempted to determine which cell compartment(s) became PtdIns-depleted when cells were stimulated, again with very varying results (for references see Michell *et al.* 1981). However, at this time it became recognized that receptor-activated PIC has PtdIns(4,5)P_2 as its major or only substrate (Michell *et al.* 1981; Kirk *et al.* 1981). Once again it could be argued that the primary PIC-catalysed attack on PtdIns(4,5)P_2 is at the plasma membrane, but that cell-wide stores of PtdIns are called upon to replenish the depleted PtdIns(4,5)P_2 pool after stimulation.

By about 1980, therefore, most of the available evidence was easily reconciled with the idea that receptor-mediated stimulation of inositol lipid hydrolysis at the cell surface leads to a rather generalized depletion of PtdIns throughout the various membrane compartments of stimulated cells, and in some 'secondary' way to a resynthesis of labelled PtdIns that also becomes distributed throughout the cell. A natural corollary of this view was the rejection of any suggested mechanism that invoked receptor-triggered hydrolysis of a limited inositol lipid pool that was not in rapid metabolic equilibrium with the remainder of the inositol lipids of the cell. This was, however, exactly the conclusion that emerged from two sets of experiments at about that time.

First, Fain & Berridge (1979) showed that if the PtdIns of the salivary gland of blowflies is labelled both to equilibrium with ^{32}P and briefly using [^{3}H]inositol, then there is a preferential breakdown of the recently labelled lipid when the glands are stimulated with 5-hydroxytryptamine. Secondly, Monaco labelled WRK1 mammary tumour cells with ^{32}P in an initial incubation either 'at rest' or during stimulation by vasopressin, and then stimulated them during a second incubation period with vasopressin. In these experiments, the second period of vasopressin stimulation led to a much more rapid degradation of the PtdIns synthesized during the initial stimulation than of the PtdIns labelled in previously unstimulated cells (Monaco 1982). Both of these studies clearly suggested that recently synthesized PtdIns was much more likely to be degraded as a result of stimulation than PtdIns laid down many hours or days previously, and the data of Monaco suggested that the PtdIns formed by

'compensatory resynthesis' during a first period of receptor-stimulated inositol lipid breakdown was more likely to be degraded in response to a second stimulus than the labelled PtdIns of unstimulated cells. These observations were in many ways similar to the original observation on the avian salt gland by Hokin & Hokin (1964), and clearly revived the idea that only a small proportion of the total inositol lipid of cells (approximately one sixth in the mammary tumour cells) might be accessible to hormone-stimulated PIC. The most obvious site of such a hormone-sensitive pool would be in the plasma membrane, and maybe also in other membranes such as endosomes that are in dynamic equilibrium with the plasma membrane.

Based largely on these results, Downes & Michell (1985) proposed an alternative model of the cellular events in hormone-stimulated inositol lipid metabolism. This model envisaged that cellular inositol lipids are compartmentalized into a minimum of two metabolic pools that do not mix to any substantial extent over a timescale of minutes or a few hours, and that only one of these pools, which is usually or always the smaller, is susceptible to attack by hormone-stimulated PIC. One immediate requirement of any such model is a set of biosynthetic enzymes for PtdIns synthesis that is located at the plasma membrane, and Imai & Gershengorn (1987) have recently presented evidence for the existence of such enzymes. However, an attempt by Monaco (1987) to confirm this result was unsuccessful.

