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Rapid breakdown of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate in rat hepatocytes stimulated by vasopressin and other Ca^{2+}-mobilizing hormones

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Rat hepatocytes rapidly incorporate [^{32}P]P_i into phosphatidylinositol 4-phosphate (PtdIns4*P*) and phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2]; their monoester phosphate groups approach isotopic equilibrium with the cellular precursor pools within 1 h. Upon stimulation of these prelabelled cells with Ca^{2+}-mobilizing stimuli (V_1-vasopressin, angiotensin, α_1-adrenergic, ATP) there is a rapid fall in the labelling of PtdIns4*P* and PtdIns(4,5)P_2. Pharmacological studies suggest that each of the four stimuli acts at a different population of receptors. Insulin, glucagon and prolactin do not provoke disappearance of labelled PtdIns4*P* and PtdIns(4,5)P_2. The labelling of PtdIns4*P* and PtdIns(4,5)P_2 in cells stimulated with vasopressin or angiotensin initially declines at a rate of 0.5–1.0% per s, reaches a minimum after 1–2 min and then returns towards the initial value. The dose–response curves for the vasopressin- and angiotensin-stimulated responses lie close to the respective receptor occupation curves, rather than at the lower hormone concentrations needed to evoke activation of glycogen phosphorylase. Disappearance of labelled PtdIns4*P* and PtdIns(4,5)P_2 is not observed when cells are incubated with the ionophore A23187. The hormone-stimulated polyphosphoinositide disappearance is reduced, but not abolished, in Ca^{2+}-depleted cells. These hormonal effects are not modified by 8-bromo cyclic GMP, cycloheximide or δ-hexachlorocyclohexane. The absolute rate of polyphosphoinositide breakdown in stimulated cells is similar to the rate previously reported for the disappearance of phosphatidylinositol [Kirk, Michell & Hems (1981) *Biochem. J.* **194**, 155–165]. It seems likely that these changes in polyphosphoinositide labelling are caused by hormonal activation of the breakdown of PtdIns(4,5)P_2 (and may be also PtdIns4*P*) by the action of a polyphosphoinositide phosphodiesterase. We therefore suggest that the initial response to hormones is breakdown of PtdIns(4,5)P_2 (and PtdIns4*P*?), and that the simultaneous disappearance of phosphatidylinositol might be a result of its consumption for the continuing synthesis of polyphosphosinositides.

In many stimulated cells, there is a striking association between enhanced inositol lipid metabolism and elevation of cytosol Ca^{2+} concentrations. Amongst the affected cells are hepatocytes stimulated by angiotensin, vasopressin or α_1-adrenergic stimuli (for reviews, see Kirk *et al.*, 1980; Exton, 1981; Williamson *et al.*, 1981). Whether stimulated inositol lipid metabolism plays any general role in receptor-stimulated Ca^{2+} mobilization (Michell, 1975, 1979*a*,*b*; Michell *et al.*, 1977, 1981; Downes & Michell, 1982*a*) is at present a matter of disagreement (Cockcroft, 1981; Hawthorne, 1982; Michell & Kirk, 1982; Michell, 1982*a*). However, the evidence obtained so far is compatible with such a role in hepatocyes (Kirk *et al.*, 1977, 1978, 1979, 1980, 1981*a*; Billah & Michell, 1979; Tolbert *et al.*, 1980).

Details of the mechanisms that are responsible for stimulated inositol lipid metabolism in hepatocytes and elsewhere remain obscure, but recent work has consistently pointed to a phospholipase C-catalysed breakdown of PtdIns as the initiating reaction (for

Abbreviations used: PtdIns, 1(3-*sn*-phosphatidyl)-L-*myo*-inositol; PtdIns4*P*, 1-(3-*sn*-phosphatidyl)-L-*myo*-inositol 4-phosphate; PtdIns(4,5)P_2, 1-(3-*sn*-phosphatidyl)-L-*myo*-inositol 4,5-bisphosphate.

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reviews, see Michell, 1975; Michell *et al.*, 1977; Jones *et al.*, 1979). However, most of the published studies have ignored PtdIns4*P* and PtdIns(4,5)P_2, two lipids that are usually present in much smaller quantities than PtdIns but which are metabolically very active. Rapid labelling of PtdIns4*P* and PtdIns(4,5)P_2 in rat liver was reported many years ago (Hölzl & Wagner, 1964; Santiago-Calvo *et al.*, 1964), but the only published comment on their metabolism in hepatocytes suggested that this was unaffected by hormones (Tolbert *et al.*, 1980). By contrast, our recent experiments, which are reported here, suggest that breakdown of PtdIns(4,5)P_2 (and perhaps also PtdIns4*P*) may precede the previously reported PtdIns 'breakdown'. Some of these studies have been described briefly in the proceedings of recent meetings (Michell *et al.*, 1981, 1982; Kirk *et al.*, 1981*b*; Creba *et al.*, 1981; Creba & Kirk, 1982).

Materials and methods

Materials and animals (Wistar rats) were, unless otherwise noted, of the type and from the source specified previously (Michell & Jones, 1974; Billah & Michell, 1979; Kirk *et al.*, 1979, 1981*a*). Yohimbine was from Sigma Chemical Co., insulin from Burroughs–Wellcome, glucagon from Eli Lilly, Indianapolis, IN, U.S.A., and prazosin from Pfizer, Sandwich, Kent, U.K. [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),8-arginine]-Vasopressin ([1-{α-(1-mercaptocyclohexyl)acetic acid},8-arginine]vasopressin) was a gift from Professor M. Manning, Medical College of Ohio, Toledo, OH, U.S.A.

Isolation of hepatocytes

Hepatocytes were prepared as described by Wakelam & Walker (1980), except that: (i) collagenase was added to a final concentration of 0.3 mg/ml; (ii) except where otherwise stated, the perfusion medium contained 10 mM-glucose; and (iii) bovine serum albumin (20 mg/ml) was included in the perfusion, isolation and resuspension media for some experiments (see the Results section).

Incubation of hepatocytes and extraction of lipids

Hepatocyte suspensions were usually incubated in bulk with [^{32}P]P_i (10–15 μCi/ml) for 70 min in a shaking incubator under the conditions described by Kirk *et al.* (1981*a*). They were then distributed in 0.5 ml samples to small plastic vials for incubation with hormones, antagonists or other additions. Both the bulk incubations and incubations for more than 2 min in plastic vials were gassed wih O_2/CO_2 (19:1, v/v) and sealed. When the effects of antagonists on hormone responses were being tested then the antagonists were added 3 min before the hormone.

Reactions were terminated with 0.5 ml of cold trichloroacetic acid (20%, w/v) and, when absent from the incubations, 15 mg of bovine serum albumin to facilitate handling of the trichloroacetic acid-precipitated material. The precipitate was washed once with 1 ml of 5% (w/v) trichloroacetic acid containing 1 mM-EDTA and once with 1 ml of water. Lipid extraction was by sequential treatment, twice with 1.5 ml of chloroform/methanol/12 M-HCl (100:100:1, by vol.) and once with 1.0 ml of chloroform/methanol/12 M-HCl (200:100:1, by vol.). The pellet from each extraction was collected by centrifugation. The three extracts were combined, and 1.5 ml of chloroform and 1.1 ml of 0.1 M-HCl were added. After mixing and centrifugation, the lower phase was washed once with new upper phase, collected and dried *in vacuo*. Of the methods tested, this procedure, based on that of Griffin & Hawthorne (1978), gave the largest yields of extracted PtdIns4*P* and PtdIns(4,5)P_2 (see Downes & Michell, 1982*a*).

Deacylation of lipids and separation of glycerophosphoesters

The method was based on that of Ellis *et al.* (1963). The dried lipids were dissolved in 1 ml of chloroform, to which was added 0.2 ml of methanol and 0.2 ml of 1 M-NaOH in methanol/water (19:1, v/v). After 20 min at room temperature 1 ml of chloroform, 0.6 ml of methanol and 0.6 ml of water were added. The samples were mixed thoroughly and centrifuged. Of the upper, aqueous phase 1 ml was removed and neutralized with boric acid. Samples were then diluted to 5 ml, with the addition of ammonium formate and additional sodium tetraborate so as to give final concentrations of 0.18 M and 5 mM respectively. When this deacylation procedure was applied to the lipids of ^{32}P-labelled erythrocyte ghosts (Downes *et al.*, 1982*a*) it converted ⩾99% of the ^{32}P of the lipid extract into a water-soluble form. After desalting and high-voltage electrophoresis (see below) only three radioactive products were detected, and these had the mobilities expected of glycero-3-phosphate (from phosphatidate) and of deacylated PtdIns4*P* and PtdIns(4,5)P_2.

The mixture of deacylated phospholipids was loaded on to a small column (4 cm × 0.6 cm) in a Pasteur pipette of Dowex 1 (X10; 100–200 mesh; formate form) and eluted with 20 ml of 0.18 M-ammonium formate/0.005 M-tetraborate. The combined eluates from loading the column and the application of this first eluant contained the water-soluble deacylation products of glycerolipids other than PtdIns4*P* and PtdIns(4,5)P_2. Glycerophosphoinositol 4-phosphate (from PtdIns4*P*) was next eluted with 20 ml of 0.3 M-ammonium formate/0.1 M-formic acid, and the glycerophosphoinositol

4,5-bisphosphate [from PtdIns(4,5)P_2] with 20ml of 0.75M-ammonium formate/0.1M-formic acid. Columns were regenerated after each experiment by passing through 15ml of 2M-ammonium formate/0.1M-formic acid, followed by 10ml of water, and they were discarded after five experiments.

Eluates were collected directly into scintillation vials and their ^{32}P content measured by Čerenkov counting.

By using these simple methods for extraction and analysis of PtdIns4*P* and PtdIns(4,5)P_2 it was possible routinely to complete an experiment involving 50–60 incubations within 2 days.

