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Noradrenaline stimulation of the phosphoinositide system: evidence for a novel hydrophobic inositol-containing compound in resistance arterioles

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1 Five inositol phosphates were extracted from adult rat resistance arterioles and separated by ion-exchange high performance liquid chromatography.

2 By use of this technique, inositol phosphates liberated were identified as inositol 1-phosphate, inositol 1,4-bisphosphate, inositol 1,3,4-trisphosphate, inositol 1,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate. Stimulation of phosphoinositide hydrolysis with noradrenaline produced increases in inositol phosphate production.

3 Three inositol-containing phospholipids extracted from resistance arterioles were measured as their glycerol esters following deacylation, thereby permitting an analysis of both membrane and cytosolic components of the phosphoinositide signalling system.

4 A substantial agonist-sensitive pool of a previously undescribed inositol but not glycerol-containing lipid extract component was also identified in this tissue.

5 These experiments for the first time allow a precise description of phosphoinositide metabolism in resting and agonist-stimulated resistance arterioles and provide data on a novel compound possibly similar to that recently described in other tissues.

Introduction

There has been considerable recent interest in the metabolism of a relatively minor fraction of phospholipids located within the inner lamella of the plasma membrane. This has been generated as a result of the increasing recognition that many hormones and agonists utilize these phosphoinositide lipids in transmembrane stimulus-effector coupling (Berridge & Irvine, 1984). Indeed, following agonist receptor binding two second messenger molecules are generated: inositol 1,4,5-trisphosphate (Ins 1,4,5-P_3) and 1,2-diacylglycerol (1,2-DAG) are able individually and synergistically to induce contractions in vascular tissue by modulating the concentration of intracellular ionized calcium (Somlyo *et al.*, 1985; Itoh & Lederis, 1987). In addition to the contribution to vasoconstriction, the phosphoinositide signalling system may also play a primary role in the modulation of cellular growth and differentiation, a function which may be of particular relevance in diseases such as hypertension which is characterized by alterations in resistance vessel structure (Mulvany, 1987).

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There have been a number of investigations of phosphoinositide metabolism in arteries (Campbell *et al.*, 1985; Heagerty *et al.*, 1986; Rapoport, 1986) as well as in cells cultured from such tissue (Griendling *et al.*, 1986). The gross accumulation of inositol phosphates observed when experiments are conducted in the presence of lithium ions provides a sensitive assay of inositol phospholipid hydrolysis (Berridge *et al.*, 1982) and facilitates the characterization of pharmacologically active agents, which may stimulate or have inhibitory roles upon the hydrolysis of phosphoinositide and vascular contraction (Rapoport, 1986; Fox & Friedman, 1987). Recently, we have demonstrated abnormalities in basal and α_1-adrenoceptor agonist-stimulated vascular phosphoinositide hydrolysis in the early and established stages of hypertension in genetically hypertension-prone rats (Heagerty *et al.*, 1986), suggesting a possible role for this system in the genesis of this disease.

The metabolism of cellular inositol-containing compounds is complex and it is becoming clear that Ins 1,4,5-P_3 is not the only inositol phosphate to possess a role in intracellular events triggered by

activation of phosphoinositide phospholipase C (Wilson et al., 1985; Michell, 1986), and, in order to explore this signalling system, it is necessary to account for individual inositol-containing metabolites. Furthermore, in studies of cardiovascular diseases it is necessary to examine this system in contractile vascular tissue rather than in conduit vessels where the majority of resistance to blood flow is located. Conventional low pressure ion-exchange techniques used to separate phosphorylated components of this signalling system are not adequate to resolve structural isomers of inositol phosphates. Therefore, in order to describe qualitative and quantitative agonist-evoked changes in phosphoinositide signalling in resistance arterioles, we have applied high performance ion-exchange chromatography to try and facilitate examination of inositol-containing components of this system in such tissue.

Methods

Animals

Adult female Wistar rats (body weight 250–300 g) were used for all the experiments described.

Dissection and incubation

Four subcutaneous resistance arterioles supplied by the axillary artery (length 2–3 cm: internal diameter 100–150 μm) were dissected from two rats, and cleaned of adherent fat and connective tissue. The vessels were placed in tissue culture medium (M199) at 37°C and after 30 min of equilibration the arterioles were transferred to a tube containing 50 μl M199 and 60 μM [^{3}H]-myoinositol (specific activity 17.1 Ci mmol^{-1}). The vessels were left at 37°C for 120 min in a shaking waterbath to allow incorporation of radiolabel into inositol-containing compounds. Following this, 50 μl of pre-warmed vehicle or noradrenaline was added to give a final concentration of 1 mM and left for 20 s before removal of the tissue for homogenization in 0.5 ml 10% (w/v) ice-cold trichloroacetic acid in a glass/glass tissue grinder. Noradrenaline was applied at such concentrations to ensure maximal activation of phosphoinositide-linked receptors (Fox & Friedman, 1987).