In retrospect, it is clear that the 1975 model, which envisaged that the inositol lipid hydrolysed upon stimulation is a metabolically 'average' sample of cell lipids (Michell 1975), and the 1985 model, which invoked a small hormone-sensitive inositol lipid pool at the plasma membrane that is metabolically independent of the bulk of cell inositol lipids (Downes & Michell 1985), are to a substantial degree contradictory, and neither can be satisfactorily reconciled with all of the experimental data. What are the reasons for this uncertainty over the interpretation of such a relatively substantial and precise body of experimental work? First, it is possible that there will be detailed differences in the mechanisms responsible for inositol lipid biosynthesis and distribution in different cells. However, it seems most unlikely that these differences will be sufficiently fundamental to allow the existence of fully fledged examples of both of the above mechanisms. Secondly, some of the apparent differences in the metabolic pooling of inositol lipids in different cells may arise from a failure to distinguish between primary receptor-coupled events and as yet undefined 'secondary' processes of inositol lipid depletion. Finally, some of the apparent contradictions inherent in the information discussed above may be due to misinterpretation of the data yielded by experimental designs that are somewhat ambiguous.

A resolution of this uncertainty, with the resulting quantitative description of inositol lipid metabolism and its control both in and between the various cell compartments, will be of great importance, because control of the supply of PtdIns(4,5)P_2 to the receptor-controlled PIC of cells may sometimes play a major role in determining cellular sensitivity to natural stimuli. A full description of these processes seems most likely to come first from studies of hormone-sensitive cell lines in culture. This is primarily because such cells can readily be labelled with radioisotopic precursors both to equilibrium (so obviating the need to develop highly sensitive chemical assays for lipids that are present in cells at very low concentrations) and over the much shorter periods needed to identify metabolically dynamic lipid pools.

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The use of cultured cells doubly labelled in the inositol moiety of their phospholipids to investigate the metabolic characteristics of the inositol lipid pool(s) hydrolysed in response to stimulation

Previous attempts to discover whether the inositol lipid pool that is hydrolysed when cells are stimulated is metabolically distinct from the bulk of the cell lipids have employed single isotopes to label either the inositol or phosphodiester phosphate groups of these lipids or have introduced labels into these two parts of the lipid molecules simultaneously. The study by Fain & Berridge (1979) was an important example of the latter type. Studies with a single isotopic label are often difficult to interpret unambiguously. For example, it is often difficult to distinguish between effects that might reflect the coexistence of multiple phospholipid pools and effects caused by uncontrolled changes in the isotopic status of lipid precursor pools. Although studies that incorporate two isotopes into different parts of the phospholipid headgroup can be more informative, they bring into play the additional unknown variable of two or more precursor pools, each of which has undefined labelling characteristics when using externally added precursors.

We reasoned that one way around this problem might be to label cells using the same chemical precursor (inositol) labelled with two different radioactive isotopes (^{3}H attached to the 2-carbon, and ^{14}C uniformly distributed in the inositol ring). One form of labelled inositol could then be employed to equilibrium label all of the inositol-containing constituents of the cells during several days of culture, and the second type of labelled inositol could be used for brief labelling of the same cells just before stimulation (either in the presence or absence of a prior stimulus). To identify unequivocally the products of hormone-stimulated inositol lipid turnover, we would then harvest the labelled inositol phosphates that accumulate during stimulation, and the lipids from which they are derived, and examine their content of the two isotopic forms of inositol.

We have so far done relatively few experiments using this technique. However, experiments have been done on two cell-types in parallel, with very similar results. These are: (*a*) vasopressin-stimulated WRK1 rat mammary tumour cells, the cell-line in which Monaco has done her extensive studies of the apparent existence of a relatively small and metabolically independent hormone-sensitive inositol lipid pool; and (*b*) the T15+ subline of 3T3 fibroblasts, which have been engineered to overexpress the N*ras* proto-oncogene in response to glucocorticoids and which exhibit inositol lipid hydrolysis in response to bombesin (Wakelam *et al.* 1986) or prostaglandin $F_{2\alpha}$ (M. J. O. Wakelam, personal communication).