Isolation and analysis of cyclohexylamine salts of glycerophosphoinositol 4-phosphate and glycerophosphoinositol 4,5-bisphosphate

When samples were to be used for isolation of these esters, an unlabelled sample of the Folch fraction I/II mixed-inositol-lipid preparation (Folch, 1949) was added before deacylation to act as a carrier for the highly labelled esters from hepatocytes. After elution from the Dowex-1 (formate form) column the eluates were diluted 4-fold with water and applied to 0.2ml columns of Dowex-1 (chloride form). After initial elution with 5ml of 5mM-LiCl, the esters were quantitatively removed with 6ml of 2M-LiCl and a few mg of mannitol were added (as a carrier to prevent the adsorption of the phosphate esters to the vessel walls). After freeze-drying, the LiCl was removed with 4×4ml of ethanol and the salt-free samples dried under vacuum, dissolved in water, adjusted to pH7 with cyclohexylamine and stored frozen.

High-voltage electrophoresis was at pH3.5 and 60V/cm for 75min (Dawson & Clarke, 1972), followed by autoradiography using Kodak Blue brand film.

Analysis of the distribution of ^{32}P in the 4- and 5-phosphate groups of PtdIns(4,5)P_2

Erythrocyte membranes contain an inositol trisphosphate phosphomonoesterase that will selectively remove the 5-phosphate from glycerophosphoinositol 4,5-bisphosphate (Downes *et al.*, 1982*a*). This enzyme is stable to freezing and active in 0.1M-ammonium formate (Downes *et al.*, 1982*b*). At low substrate concentrations ($\leqslant 1\mu M$) its attack upon glycerophosphoinositol 4,5-bisphosphate displays first-order kinetics and can be used to assay the proportion of the total radioactivity that is present in the 5-phosphate.

Column eluants containing glycerophosphoinositol 4,5-bisphosphate were collected, diluted to a final concentration of 0.1M-ammonium formate and neutralized. Incubation was with 30mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH7.4, 2mM-$MgCl_2$ and erythrocyte ghosts (0.9mg of protein) in a final volume of 5ml. After incubation for 75min, which under these conditions gave approx. 50–60% hydrolysis, the released radioactive P_i (5-phosphate) was assayed as described by Downes *et al.* (1982*b*). Calibration was achieved by simultaneously measuring attack upon a sample of labelled glycerophosphoinositol 4,5-bisphosphate from erythrocytes (Downes *et al.*, 1982*b*), followed by separation and analysis of the released glycerophosphoinositol 4-phosphate (4-phosphate) and P_i (5-phosphate).

Activation of glycogen phosphorylase

This was assayed as described by Kirk *et al.* (1979) using hepatocytes that had been isolated in glucose-free conditions and then pre-incubated for 40min in the presence of 10mM-glucose.

Results

Extraction of polyphosphoinositides

In the past, various methods have been used for extracting polyphosphoinositides from tissues and cells, amongst them neutral solvents in the presence of high concentrations of salt (e.g. Garbus *et al.*, 1963; Michell *et al.*, 1970), hot aqueous ethanol (Hanson & Lester, 1980) and various acidic solvent mixtures (e.g. Yagihara *et al.*, 1972; Griffin & Hawthorne, 1978). The results obtained with different cells have varied in unexplained ways (see, for example, Michell *et al.*, 1970; Hanson & Lester, 1980): for a review, see Downes & Michell (1982*b*). When we tested these three techniques on ^{32}P-labelled hepatocytes it was clear that they all extracted PtdIns4*P* from liver cells with approximately equal facility but that only the acidified solvent achieved a substantial extraction of PtdIns(4,5)P_2 (Downes & Michell, 1982*a*).

This procedure was therefore adopted as it gave both the highest and the most consistent value for PtdIns4*P* and PtdIns(4,5)P_2 labelling. We must emphasize, however, that we do not know whether this procedure routinely extracts all of the polyphosphoinositides or simply a very constant proportion of these lipids. In this regard, it should be remembered that in liver a small quantity of inositol, of unknown chemical nature, remains associated with the tissue residue even after exhaustive extraction with acidified chloroform/methanol (Michell *et al.*, 1970).

Identification of labelled lipids

When liver cells were incubated for 70min with [^{32}P]P_i, about 20% of the total radioactivity that was incorporated into lipids was found in molecules that, after deacylation, were eluted from columns of Dowex-1 anion-exchange resin (see the Materials and methods section) together with unlabelled

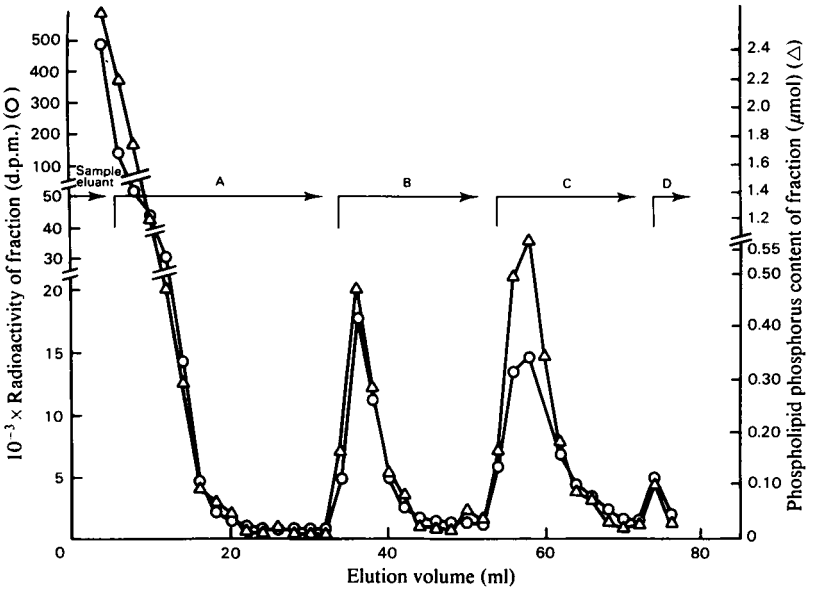

Fig. 1. *Chromatographic separation of glycerophosphoinositol esters derived from hepatic lipids and Folch fraction I/II*
Hepatocytes were incubated with [^{32}P]P_i for 70min and lipids were extracted from 0.25ml of packed cells as described in the text. A small sample of Folch fraction I/II (Folch, 1949) was added to lipid samples which were then deacylated. The water-soluble products thus obtained were applied to a Dowex 1 (formate form) column and eluted in 2ml fractions as described in the Materials and methods section. Fractions were assayed for radioactivity (O) and total phosphorus (△). Elution conditions: A, 5mM-$Na_2B_4O_7$/0.18M-ammonium formate; B, 0.1M-formic acid/ 0.3M-ammonium formate; C, 0.1M-formic acid/ 0.75M-ammonium formate; D, 0.1M-formic acid/2.0M-ammonium formate.

glycerophosphoinositol 4-phosphate (deacylated PtdIns4*P*) and glycerophosphoinositol 4,5-bisphosphate [deacylated PtdIns(4,5)P_2] derived from a Folch fraction I/II lipid mixture (Fig. 1).

When the esters eluted from these resin columns were isolated and subjected to high-voltage electrophoresis (see the Materials and methods section) more than 90% of the radioactivity in the 'glycerophosphoinositol 4,5-bisphosphate' fraction migrated as a single component with the appropriate mobility. In addition, the rate constant for the first-order degradation of this compound by the inositol 1,4,5-trisphosphate 5-phosphomonoesterase of the erythrocyte membrane (Downes *et al.*, 1982*a*,*b*) was identical with that for the simultaneous degradation of glycerophosphoinositol 4,5-bisphosphate derived from the PtdIns(4,5)P_2 of erythrocytes (results not shown).

Approx. 80% of the ^{32}P in the isolated 'glycerophosphoinositol 4-phosphate' fractions was in a single component of the appropriate mobility; the contaminants in this fraction were not identified. In general the changes that were observed in PtdIns4*P* labelling were similar to the changes in PtdIns(4,5)P_2 labelling, though they were often of somewhat smaller magnitude (see below).

Equilibrium labelling of PtdIns4P and PtdIns(4,5)P_2

The concentrations of PtdIns4*P* and PtdIns(4,5)P_2 in liver are much lower than that of PtdIns. Measurements by Michell *et al.* (1970) suggest that each may constitute 1–2% of the total inositol lipids in whole liver. Such concentrations are too low for reliable assay by available chemical assay methods, and so we have assayed changes in the steady-state labelling of these lipids in hepatocytes in order to provide fairly reliable estimates of changes in their concentrations.

In hepatocytes, the γ-phosphate of ATP reaches isotopic equilibrium with extracellular [^{32}P]P_i in 30–60min (Garrison *et al.*, 1979; P. T. Hawkins, unpublished work). Similarly, the labelling of PtdIns4*P* and PtdIns(4,5)P_2 approaches a constant value in about 70min. This suggests rapid labelling of the monoester phosphates to equilibrium with the γ-phosphate of ATP, with very little ^{32}P in the diester phosphate derived from PtdIns. This was tested by treatment of the isolated glycerophosphoinositol 4,5-bisphosphate with alkaline phosphatase; this treatment released >99% of its radioactivity as [^{32}P]P_i.

This being the case, the total radioactivities of PtdIns4*P* and PtdIns(4,5)P_2 after 70min incubation

of cells with [^{32}P]P_i will give a guide to their relative concentrations [remembering that PtdIns4*P* and PtdIns(4,5)P_2 will have incorporated, respectively, one and two molecules of labelled phosphate]. In most cell preparations the labelling of PtdIns(4,5)P_2 after 70min was almost twice that of PtdIns4*P*, suggesting that PtdIns4*P* and PtdIns(4,5)P_2 are present at very similar concentrations.

Effects of receptor-directed stimuli

Cells incubated with [^{32}P]P_i for 70min were briefly exposed to various agonists that stimulate hepatocytes via cell-surface receptors. These were vasopressin, angiotensin, an α_1-adrenergic stimulus (adrenaline in the presence of pindolol or propranolol to block β-receptors and sometimes yohimbine to block α_2-receptors), ATP, glucagon, insulin and prolactin. The former four stimuli all mobilize Ca^{2+} in liver cells (Kirk *et al.*, 1980; Exton, 1981; Williamson *et al.*, 1981; Burgess *et al.*, 1981), glucagon activates adenylate cyclase and insulin and prolactin act by unknown mechanisms.