Both in the study of blowfly salivary glands by Fain & Berridge (1979) and in the much more extensive investigations of WRK1 cells by Monaco (Monaco 1982, 1987; Monaco & Woods 1983; Koreh & Monaco 1986), a limited hormone-sensitive inositol pool appeared to turn over relatively more rapidly than the 'bulk' inositol lipid of the cells, even in the absence of a stimulus. A clear prediction that arises from this apparent metabolic pooling is that a large proportion of the inositol phosphates liberated by PIC action during stimulation should be derived from recently synthesized inositol lipid rather than from lipid synthesized during a prolonged period of labelling. To test this prediction, we first labelled cells to equilibrium with [^{14}C]inositol during several days in culture. [^{3}H]inositol was then added to the cultures for 140 min to label selectively any inositol lipid pools that have a particularly rapid turnover, and the cells were stimulated for 10 min. To maximize the recovery of the inositol phosphates

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formed during stimulation, 10 mM LiCl was added to the cultures for the final 20 min of culture (i.e. it was added 10 min before the hormone). The cells were killed, and the (deacylated) lipids and inositol phosphates were examined by anion-exchange chromatography for changes in their contents of the two isotopic forms of inositol. The results of such an experiment on WRK1 cells are shown in table 1.

TABLE 1. VASOPRESSIN-STIMULATED INOSITOL LIPID DEPLETION AND ACCUMULATION OF INOSITOL PHOSPHATES IN WRK1 MAMMARY TUMOUR CELLS THAT HAD BEEN LABELLED TO EQUILIBRIUM WITH [^{14}C]INOSITOL AND BRIEFLY WITH [^{3}H]INOSITOL

(WRK1 cells were grown for 3 days in inositol-free medium containing dialysed foetal calf serum and dialysed rat serum as described by Wong *et al.* (1988). [U-^{14}C]inositol was present throughout the cultures, and [2-^{3}H]inositol was added for the final 140 min. 10 mM LiCl was added to all cultures 20 min before quenching, and 220 nM 8-arginine-vasopressin was added to the stimulated cultures for the final 10 min. Cultures were quenched, and lipids and water-soluble inositol phosphates extracted. The inositol phosphates were separated into mixed fractions containing Ins$P$ plus InsP_2, InsP_3 plus InsP_4, and InsP_5 plus InsP_6 by chromatography on small Dowex 1 columns (formate form).)

	control cells			vasopressin-stimulated cells		
	^{3}H (d.p.m.)†	^{14}C (d.p.m.)†	^{3}H:^{14}C ratio	^{3}H (d.p.m.)†	^{14}C (d.p.m.)†	^{3}H:^{14}C ratio
Lipids	213000 ± 30700	45900 ± 3250	4.64	96700 ± 11700	27400 ± 6000	3.52
			change on stimulation:	*−116400*	*−18500*	*6.3*
Ins$P$ + InsP_2	13100 ± 1800	2700 ± 100	4.85	77800 ± 8800	16100 ± 1860	4.83
			change on stimulation:	*+64700*	*+13400*	*4.82*
InsP_3 + InsP_4	3900 ± 150	1440 ± 60	2.68	36500 ± 1940	8100 ± 330	4.49
			change on stimulation:	*+32600*	*+6700*	*4.87*
InsP_5 + InsP_6	500 ± 80	2140 ± 220	0.23	680 ± 150	2440 ± 520	0.28
			change on stimulation:	*+180*	*+300*	*(0.6)*

† D.p.m., disintegrations per minute. 60 d.p.m. = 1 Bq.

The first notable result is that stimulation with vasopressin caused a large decrease in the labelling of total inositol lipids with both isotopes, clearly demonstrating the occurrence of vasopressin-stimulated inositol lipid degradation. As in other cells, it seems likely that the initiating reaction in this response is PtdIns(4,5)P_2 hydrolysis by PIC (Kirk *et al.* 1986). Although the ^{3}H:^{14}C ratio in the lipids degraded (which was 6.3) appeared to be slightly greater than that of the lipids of the cells before stimulation (4.64), there was no indication of any highly selective degradation of the ^{3}H-labelled lipids that had been synthesized during the 140 min before the addition of vasopressin.