The four Ca^{2+}-mobilizing ligands all caused a significant decrease in the radioactivity of PtdIns4*P* and PtdIns(4,5)P_2 within 1min (Table 1), whereas 1μM-glucagon, 1μM-insulin and 2ng of prolactin/ml were without significant effect in such brief incubations. It therefore seems that a very rapid decrease in the concentrations of PtdIns4*P* and PtdIns(4,5)P_2 is, like the rapid and extensively studied depletion of PtdIns produced by these agonists, a characteristic cellular response to ligands that raise cytosol Ca^{2+} concentrations and not to hormones with other mechanisms of action.

Responses to four separate Ca^{2+}-mobilizing receptors?

It has previously been shown that the PtdIns labelling and 'breakdown' responses of rat hepatocytes to angiotensin, vasopressin and catecholamines are mediated by separate angiotensin, V_1-vasopressin and α_1-adrenergic receptors (Kirk *et al.*, 1977, 1979, 1981*a*; Billah & Michell, 1979; Tolbert *et al.*, 1980). We have therefore done simple pharmacological experiments to check that this is also true of the rapid receptor-controlled depletion of PtdIns(4,5)P_2. The actions of vasopressin at Ca^{2+}-mobilizing V_1-receptors, but not at adenylate cyclase-coupled V_2-receptors, can be antagonized by [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid),8-arginine]vasopressin (Kruszynski *et al.*, 1980; Kirk *et al.*, 1981*a*; Takhar & Kirk, 1981). This compound partially inhibited the vasopressin-stimulated depletion of PtdIns(4,5)P_2, but had no effect on the response to angiotensin (Table 2).

Receptor-mediated effects of angiotensin, including stimulation of PtdIns metabolism (Billah & Michell, 1979), are susceptible to blockade by [1-sarcosine,8-isoleucine]angiotensin. This compound also partially inhibited the angiotensin-stimu-

Table 1. *The influence of various agonists upon polyphosphoinositide concentrations in hepatocytes*

Hepatocytes were incubated with [^{32}P]P_i for 70min before the addition of agonists. Incubations were terminated 60s after agonist addition and the radioactivity of the polyphosphoinositides was determined. Results are means ± S.E.M. for the numbers of separate hepatocyte preparations shown in parentheses. **P* versus agonist-free controls < 0.01.

Agonist	^{32}P radioactivity remaining in polyphosphoinositides (% of control)	
	PtdIns4*P*	PtdIns(4,5)P_2
[Arg^8]Vasopressin (0.23μM)	89.3 ± 1.6 (23)*	76.0 ± 1.5 (23)*
Angiotensin (1μM)	87.6 ± 1.4 (17)*	81.4 ± 1.4 (17)*
Adrenaline (30μM), propranolol (1μM) and yohimbine (1μM)	98.4 ± 1.2 (8)	88.0 ± 1.3 (8)*
ATP (100μM)	93.2 ± 1.3 (8)*	88.3 ± 1.0 (8)*
Glucagon (1μM)	101.6 ± 3.0 (3)	101.4 ± 1.4 (3)
Insulin (0.1μM)	100.5 ± 3.0 (3)	100.0 ± 2.8 (3)

Table 2. *Pharmacology of hormone-stimulated PtdIns(4,5)P_2 depletion in hepatocytes*

Incubations were performed as described in the legend to Table 1, except that, where appropriate, antagonists were added 3min before the addition of hormones. Results are means ± S.E.M. for the numbers of separate hepatocyte preparations shown in parentheses. *P* versus the value in the absence of antagonist: *, < 0.01; †, < 0.02; ‡, < 0.05 (paired *t*-test).

Further additions	^{32}P radioactivity remaining in PtdIns(4,5)P_2 (% of control) in the presence of:		
	Vassopressin (0.23μM)	Angiotensin (1μM)	Adrenaline (30μM)
None	83.1 ± 1.7 (5)	83.2 ± 1.7 (4)	86.1 ± 2.1 (6)
[1-(β-mercapto-β,β-cyclopentamethylene-propionic acid),8-arginine]Vasopressin (4μM)	91.7 ± 1.6 (5)*	82.2 ± 1.5 (4)	—
[1-sarcosine,8-isoleucine]Angiotensin (10μM)	83.2 ± 0.5 (3)	92.1 ± 1.4 (4)‡	—
Prazosin (1μM)	81.0 ± 4.4 (3)	84.4 ± 2.4 (3)	96.5 ± 2.7 (6)†
Proprandol (1μM)	—	—	89.1 ± 1.6 (4)
Yohimbine (1μM)	—	—	84.0 ± 4.2 (3)

lated depletion of PtdIns(4,5)P_2 but failed to modify the response to vasopressin (Table 2). Although this confirms that PtdIns(4,5)P_2 disappearance is a response to activation of specific angiotensin receptors it does not establish that these are the angiotensin receptors that control Ca^{2+}-mobilization. This is because angiotensin, unlike vasopressin, both stimulates Ca^{2+} mobilization (Keppens *et al.*, 1977) and causes inhibition of adenylate cyclases in rat hepatocytes (Jard *et al.*, 1981).

Responses of liver cells to catecholamine receptors are even more complex, since rat hepatocytes possess β-adrenergic receptors whose stimulation activates adenylate cyclase, α_1-adrenergic receptors that stimulate PtdIns metabolism (De Torrentegui & Berthet, 1966; Kirk *et al.*, 1977; Tolbert *et al.*, 1980) and mobilize Ca^{2+}, and α_2-adrenergic receptors that cause inhibition of adenylate cyclase (for review, see Exton, 1981). Our data (see Table 3) show no significant blockade of the PtdIns(4,5)P_2 depletion response by propranolol (a β-antagonist) or yohimbine (an α_2-antagonist), but substantial inhibition by prazosin (an α_1-antagonist). These results are compatible with the view that the adrenergic receptor responsible for the adrenaline-stimulated decrease in PtdIns(4,5)P_2 concentration is of the α_1-type, i.e. of the type that causes mobilization of Ca^{2+}. Prazosin, the effective α_1-antagonist, did not change the responses to vasopressin or angiotensin.

Characteristics of vasopressin-stimulated depletion of PtdIns(4,5)P_2

Of the three well-characterized stimuli that evoke an increased rate of inositol lipid metabolism in hepatocytes, vasopressin is, for two reasons, the most easily investigated. First, it gives the largest stimulation of PtdIns labelling and 'breakdown' (Billah & Michell, 1979; Kirk *et al.*, 1981*a*) and of PtdIns(4,5)P_2 depletion (Table 1). Secondly, it seems, in contrast with angiotensin and adrenaline, to evoke all of its intracellular responses in the liver as a result of the mobilization of Ca^{2+} by a single class of receptors. We therefore chose initially to study this PtdIns(4,5)P_2 breakdown response in greatest detail, since it seemed that it would provide the most easily interpretable results.

Time course. Fig. 2 shows the time course of the decrease in PtdIns4*P* and PtdIns(4,5)P_2 concentrations in response to a concentration of vasopressin that is sufficient to fully occupy the V_1-receptors of hepatocytes (see Cantau *et al.*, 1980). If we start by considering the effects on PtdIns(4,5)P_2 then two notable features emerge. First, this response is initiated rapidly and its initial rate, sustained for approx. 30s, represents disappearance of approx. 0.7% of the PtdIns(4,5)P_2 per s. Note, however, that if the rate of PtdIns(4,5)P_2 synthesis matches a substantial fraction of the rate at which it disappears then this apparent rate of breakdown will significantly underestimate the true rate of depletion of PtdIns(4,5)P_2.

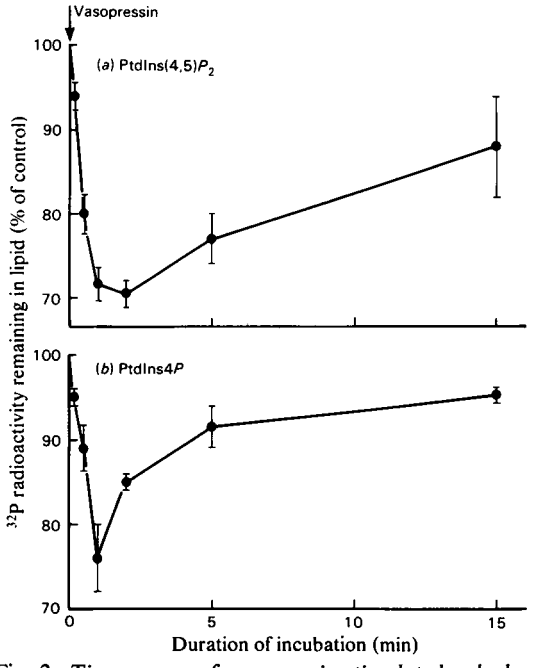

Fig. 2. *Time course of vasopressin-stimulated polyphosphoinositide depletion in hepatocytes*
Hepatocytes were incubated with [^{32}P]P_i for 70min before the addition of vasopressin. Incubations were terminated at the times indicated and the radioactivity present in (*a*) PtdIns(4,5)P_2 and (*b*) PtdIns4*P* was determined. Results are means ± S.E.M. for four to six separate hepatocyte preparations.

Secondly, the rapid loss of radioactivity that occurs during the first minute or so of stimulation is followed by a much slower recovery of the PtdIns(4,5)P_2 labelling towards its initial level. It therefore seems that vasopressin-stimulated breakdown is transient, and that it is rapidly followed by a resynthesis of PtdIns(4,5)P_2 to pre-stimulation levels. This may be related to the rapid receptor desensitization that occurs when hepatocytes are continuously exposed to high concentrations of vasopressin (Bréant *et al.*, 1981; Kirk & Creba, 1982).

The data for PtdIns4*P* are quite similar to those for PtdIns(4,5)P_2 (Fig. 2). In particular, they show a rapid decline in radioactivity followed by a return to control levels. This appears to eliminate one possible route for the disappearance of radioactivity from PtdIns(4,5)P_2, namely its conversion into PtdIns4*P* by the 5-phosphate-specific PtdIns(4,5)P_2 phosphomonoesterase (see, for example, Roach & Palmer,

1981). If this reaction was stimulated then it would lead to a rapid increase in PtdIns4*P* labelling, i.e. to the opposite of the observed result. Other possible routes of breakdown are considered in the Discussion section.

Dose–response relationships. Fig. 3 shows the dose–response curve for vasopressin-stimulated depletion of PtdIns(4,5)P_2. These data represent measurements made over 30s and will be close to measurements of the initial rate of change from the previous steady-state concentration of this lipid. They are plotted together with data on the activation of glycogen phosphorylase and with previously published data on the disappearance of ^{3}H-labelled PtdIns (Kirk *et al.*, 1981*a*) and the binding of [8-lysine]vasopressin to V_1-receptors in intact hepatocytes (Cantau *et al.*, 1980). It is clear that maximum stimulation of the depletion of PtdIns(4,5)P_2 requires occupation by agonist of all of the V_1-vasopressin receptors, as did the disappearance of ^{3}H-labelled inositol from prelabelled hepatocytes in our previous experiments (Kirk *et al.*, 1981*a*). In contrast is the ability of much smaller concentrations of vasopressin to evoke a maximum Ca^{2+}-mediated activation of glycogen phosphorylase.

Ca^{2+} and the control of polyphosphoinositide breakdown. The similarity between the PtdIns(4,5)P_2-depletion dose–response curve and the receptor-occupation curve, as contrasted with the large 'receptor reserve' for activation of phosphorylase, can be simply interpreted in either of two ways. In the first alternative, it is envisaged that the modes of control of the two types of response are fundamentally similar, with both being controlled by the rise in cytosol Ca^{2+} concentration that is brought about by activating vasopressin receptors. If this were to be correct then it is clear from the relative positions of the dose–response curves that a much greater rise in cytosol Ca^{2+} would be needed to activate inositol lipid breakdown than to activate phosphorylase kinase; the arguments in support of this view have been developed in detail previously (Jafferji & Michell, 1976; Michell & Kirk, 1981*a*; Michell *et al.*, 1981). In the second interpretation, a direct coupling at the plasma membrane between activated receptors and polyphosphoinositide breakdown would be envisaged.

We have approached this question in two ways; by facilitating Ca^{2+} entry using the ionophore A23187 and by Ca^{2+}-deprivation of cells. Only modest concentrations of A23187 need to be added to hepatocyte suspensions in order to admit sufficient Ca^{2+} to bring about a rapid activation of glycogen phosphorylase (Keppens *et al.*, 1977). But if the depletion of PtdIns4*P* and PtdIns(4,5)P_2 were to be Ca^{2+}-triggered then, as noted above, it seems certain that it would require much more Ca^{2+} for its activation to occur. Neither a much higher concentration of A23187 nor ethanol, the solvent in which it was added, caused any diminution in the labelling of the polyphosphoinositides (Table 3). In previous experiments, reported briefly at a meeting (Creba *et al.*, 1981), we mistakenly thought that A23187 treatment caused loss of label from polyphosphoinositides. However, this was an effect of the addition of small quantities of dimethyl sulphoxide, the solvent in which A23187 was dissolved in those experiments (Table 3). At present, we have no further information on the characteristics of this effect of dimethyl sulphoxide.

When liver cells are deprived of Ca^{2+}, either by omitting Ca^{2+} from the extracellular medium or, more severely, by the addition of extracellular EGTA, then the Ca^{2+}-mediated rise in phosphorylase activity in vasopressin-stimulated cells rapidly declines or disappears (Whitton *et al.*, 1977). If the diminution of PtdIns4*P* and PtdIns(4,5)P_2 concentrations were to be mediated by a relatively large

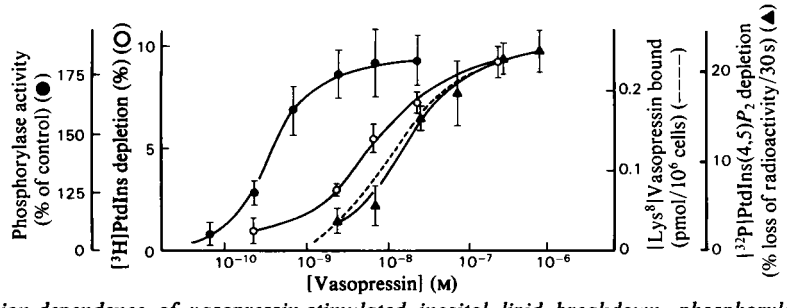

Fig. 3. *Concentration-dependence of vasopressin-stimulated inositol lipid breakdown, phosphorylase activation and receptor occupation in hepatocytes*

Hepatocytes were prepared and incubated as described in the legend to Fig. 2 or, when phosphorylase *a* activity was measured, as described by Kirk *et al.* (1979). Phosphorylase *a* activity (●) and [^{32}P]PtdIns(4,5)P_2 depletion (▲) were assessed 60s and 30s after the addition of vasopressin respectively. Results are means ± S.E.M. for three to six separate hepatocyte preparations. Sources of other data are: PtdIns depletion (○), Kirk *et al.* (1981*a*); vasopressin binding (----), Cantau *et al.* (1980), with permission of Marcel Dekker, New York).

rise in cytosol [Ca^{2+}], as proposed in the first of our possible interpretations, then this response should be much more sensitive to Ca^{2+}-deprivation than is the activation of phosphorylase. Table 4 shows that this was not the case. The magnitude of stimulated PtdIns(4,5)P_2 depletion was reduced by 50% in the presence of EGTA, but a highly significant decrease in the concentration of this lipid was still seen even after 15 min of Ca^{2+}-deprivation. In similar experiments, the increment in phosphorylase activity provoked by vasopressin was eliminated in the presence of EGTA.

These experiments appear to rule out the possibility that vasopressin-stimulated depletion of PtdIns4*P* and PtdIns(4,5)P_2 is simply a consequence of the rise in cytosol Ca^{2+} that is brought about by vasopressin treatment. However, the reduction, but not the loss, of the response during Ca^{2+} deprivation leaves open the possibility that the enzyme(s) responsible for the disappearance of these lipids may have a requirement for Ca^{2+} (see Michell *et al.*, 1981).

Possible inhibitors. It would be invaluable, in future studies, if a specific inhibitor of the reaction responsible for disappearance of the polyphosphoinositides was available, since any such inhibitor would be expected to abolish all cell functions in which this process plays an essential role. Several compounds have in the past been reported to inhibit one or other of the various events involved in stimulated inositol lipid metabolism, and we have looked for effects of these on vasopressin-stimulated depletion of polyphosphoinositides.

It has long been known that an elevation of cytosol cyclic GMP concentration frequently occurs in stimulated cells in which the cytosol [Ca^{2+}] is elevated (Berridge, 1975; Goldberg & Haddox, 1977), but cyclic GMP does not appear to be a primary intracellular mediator of hormone action.

Table 3. *Influence of ionophore A23187, ethanol and dimethyl sulphoxide on hepatocyte polyphosphoinositide concentrations*

Hepatocytes were incubated as described in the legend to Table 1. Radioactivity in polyphosphoinositides was assessed in incubations terminated 60 s after appropriate additions had been made. Results are means ± S.E.M. for the numbers of separate hepatocyte preparations shown in parentheses. **P* versus control incubations, < 0.01 (paired *t*-test).

Additions	^{32}P radioactivity remaining in polyphosphoinositides (% of control)	
	PtdIns4*P*	PtdIns(4,5)P_2
A23187 (20 μM)	105.6 ± 3.5 (3)	101.7 ± 0.9 (3)
Ethanol (2%, v/v)	101.3 ± 3.2 (3)	101.0 ± 3.0 (3)
Dimethyl sulphoxide (2%, v/v)	103.0 ± 0.7 (3)	79.4 ± 0.3 (3)*

Table 4. *The Ca^{2+}-sensitivity of vasopressin-stimulated PtdIns(4,5)P_2 depletion and phosphorylase activation in hepatocytes*

Hepatocytes were incubated in the presence of 1.3 mM-Ca^{2+} as described in the legend to Table 1 or, when phosphorylase activity was to be measured, as described by Kirk *et al.* (1979). Phosphorylase activity and [^{32}P]-PtdIns(4,5)P_2 depletion were assessed 30 s and 60 s after the addition of hormone respectively. These parameters were measured in hepatocytes incubated in Ca^{2+}-containing medium (1) and after the further treatments described in the Table (2 and 3). Results are means ± S.E.M. for four separate hepatocyte preparations. The washing media employed in conditions (2) and (3) did not contain ^{32}P and thus the ^{32}P radioactivity associated with PtdIns(4,5)P_2 diminished during the washing and resuspension procedures described above: the control values at stages 2 and 3 were, respectively, 70% ± 1% and 59% ± 4% of the values at stage 1. Hence it is not appropriate to compare the radioactivity of this lipid between the different incubation conditions, and the control radioactivity in PtdIns(4,5)P_2 for each condition has been designated at 100%. Statistical significance for [^{32}P]PtdIns(4,5)P_2 determinations was assessed by a paired Student's *t*-test. An unpaired Student's *t*-test was used with the measurements of phosphorylase activity. Abbreviation used: N.S., not significant.