In these initial experiments, the inositol phosphates extracted from the cells were separated only into three mixed fractions, namely: (*a*) the highly phosphorylated InsP_5 and InsP_6, which turn over slowly and whose metabolism is probably unaffected by the hormonal stimulation of PtdIns(4,5)P_2 (at least over a timescale of a few minutes); (*b*) an InsP_3 plus InsP_4 fraction, which should contain the Ins(1,4,5)P_3 liberated during PtdIns(4,5)P_2 hydrolysis, together with its key early metabolites Ins(1,3,4,5)P_4 and Ins(1,3,4)P_3; and (*c*) an Ins$P$ plus InsP_2 fraction that should include a variety of inositol bisphosphates and monophosphates that are secondary products formed from the various inositol tris- and tetrakisphosphates.

Although the InsP_5 plus InsP_6 fraction contained a substantial amount of ^{14}C, it incorporated very little ^{3}H either in control or stimulated cells. This is in accord with the expectation that the phosphates in this fraction would be metabolically inert and independent, at least in the short term, of the phosphates derived from phospholipids.

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As expected, a substantial fraction (about one third) of the radioactivity released from lipids on stimulation was recovered in the InsP_3 plus InsP_4 fraction. The material composing this fraction before stimulation had a fairly low $^3H:^{14}C$ ratio. The predominant compound in these samples was almost certainly Ins(3,4,5,6)P_4. This was recently discovered as a major InsP_4 species in other mammalian cells (Stephens *et al.* 1988*a*) where it is the precursor of Ins(1,3,4,5,6)P_5 (Stephens *et al.* 1988*b*). We have shown that it is the major InsP_4 isomer present in WRK1 cells and is unaffected by stimulation with vasopressin (A. J. Morris & C. J. Barker, unpublished data). It is notable that the $^3H:^{14}C$ ratio of the material recruited into this fraction during stimulation with vasopressin was almost identical with that of the total lipid fraction of the cells.

An even larger quantity of the liberated water-soluble label, equivalent to at least half of that lost from the lipids on stimulation, found its way into the Ins$P$ plus InsP_2 fraction and appears to have been effectively trapped by Li^+ inhibition of the phosphatases that degrade these inositol phosphates. Once again the $^3H:^{14}C$ ratio of the accumulated phosphates was very similar to that of the whole cell lipids.

From Monaco's studies, we anticipated that the inositol phosphates liberated by stimulation in the cells that had been doubly labelled with inositol would come from a rapidly turned-over inositol lipid pool, and would therefore have a higher $^3H:^{14}C$ ratio than the total lipids of the cells in which they were formed. However, our initial results have not shown this effect either in WRK1 cells (table 1) or in T15+ cells (results not shown). The more precise of the three estimates in table 1 of the $^3H:^{14}C$ ratio of the degraded lipids are almost certainly those derived from the analysis of the accumulated inositol phosphates, and it is striking that these figures (4.82 and 4.87) are very close to the equivalent ratio for the total cell lipids (4.64). We are now doing a much more detailed analysis of the behaviour of the lipids of these doubly labelled cells, particularly by using cells in which a stimulus is applied twice, first to selectively introduce ^3H into the putative hormone-sensitive pool and on the second occasion to 'chase' label out of this putative pool selectively.

Concluding comments

The existing literature, on a variety of cells studied by a substantial range of techniques, presents a contradictory picture of the metabolic behaviour of the inositol lipids that are used as a substrate for transmembrane signalling. In particular, it is not clear whether they are drawn relatively randomly from all of the inositol lipids of the stimulated cells or are somehow held in a metabolically segregated state waiting to fulfil this special function. We believe that the new technique described here will allow this question to be answered with much greater certainty than hitherto. Our preliminary results with this analytical method, typified by those in table 1, have suggested that the inositol phosphates liberated in cells during stimulation are not preferentially formed from a rapid turnover pool of inositol lipid molecules, even in the WRK1 mammary tumour cell line in which the existence of such a discrete hormone-sensitive inositol lipid pool has appeared to be most firmly established.

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