		Radioactivity in [^{32}P]PtdIns(4,5)P_2		Phosphorylase activity				
					Significance of difference from:			
Incubation conditions	[Vasopressin]	(% of control)	Significance of difference from paired control	(μmol/min per g dry wt.)	Paired control	1*a*	1*b*	2*b*
(1) 1.3 mM-Ca^{2+}	(*a*) —	100		23.0 ± 2.2				
	(*b*) 0.23 mM	72 ± 2.9	*P* < 0.01	39.7 ± 5.0	*P* < 0.01			
(2) As (1) then washed Ca^{2+}-free + 0.2 mM-EGTA for 15 min before challenge	(*a*) —	100		10.0 ± 1.4		*P* < 0.01		
	(*b*) 0.23 μM	86 ± 2.4	*P* < 0.01	12.5 ± 1.5	N.S.		*P* < 0.01	
(3) As (2) then 1.5 mM-Ca^{2+} for 15 min before challenge	(*a*) —	100		16.0 ± 2.0		N.S.		
	(*b*) 0.23 μM	86 ± 3.4	*P* < 0.01	27.6 ± 3.7	*P* < 0.05		N.S.	*P* < 0.01

Table 5. *Effects of 8-bromo cyclic GMP, delthexane and cycloheximide on vasopressin-stimulated depletion of PtdIns4P and PtdIns(4,5)P_2*

Hepatocytes were incubated with [^{32}P]P_i and stimulated with vasopressin for 1 min as described in the legend to Table 1. Results are means ± S.E.M. (for the numbers of incubations from individual experiments shown in parentheses. Cells were pre-incubated with potential inhibitors for: (*a*) 6 min; (*b*) 1 min; (*c*) 20 min. Essentially similar results were obtained with 1 mM-8-bromo cyclic GMP and 1 min of pre-incubation, with 50 μM- or 100 μM-delthexane (1 min or 10 min pre-incubation), and with 0.1 mM-cycloheximide and 3 min of pre-incubation. Abbreviation used: n.d., not determined.

	Radioactivity (% of control)	
	PtdIns4*P*	PtdIns(4,5)P_2
Expt. (*a*)		
230 nM-Vasopressin	86.0 ± 2.9 (4)	70.8 ± 2.9 (4)
1 mM-8-Bromo cyclic GMP	106.0 ± 4.1 (5)	99.9 ± 3.4 (4)
1 mM-8-Bromo cyclic GMP and 230 nM-vasopressin	92.7 ± 3.9 (5)	78.1 ± 6.1 (5)
Expt. (*b*)		
230 nM-Vasopressin	n.d.	79.2 ± 2.8 (3)
500 μM-Delthexane	n.d.	96.8 ± 10.9 (3)
500 μM-Delthexane and 230 nM-vasopressin	n.d.	77.9 ± 5.0 (3)
Expt. (*c*)		
230 nM-Vasopressin	88.6 ± 1.7 (3)	81.0 ± 0.8 (4)
0.1 mM-Cycloheximide	99.9 ± 2.1 (4)	97.9 ± 5.3 (3)
0.1 mM-Cycloheximide and 230 nM-vasopressin	91.4 ± 1.7 (4)	71.9 ± 2.0 (4)

More recently it has been suggested that cyclic GMP plays a feedback role, countering the actions of Ca^{2+}-mobilization in cells (Schultz *et al.*, 1977; Mellian *et al.*, 1981). In particular, Nishizuka and his colleagues have provided evidence that the ability of cyclic GMP to inhibit platelet aggregation might be a result of inhibition by cyclic GMP of the thrombin-stimulated formation of diacylglycerol from inositol lipids (Takai *et al.*, 1981). However, in the present study neither 1 mM-8-bromo cyclic GMP (Table 5) nor 1 mM-dibutyryl cyclic GMP (results not shown) had any effect on polyphosphoinositide depletion in vasopressin-stimulated liver cells.

Both γ-hexachlorocyclohexane (gammexane) and δ-hexachlorocyclohexane (delthexane) have been reported to block receptor-stimulated inositol lipid metabolism and subsequent cell responses (Hokin & Brown, 1969; Fisher & Mueller, 1971; Hoffman *et al.*, 1980). The fullest published data were obtained with delthexane, in which the disposition of chlorides on the cyclohexane ring is identical with the disposition of hydroxy groups in *myo*-inositol. In our experiments neither 0.05–0.5 mM-delthexane nor ethanol, the solvent in which it was added, had any effect on vasopressin-stimulated diminution of polyphosphoinositide concentrations in hepatocytes (Table 5).

In an extensive recent series of papers, Farese and his colleagues have reported striking effects of corticotropin, angiotensin and other hormones upon polyphosphoinositide metabolism in steroidogenic tissues such as adrenal cortex. A very important aspect of these experiments was the rapid blockade of these effects by cycloheximide, suggesting that they require the participation of a protein with a turnover time of only a few minutes (e.g. Farese *et al.*, 1980, 1981). Pre-incubation of liver cells with 0.1 mM-cycloheximide for 3 or 20 min had no effect upon vasopressin-stimulated polyphosphoinositide depletion (Table 5).

Characteristics of angiotensin-stimulated PtdIns(4,5)P_2 breakdown

Fig. 4 shows the time course of angiotensin-stimulated polyphosphoinositide breakdown, and Fig. 5 the dose–response curves for activation by angiotensin of glycogen phosphorylase and of PtdIns(4,5)P_2 depletion. The time course is very similar to that observed with vasopressin (Fig. 2), except that angiotensin usually evoked a slightly smaller rate and extent of polyphosphoinositide depletion. Again this was followed by a relatively rapid return to control levels of the lipids. Another similarity to the vasopressin data is seen in the dose–response curves, where half-maximal activation of glycogen phosphorylase required very much less angiotensin than did half-maximal diminution of polyphosphoinositide concentrations. The concentration of angiotensin required to provoke the latter effect (60 nM) is similar to that reported to cause 50% occupation of hepatocyte angiotensin receptors (30 nM; Keppens *et al.*, 1982).

The polyphosphoinositide depletion in response to angiotensin was not mimicked by A23187 (Table 3) and was reduced, but not abolished, by Ca^{2+} deprivation of the cells (results not shown). By using the same arguments as were applied above to the data obtained with vasopressin, we conclude that the

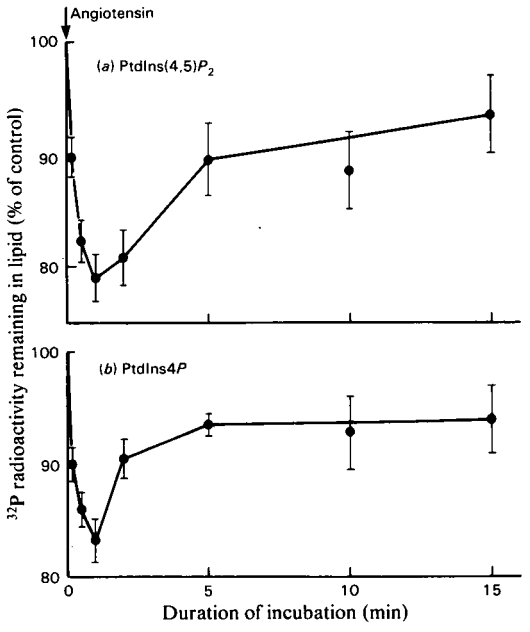

Fig. 4. *Time course of angiotensin-stimulated polyphosphoinositide depletion in hepatocytes*

Hepatocytes were incubated with [^{32}P]P_i for 70min before the addition of 1μM-angiotensin. Incubations were terminated and the radioactivity present in (*a*) PtdIns(4,5)P_2 and (*b*) PtdIns4P was determined. Results are means ± S.E.M. for four to six separate hepatocyte preparations.

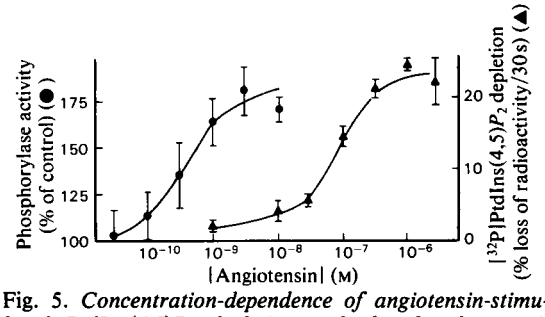

Fig. 5. *Concentration-dependence of angiotensin-stimulated PtdIns(4,5)P_2 depletion and phosphorylase activation in hepatocytes*

Hepatocytes were prepared and incubated as described in the legend to Fig. 4 or, when phosphorylase *a* activity was to be measured, as described by Kirk *et al.* (1979). Phosphorylase *a* activity (●) and [^{32}P]PtdIns(4,5)P_2 depletion (▲) were assessed 60s and 30s after the addition of angiotensin respectively. Results are means ± S.E.M. for three to six separate hepatocyte preparations.

angiotensin-stimulated diminution of PtdIns4P and PtdIns(4,5)P_2 concentrations is not controlled by hormone-induced changes in cytosolic Ca^{2+} concentration.

Discussion

When these studies were initiated it appeared that the metabolism of polyphosphoinositides was subject to few very rapid perturbations by hormones or neurotransmitters (for review, see Downes & Michell, 1982*a*). However, it has since become apparent that this was an incorrect view; several stimuli provoke very rapid breakdown and/or turnover of PtdIns(4,5)P_2, and maybe also PtdIns4P, in a variety of cells (Abdel-Latif *et al.*, 1977, 1978; Kirk *et al.*, 1981*b*; Michell *et al.*, 1981; Downes & Michell, 1982*a*; Creba & Kirk, 1982; Kirk & Creba, 1982; Soukup & Schanberg, 1982; Billah & Lapetina, 1982; Weiss *et al.*, 1982; Kirk, 1982; Berridge, 1982; Putney, 1982; Berridge *et al.*, 1983; Putney *et al.*, 1983). The effective ligands are those that both mobilize Ca^{2+} in the cytosol of stimulated cells and cause the disappearance of some fraction of the cell's PtdIns (previously assumed to indicate its breakdown).

The effects of Ca^{2+}-mobilizing stimuli upon polyphosphoinositide metabolism

Rapid effects of Ca^{2+}-mobilizing stimuli upon the metabolism of polyphosphoinositides have been reported a number of times in the past, but have not been readily interpretable. This has usually been because the studies did not employ conditions of close-to-equilibrium labelling, so making it impossible to distinguish between control of synthesis and of breakdown (for a review, see Downes & Michell, 1982*a*).

The only previous studies that have characterized the first event as a breakdown of one or both of the polyphosphoinositides employed either iris muscle that has been incubated with 2-deoxyglucose to reduce its ATP concentration (Abdel-Latif *et al.*, 1977, 1978; Akhtar & Abdel-Latif, 1980) or cells loaded with unphysiologically large quantities of Ca^{2+} by the use of the ionophore A23187 (Lang *et al.*, 1977; Griffin & Hawthorne, 1978; Allan & Thomas, 1981); these studies have been reviewed by Downes & Michell (1982*a*). We believe that the studies reported here, which demonstrate a very rapid hormonal stimulation of the disappearance of PtdIns(4,5)P_2 and PtdIns4P that is not mediated by a rise in cytosol Ca^{2+} concentration, have revealed a process that is fundamentally different from these unphysiological events. This view is in accord with the recent studies of Downes & Michell (1982*b*), who concluded that normal physiological fluctuations in cytosol Ca^{2+} concentrations do not influence the polyphosphoinositide phosphodiesterase of isolated erythrocyte plasma membranes.

Another conclusion from our experiments is that each of the ligands we studied probably acts at a different type of Ca^{2+}-mobilizing receptor; these are

V_1-vasopressin receptors, α_1-adrenergic receptors, saralasin-sensitive angiotensin receptors and ATP receptors. We have not observed stimulation of the breakdown of the polyphosphoinositides with hormones that do not utilize Ca^{2+} as their main intracellular signal. As with the previously reported disappearance of PtdIns from stimulated liver cells (Billah & Michell, 1979; Kirk *et al.*, 1981*a*; Lin & Fain, 1981; Takenawa *et al.*, 1982), maximal rates of depletion of PtdIns4*P* and PtdIns(4,5)P_2 occur only at high ligand concentrations that are able fully to occupy the cells' receptor populations.

Is polyphosphoinositide breakdown a general response to Ca^{2+}-mobilizing stimuli?

It seems, therefore, that the well-known stimulation of PtdIns metabolism by Ca^{2+}-mobilizing hormones (for reviews, see Michell *et al.*, 1981; Michell & Kirk, 1981*a*; Berridge, 1980, 1981; Irvine *et al.*, 1982) is accompanied by even more dramatic changes in the metabolism of polyphosphoinositides, at least in liver cells (Kirk *et al.*, 1981*b*; Michell *et al.*, 1981, 1982; and results herein). It is now becoming apparent that such rapid changes in polyphosphoinositide metabolism are a frequent, and maybe ubiquitous, accompaniment to the long-studied changes in PtdIns metabolism. In addition to the various tissues noted above that show uncharacterized changes in polyphosphoinositide metabolism on stimulation, very recent studies have shown that rapid receptor-stimulated disappearance of these lipids is a widespread response of healthy cells to Ca^{2+}-mobilizing stimuli. Effective stimuli are muscarinic cholinergic, substance P or α-adrenergic on parotid cells (Weiss *et al.*, 1982; Putney, 1982), 5-hydroxytryptamine on blowfly salivary gland (Berridge, 1982; Berridge *et al.*, 1983), acetylcholine or pancreozymin on exocrine pancreas (Putney *et al.*, 1983) and thrombin on platelets (Billah & Lapetina, 1982, 1983). In each of these tissues, this response seems to occur normally without the intervention of any change in cytosol Ca^{2+} concentration: this was demonstrated particularly elegantly by Weiss *et al.* (1982).

The relationship between stimulation of polyphosphoinositide disappearance and stimulation of PtdIns metabolism

So what is likely to be the relationship between all of these different processes [receptor activation, disappearance of PtdIns, PtdIns4*P* and PtdIns(4,5)P_2 and mobilization of Ca^{2+} in the cytosol] that accompany one another in so many different stimulated cells?

First, how do the characteristics of the polyphosphoinositides in liver compare with those of PtdIns, their metabolic precursor? PtdIns is present in liver tissue and in isolated liver cells in quite substantial quantities: approx. 1.7 μmol per g of tissue in liver (Michell *et al.*, 1970) and approx. 2 μmol per ml of cells in isolated hepatocytes (based on 20 μmol per g of cell protein; Kirk *et al.*, 1981*a*). PtdIns is present in appreciable quantities in all cell organelles. Polyphosphoinositides were originally thought to be components only of nervous tissue, but were then found in trace quantities in other tissues (for references, see Michell, 1975; Downes & Michell, 1982*a*). Early studies established the presence of trace quantities of both PtdIns4*P* and PtdIns(4,5)P_2 in liver (Hölzl & Wagner, 1964; Kfoury & Kerr, 1964; Santiago-Calvo *et al.*, 1964), but the only published report that might offer a good guide to their concentrations is that of Michell *et al.* (1970). These authors found, by measuring either acid-extractable lipid inositol or the inositol phosphates released by alkaline hydrolysis, evidence for the presence of approx. 25–50 nmol of polyphosphoinositides per g of guinea-pig liver; a slight predominance of inositol trisphosphate over inositol bisphosphate suggested the presence of rather more PtdIns(4,5)P_2 than PtdIns4*P*. These values suggested that between 1 and 3% of total liver inositol lipids exist as polyphosphoinositides. L. M. Jones (unpublished work) has recently approached the same question by injecting [2-^{3}H]inositol into rats 18 h before the isolation of liver cells; it was assumed that the three inositol lipids would reach isotopic equilibrium and that the ratio of their radioactivities would therefore approximate to the ratio of their concentrations. These experiments suggested that polyphosphoinositides constitute 1–2% of total inositol lipids in the hepatocyte. A third estimate has recently been obtained from the radioactivities of PtdIns4*P* and PtdIns(4,5)P_2 in liver cells that were labelled to equilibrium with [^{32}P]P_i and the specific radioactivity of the γ-phosphate of ATP determined (P. T. Hawkins, unpublished work). In this case it appeared that the concentrations of PtdIns4*P* and PtdIns(4,5)P_2 were equal, each being approx. 60 nmol/g dry weight of cells; i.e. each was present at about 0.5% of the concentration of PtdIns.

When the above values are combined with the fractional rates of 'breakdown' of the inositol lipids in stimulated cells (the present results; Kirk *et al.*, 1981*a*), then they yield approximate estimates for the minimum initial rates of depletion of 0.4 nmol/g of packed cells per s for PtdIns and of 0.1–0.3 nmol/g of packed cells per s for PtdIns4*P* and for PtdIns(4,5)P_2. Given the imprecision of the available data on lipid concentrations, the latter two values must be regarded as approximations. In view of the very different concentrations of the three inositol lipids the similarity of these rates is striking, as is the fact that the disappearance of all three lipids seems to be controlled by mechanisms that do not

use Ca^{2+} as a mediator and that are coupled directly to the occupation of Ca^{2+}-mobilizing receptors.

An obvious and mechanistically economical possibility is that the activation of a single metabolic reaction leads to the decreases in the concentrations of all three inositol lipids. As realised first by Durell *et al.* (1969), the two most likely candidates for this primary step are breakdown of PtdIns catalysed by the PtdIns-specific phospholipase C of the cytosol (Michell, 1975; Irvine *et al.* 1982; Shukla, 1982) or breakdown of polyphosphoinositides catalysed by a plasma-membrane phosphodiesterase similar to that of the erythrocyte (Abdel-Latif *et al.*, 1977, 1978; Hawthorne & Pickard, 1979; Kirk *et al.*, 1981*b*; Michell *et al.*, 1981; Downes & Michell, 1982*a*,*b*). Final confirmation that a phosphodiesterase is indeed implicated was recently obtained by Berridge *et al.* (1982).

The present experimental results do not distinguish unambiguously between primary attack upon PtdIns and upon polyphosphoinositides, but they are most easily reconciled with a mechanism involving primary activation of a polyphosphoinositide phosphodiesterase. First, a role in receptor function would provide a rationale for the fact that polyphosphoinositides seem generally to be concentrated at the cytoplasmic surfaces of plasma membranes (for review, see Downes & Michell, 1982*a*). Although the subcellular localization of these lipids in hepatocytes has yet to be investigated, it was shown many years ago that a substantial proportion of the PtdIns kinase of liver is located at the plasma membrane (Michell & Hawthorne, 1966; Michell *et al.*, 1967). Secondly, direct coupling to receptors can readily be envisaged if PtdIns4*P* and/or PtdIns(4,5)P_2 are attacked by a phosphodiesterase at the plasma membrane. Thirdly, there is no reason why a primary breakdown of PtdIns should lead to much larger fractional rates of depletion of the small cellular pools of polyphosphoinositides. Fourthly, a sustained breakdown of PtdIns4*P* and/or PtdIns(4,5)P_2 at the rates reported here would lead inevitably to a continuous withdrawal of PtdIns from the cellular reserves in order to restore the depleted pools of polyphosphoinositides.

We therefore suggest that phosphodiesterase-catalysed breakdown of PtdIns(4,5)P_2 (and maybe also of PtdIns4*P*) is the primary receptor-coupled event and that depletion of PtdIns (previously incorrectly termed its breakdown) represents its consumption during polyphosphoinositide synthesis. If this is correct, then the released lipid head-groups should appear in the cytosol of stimulated cells as inositol trisphosphate and/or bisphosphate. Although we have not yet done experiments with liver cells to test this prediction, these inositol polyphosphates do accumulate in stimulated synaptosomal fractions (Durell & Garland, 1969), tris muscle (Akhtar & Abdel-Latif, 1980), rat parotid fragments, blowfly salivary gland and brain slices (Berridge *et al.*, 1983).

In this context, we must always be aware that the observed rates of depletion ('breakdown') of PtdIns4*P* and PtdIns(4,5)P_2 represent minimum estimates, since lipid synthesis is a continuous process. As yet, we have no clear indication of the extent of this inevitable underestimation, but this information may soon become available from techniques that allow kinetic measures of the rate of release of inositol phosphates in stimulated cells (Berridge *et al.*, 1982, 1983). Another uncertainty is that PtdIns4*P* lies between PtdIns and PtdIns(4,5)P_2 in both the synthetic and degradative pathways, so that it is impossible from the present results to decide whether PtdIns4*P* concentration decreases only because of its consumption for PtdIns(4,5)P_2 synthesis.

Polyphosphoinositide breakdown as a primary response to Ca^{2+}-mobilizing hormonal stimuli

On the basis of our results and of recent studies elsewhere (Berridge, 1982; Berridge *et al.*, 1983; Weiss *et al.*, 1982; Putney, 1982; Putney *et al.*, 1983) we would suggest that the widely accepted general model of stimulated inositol lipid metabolism that envisages breakdown of PtdIns as its initiating event is incorrect. We propose that it should be replaced by a reaction scheme in which breakdown of PtdIns(4,5)P_2 (and maybe simultaneously of PtdIns4*P*) initiates inositol lipid turnover (i.e. Fig. 3 of Michell *et al.*, 1981). If this is correct then our recent exhortation for future studies to be focused upon inositol lipid breakdown (Michell & Kirk, 1981*b*) can be directed even more closely to the need for studies of the mechanism and function of PtdIns(4,5)P_2 breakdown. Amongst the immediate needs in this regard is a search for a polyphosphoinositide phosphodiesterase in liver plasma membranes: all that we at present know about the mechanisms available for polyphosphoinositide degradation in liver is that there is a soluble phosphodiesterase activity that attacks both PtdIns and the polyphosphoinositides (Kemp *et al.*, 1961). Even in brain, intestinal mucosa and erythrocytes, the most intensively studied tissues, the available information is limited (for reviews, see Hawthorne & White, 1975; Hawthorne & Pickard, 1979; Downes & Michell, 1982*a*).

Finally, we must consider what might be the role of receptor-stimulated breakdown of PtdIns(4,5)P_2. For the past few years a dominant idea has been that the stimulation of inositol lipid breakdown by a wide variety of receptors is a reaction essential to a general mechanism whereby these receptors mobilize Ca^{2+} in the cytosol of stimulated cells (Michell, 1975, 1979*a*,*b*; Michell *et al.*, 1977, 1981; Berridge, 1980,

1981; Michell & Kirk, 1981*a*; Putney, 1981). Although this general hypothesis has recently been subjected to several criticisms (Hawthorne & Pickard, 1979; Cockcroft, 1981, 1982; Hawthorne, 1982), we are of the opinion that these criticisms are insufficiently convincing for it to be abandoned (Michell & Kirk, 1982; Michell, 1982*a*,*b*). In particular, there is one important experimental observation that points directly to a role for inositol phospholipids in receptor-controlled mobilization of Ca^{2+}. This is the restoration of 5-hydroxytryptamine sensitivity to inositol-deprived and 'irreversibly' desensitized blowfly salivary glands when they are resupplied with inositol (Fain & Berridge, 1979). An unexpected facet of this observation was that full sensitivity could be restored by the resynthesis of a very small amount of inositol lipid. This may reflect the need only to resynthesize the lost PtdIns(4,5)P_2.

If phosphodiesterase-catalysed breakdown of PtdIns(4,5)P_2 is indeed essential to a widely disseminated mechanism for receptor-stimulated mobilization of Ca^{2+} then it must ultimately lead to at least two, and possibly three interrelated effects upon Ca^{2+} homeostasis. The first is rapid release into the cytosol of a limited cell-associated Ca^{2+} pool that may be shared by several receptors, is in relatively rapid equilibrium with extracellular Ca^{2+} and can be mobilized by receptor activation in cells made permeable by saponin treatment (Triggle, 1972; Chang & Triggle, 1973; Putney, 1979; Michell, 1979*b*, 1982*b*; Putney *et al.*, 1981; Casteels & Droogmans, 1981; Schulz *et al.*, 1981; Aub *et al.*, 1982). It is difficult to envisage that this pool is at any site other than the plasma membrane. Secondly, and associated with the release of this pool, there is an influx of Ca^{2+} through the plasma membrane, either by the opening of some sort of separate Ca^{2+} 'gate' or via transient occupation of the binding sites used by the cell-associated pool (Chang & Triggle, 1973; Michell, 1979*b*, 1982*b*; Putney, 1979; Poggioli *et al.*, 1980). Finally, both for hepatocytes and for some other cells, there are arguments in favour of rapid Ca^{2+} release from an intracellular cell-associated pool, maybe located in mitochondria (Reinhart *et al.*, 1982; for reviews, see Exton, 1981; Williamson *et al.*, 1981). The latter process, if it is important in the initiation of cellular responses to hormones, requires a hypothetical messenger to achieve communication between plasma membrane and intracellular organelle (Exton, 1981). Such a messenger would have to raise the cytosol $[Ca^{2+}]$ in the vicinity of the plasma membrane within 0.1–0.3 s (Michell, 1982*b*). A primary role for mitochondria as the major site of hormone-stimulated Ca^{2+} mobilization is made less probable by recent studies that indicate that intramitochondrial $[Ca^{2+}]$ may initially rise, rather than fall, after application of Ca^{2+}-mobilizing stimuli to liver cells (Poggioli *et al.*, 1980; Berthon *et al.*, 1981).

The most obvious models for a role of polyphosphoinositide breakdown in Ca^{2+} mobilization are those that incorporate the Ca^{2+}-binding characteristics of PtdIns(4,5)P_2 as a key feature (Hawthorne & White, 1975; Weiss *et al.*, 1982). Such mechanisms, though superficially attractive, seem unlikely to apply at the very low Ca^{2+}/Mg^{2+} ratio prevailing in the cytosol (for review, see Downes & Michell, 1982*a*). Moreover, the ion-selectivity of the sites that bind the externally accessible and receptor-sensitive cell-associated Ca^{2+} pool is different from that of PtdIns(4,5)P_2 (Aub *et al.*, 1982). Finally, stimulation of liver cells with enough vasopressin to cause breakdown of approx. 2 nmol of PtdIns(4,5)P_2/ml of cells per min brings about mobilization of at least 10 times more Ca^{2+} from the cell-associated pool (Reinhart *et al.*, 1982, Fig. 6). It seems more reasonable to propose that breakdown of PtdIns(4,5)P_2 might change the physical environment, and hence the function, of a single family of proteins that is responsible for control both of the release into the cytosol of cell-associated Ca^{2+} and of the entry of Ca^{2+}. In this context, it is notable that breakdown of PtdIns(4,5)P_2 to diacylglycerol and inositol 1,4,5-trisphosphate achieves conversion of the most polar glycerolipid of animal cells into one of the least polar and also liberates a water-soluble product with remarkable polarity and ion-binding characteristics.

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References

Abdel-Latif, A. A., Akhtar, R. A. & Hawthorne, J. N. (1977) *Biochem. J.* **162**, 61–73

Abdel-Latif, A. A., Akhtar, R. A. & Smith, J. P. (1978) in *Cyclitols and Phosphoinositides* (Eisenberg, F. & Wells, W. W., eds.), pp. 121–143, Academic Press, New York

Akhtar, R. A. & Abdel-Latif, A. A. (1980) *Biochem. J.* **192**, 783–791

Allan, D. & Thomas, P. (1981) *Biochem. J.* **198**, 441–445

Aub, D. L., McKinney, J. S. & Putney, J. W. (1982) *J. Physiol. (London)* **331**, 557–565

Berridge, M. J. (1975) *Adv. Cyclic Nucleotide Res.* **6**, 1–98

Berridge, M. J. (1980) *Trends Pharmacol. Sci.* **1**, 419–424

Berridge, M. J. (1981) *Mol. Cell. Endocrinol.* **24**, 115–140

Berridge, M. J. (1982) *Cell Calcium* **3**, 385–397

Berridge, M. J., Downes, C. P. & Hanley, M. R. (1982) *Biochem. J.* **206**, 587–595

Berridge, M. J., Dawson, R. M. C., Downes, C. P., Heslop, J. P. & Irvine, R. F. (1983) *Biochem. J.* in the press
Berthon, B., Poggioli, J., Capiod, T. & Claret, M. (1981) *Biochem. J.* **200**, 177–180
Billah, M. M. & Lapetina, E. G. (1982) *J. Biol. Chem.* **257**, 12705–12708
Billah, M. M. & Lapetina, E. G. (1983) *Biochem. Biophys. Res. Commun.* **109**, 217–222
Billah, M. M. & Michell, R. H. (1979) *Biochem. J.* **182**, 661–668
Bréant, B., Keppens, S. & de Wulf, H. (1981) *Biochem. J.* **200**, 509–514
Burgess, G. M., Claret, M. & Jenkinson, D. H. (1981) *J. Physiol. (London)* **317**, 67–90
Cantau, B., Keppens, S., de Wulf, H. & Jard, S. (1980) *J. Receptor Res.* **1**, 137–168
Casteels, R. & Droogmans, G. (1981) *J. Physiol. (London)* **317**, 263–279
Chang, K.-J. & Triggle, D. J. (1973) *J. Theor. Biol.* **40**, 125–154
Cockcroft, S. (1981) *Trends Pharmacol. Sci.* **2**, 340–342
Cockcroft, S. (1982) *Cell Calcium* **3**, 337–349
Creba, J. A. & Kirk, C. J. (1982) *Biochem. Soc. Trans.* **10**, 265–266
Creba, J. A., Downes, C. P., Kirk, C. J. & Michell, R. H. (1981) *Biochem. Soc. Trans.* **9**, 135P
Dawson, R. M. C. & Clarke, R. (1972) *Biochem. J.* **127**, 113–118
De Torrentegui, G. & Berthet, G. (1966) *Biochim. Biophys. Acta* **116**, 477–482
Downes, C. P. & Michell, R. H. (1981) *Biochem. J.* **198**, 133–140
Downes, C. P. & Michell, R. H. (1982*a*) *Cell Calcium* **3**, 467–502
Downes, C. P. & Michell, R. H. (1982*b*) *Biochem. J.* **202**, 53–58
Downes, C. P., Mussat, M. C. & Michell, R. H. (1982*a*) *Biochem. J.* **203**, 169–177
Downes, C. P., Hawkins, P. T. & Michell, R. H. (1982*b*) *Biochem. Soc. Trans.* **10**, 250–251
Durell, J. & Garland, J. T. (1969) *Ann. N.Y. Acad. Sci.* **165**, 743–754
Durell, J., Garland, J. T. & Friedel, R. O. (1969) *Science* **165**, 862–866
Ellis, R. B., Galliard, T. & Hawthorne, J. N. (1963) *Biochem. J.* **88**, 125–131
Exton, J. H. (1981) *Mol. Cell. Endocrinol.* **23**, 233–264
Fain, J. N. & Berridge, M. J. (1979) *Biochem. J.* **180**, 655–661
Farese, R. V., Sabir, M. A. & Larson, R. E. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 7189–7198
Farese, R. V., Sabir, M. A. & Larson, R. E. (1981) *Endocrinology* **109**, 1895–1901
Fisher, D. B. & Mueller, G. C. (1971) *Biochem. Pharmacol.* **20**, 2515–2518
Folch, J. (1949) *J. Biol. Chem.* **177**, 505–519
Garbus, J., Deluca, H. F., Loomans, M. E. & Strong, F. M. (1963) *J. Biol. Chem.* **238**, 7147–7156
Garrison, J. C., Borland, M. K., Florio, V. A. & Twible, D. A. (1979) *J. Biol. Chem.* **254**, 7147–7156
Goldberg, N. D. & Haddox, M. C. (1977) *Annu. Rev. Biochem.* **46**, 823–896
Griffin, H. D. & Hawthorne, J. N. (1978) *Biochem. J.* **176**, 541–552
Hanson, B. A. & Lester, R. L. (1980) *J. Lipid Res.* **21**, 309–315
Hawthorne, J. N. (1982) *Nature (London)* **295**, 281–282
Hawthorne, J. N. & Pickard, M. R. (1979) *J. Neurochem.* **32**, 5–14
Hawthorne, J. N. & White, D. A. (1975) *Vitam. Horm. (N.Y.)* **33**, 529–573
Hoffman, R., Erzberger, P., Frank, W. & Ristow, H-J. (1980) *Biochim. Biophys. Acta* **618**, 282–292
Hokin, M. R. & Brown, D. R. (1969) *J. Neurochem.* **16**, 475–483
Hölzl, J. & Wagner, H. (1964) *Biochem. Z.* **339**, 327–330
Irvine, R. F., Dawson, R. M. C. & Freinkel, N. (1982) in *Contemporary Metabolism* (Freinkel, N., ed.), vol. 2, pp. 301–342, Plenum Press, New York
Jafferji, S. S. & Michell, R. H. (1976) *Biochem. J.* **160**, 163–169
Jard, S., Cantau, B. & Jakobs, K. H. (1981) *J. Biol. Chem.* **256**, 2603–2606
Jones, L. M., Cockcroft, S. & Michell, R. H. (1979) *Biochem. J.* **182**, 669–676
Kemp, P., Hübscher, G. & Hawthorne, J. N. (1961) *Biochem. J.* **79**, 193–200
Keppens, S., De Wulf, H., Clauser, P., Jard, S. & Morgat, J.-L. (1982) *Biochem. J.* **208**, 809–817
Keppens, S., Vandenheede, J. R. & De Wulf, H. (1977) *Biochim. Biophys. Acta* **496**, 448–457
Kfoury, G. A. & Kerr, S. (1964) *Biochim. Biophys. Acta* **84**, 391–403
Kirk, C. J. (1982) *Cell Calcium* **3**, 399–411
Kirk, C. J. & Creba, J. A. (1982) *Biochem. Soc. Trans.* **10**, 264–265
Kirk, C. J., Verrinder, T. R. & Hems, D. A. (1977) *FEBS Lett.* **47**, 128–131
Kirk, C. J., Verrinder, T. R. & Hems, D. A. (1978) *Biochem. Soc. Trans.* **6**, 1031–1033
Kirk, C. J., Rodriguez, L. M. & Hems, D. A. (1979) *Biochem. J.* **178**, 493–496
Kirk, C. J., Billah, M. M., Jones, L. M. & Michell, R. H. (1980) *Horm. Cell. Regul.* **4**, 73–88
Kirk, C. J., Michell, R. H. & Hems, D. A. (1981*a*) *Biochem. J.* **194**, 155–165
Kirk, C. J., Creba, J. A., Downes, C. P. & Michell, R. H. (1981*b*) *Biochem. Soc. Trans.* **9**, 377–379
Kruszynski, M., Lammek, B., Manning, M., Seto, J., Haldar, J. & Sawyer, W. H. (1980) *J. Med. Chem.* **23**, 364–368
Lang, V., Pryhitka, G. & Buckley, J. T. (1977) *Can. J. Biochem.* **55**, 1007–1012
Lin, S.-H. & Fain, J. N. (1981) *Life Sci.* **29**, 1905–1912
Mellian, B., Ignarro, L-J., Ohlstein, E. H., Pontecorvo, E. G., Hyman, A. L. & Kadowitz, P. J. (1981) *Blood* **57**, 946–955
Michell, R. H. (1975) *Biochim. Biophys. Acta* **415**, 81–147
Michell, R. H. (1979*a*) *Trends Biochem. Sci.* **4**, 128–131
Michell, R. H. (1979*b*) in *Companion to Biochemistry* (Bull, A. T., Lagnado, J. R., Thomas, J. O. & Tipton, K. F., eds.), vol. 2, pp. 205–228, Longmans, London
Michell, R. H. (1982*a*) *Nature (London)* **296**, 492–493
Michell, R. H. (1982*b*) *Cell Calcium* **3**, 285–294

Michell, R. H. & Hawthorne, J. N. (1966) *Biochem. Biophys. Res. Commun.* **21**, 333–338
Michell, R. H. & Jones, L. M. (1974) *Biochem. J.* **138**, 47–52
Michell, R. H. & Kirk, C. J. (1981*a*) *Trends Pharmacol. Sci.* **2**, 1–4
Michell, R. H. & Kirk, C. J. (1981*b*) *Biochem. J.* **198**, 247–248
Michell, R. H. & Kirk, C. J. (1982) *Trends Pharmacol. Sci.* **3**, 140–141
Michell, R. H., Harwood, J. L., Coleman, R. & Hawthorne, J. N. (1967) *Biochim. Biophys. Acta* **144**, 649–658
Michell, R. H., Hawthorne, J. N., Coleman, R. & Karnovsky, M. L. (1970) *Biochim. Biophys. Acta* **210**, 86–91
Michell, R. H., Jafferji, S. S. & Jones, L. M. (1977) in *Function and Biosynthesis of Lipids* (Bazan, N. G., Brenner, R. R. & Giusto, N. M., eds.), pp. 447–464, Plenum Press, New York
Michell, R. H., Kirk, C. J., Jones, L. M., Downes, C. P. & Creba, J. A. (1981) *Philos. Trans. R. Soc. London Ser. B* **296**, 123–133
Michell, R. H., Kirk, C. J., Creba, J. A., Downes, C. P. & Hawkins, P. T. (1982) *Abstr. Int. Cong. Biochem. 12th, Perth*, 129
Poggioli, J., Berthon, B. & Claret, M. (1980) *FEBS Lett.* **115**, 245–246
Putney, J. W. (1979) *Pharmacol. Rev.* **30**, 209–245
Putney, J. W. (1981) *Life Sci.* **29**, 1183–1194
Putney, J. W. (1982) *Cell Calcium* **3**, 369–383
Putney, J. W., Poggioli, J. & Weiss, S. J. (1981) *Philos. Trans. R. Soc. London Ser. B* **296**, 37–45
Putney, J. W., Burgess, G. M., Halenda, S. P., McKinney, J. S. & Rubin, R. P. (1983) *Biochem. J.* in the press
Reinhart, P. H., Taylor, W. M. & Bygrave, F. L. (1982) *Biochem. J.* **208**, 619–630
Roach, P. D. & Palmer, F. B. St. C. (1981) *Biochim. Biophys. Acta* **661**, 323–333
Santiago-Calvo, E., Mulé, S., Redman, C. M., Hokin, M. R. & Hokin, L. E. (1964) *Biochim. Biophys. Acta* **84**, 550–562
Schultz, K-D., Schultz, K. & Schulz, P. (1977) *Nature (London)* **265**, 750–751
Schulz, I., Kimura, T., Wakasugi, H., Haase, W. & Kribben, A. (1981) *Philos. Trans. R. Soc. London Ser. B* **296**, 105–113
Shukla, S. D. (1982) *Life Sci.* **30**, 1323–1335
Soukup, J. F. & Schanberg, S. M. (1982) *J. Pharmacol. Exp. Ther.* **222**, 209–214
Takai, Y., Kaibuchi, K., Matsubara, T. & Nishizuka, Y. (1981) *Biochem. Biophys. Res. Commun.* **101**, 61–67
Takenawa, T., Homma, Y. & Nagai, Y. (1982) *Biochem. Pharmacol.* **31**, 2663–2667
Takhar, A. P. S. & Kirk, C. J. (1981) *Biochem. J.* **194**, 167–172
Tolbert, M. E. M., White, A. C., Aspry, K., Cutts, J. & Fain, J. N. (1980) *J. Biol. Chem.* **255**, 1938–1944
Triggle, D. J. (1972) *Adv. Membr. Surf. Sci.* **5**, 267–331
Wakelam, M. J. O. & Walker, D. G. (1980) *FEBS Lett.* **111**, 115–119
Weiss, S. J., McKinney, J. & Putney, J. W. (1982) *Biochem. J.* **206**, 555–560
Whitton, P. D., Rodrigues, L. M. & Hems, D. A. (1977) *Biochem. Soc. Trans.* **5**, 992–994
Williamson, J. R., Cooper, R. H. & Hoek, J. B. (1981) *Biochim. Biophys. Acta* **639**, 243–295
Yagihara, Y., Bleasdale, J. E. & Hawthorne, J. N. (1972) *J. Neurochem.* **21**, 173–190