Preparation of tissue aqueous and lipid extracts

Following homogenization tissue was left on ice for 15 min before centrifugation for 10 min at 2000 *g*. The supernatant containing the aqueous tissue extract was transferred to a glass test-tube and washed five times with water-saturated diethylether (Irvine et al., 1985). The resulting neutralized extract was diluted to 2 ml with a solution containing nine nucleotides to assist the identification of eluting radioactive peaks, and also to serve as an indicator of column stability in subsequent chromatography (Irvine et al., 1985; Batty et al., 1985). This standard nucleotide mixture contained cytidine mono- and diphosphate; adenosine mono-, di- and tri-phosphate; guanosine mono-, di-, tri- and tetra-phosphate.

Phospholipids were extracted from the sedimented tissue pellet by homogenization on ice in 0.5 ml chloroform:methanol:HCl (v/v 20:40:1). This homogenate was left on ice for 15 min before sequential additions of 0.5 ml chloroform and 0.5 ml water to render the extract biphasic. Following agitation and centrifugation at 2000 *g* the upper aqueous layer was discarded and the lower phospholipid-containing organic phase was transferred to a glass test-tube leaving the residual tissue pellet, and evaporated to dryness under a stream of oxygen-free nitrogen (Downes & Wusterman, 1983).

Deacylation of dried lipid extract

Dried lipid was re-constituted in 1 ml of methylamine reagent comprising 25% methylamine solution:methanol:butan-1-ol (v/v 4:4:1) and placed in a waterbath for 45 min at 53°C. After this time the solution was evaporated to dryness and re-constituted in 0.7 ml water. The acyl moieties were then removed by washing twice with a butanol solution which contained butan-1-ol:light petroleum:ethyl formate (v/v 20:4:1) and the remaining aqueous phase containing deacylated lipid was diluted to 2 ml with a solution containing nine nucleotides before chromatography (Hawkins et al., 1986).

Quantitation

Solubilization of the residual tissue pellet after removal of the lipid-rich organic phase before deacylation was achieved using 2 M NaOH. This allows the estimation of protein content by the method of Lowry et al. (1951) and subsequent chromatographic data normalization with respect to tissue protein content.

Chromatography

Resistance arteriole aqueous and deacylated lipid extracts containing the nine marker nucleotides were injected onto a Partisil 10 SAX h.p.l.c. column (250 × 4.6 mm, Technicol, Stockport, U.K.) through a sample injector fitted with a 2 ml sample loop. The

column was eluted at 1.2 ml min^{-1} with a non-linear pre-programmed gradient of water/1.7 M ammonium formate/orthophosphoric acid (pH 3.7). On-line u.v. monitoring at 254 nm allowed measurement of retention times for nucleotide markers and the whole of the gradient was fractionated into scintillation vials (Irvine *et al.*, 1985; Batty *et al.*, 1985). Water (1.15 ml) and OptiPhase X scintillation fluid (16 ml) were added to 0.6 ml fractions of column eluate before liquid scintillation counting, and quantitation of eluting radioactive peaks was achieved using appropriate quenched standards.

Materials

Noradrenaline hydrochloride, sodium periodate and standard nucleotides were purchased from Sigma (Poole, Dorset, U.K.). [^{3}H]-myoinositol and [^{3}H]-myoinositol 1,4,5-trisphosphate were supplied by New England Nuclear (Boston, Massachusetts, U.S.A.), OptiPhase X scintillant was purchased from LKB Instruments (South Croydon, Surrey, U.K.), methylamine solution from BDH Chemicals (Poole, Dorset, U.K.) and 1,1-dimethylhydrazine from Aldrich (Gillingham, Dorset, U.K.). Tissue culture M199 was supplied by Gibco (Paisley, Scotland) and contained (mM): NaCl 137, KCl 5.4, $MgSO_4.7H_2O$ 0.81, $Na_2HPO_4.7H_2O$ 0.36, $CaCl_2$ 1.26, $NaHCO_3$ 4.2, KH_2PO_4 0.44, $MgSO_4.7H_2O$ 0.81, $Fe(NO_3)9H_2O$ 0.02.

Statistics

Five separate incubations were performed with and without noradrenaline. Results are expressed as means and means $\pm$ s.e. mean and comparisons between basal and agonist-induced changes in [^{3}H]-inositol-containing compounds were made by means of Student's unpaired *t* test.

Results

Aqueous tissue extracts

Chromatographic analysis of aqueous tissue extracts revealed the presence of six radioactive inositol-containing peaks. These were identified by way of their relative retention times to cytidine, adenosine and guanosine phosphate markers as glycerophosphoinositol (GroP-Ins), inositol 1-phosphate (Ins 1-P), inositol 1,4-bisphosphate (Ins 1,4-P_2), inositol 1,3,4-trisphosphate (Ins 1,3,4-P_3), inositol 1,4,5-trisphosphate (Ins 1,4,5-P_3) and inositol 1,3,4,5-tetrakisphosphate (Ins 1,3,4,5-P_4) (Figure 1). Authentic [^{3}H]-Ins 1,4,5-P_3 was used to confirm the elution position of this compound.

Following stimulation with 1 mM noradrenaline for 20 s, radioactivity associated with aqueous inositol-containing compounds changed dramatically. An increase in the radioactivity associated with all five inositol phosphates was noted and this was highly significant for Ins 1,4-P_2 ($P < 0.001$) and Ins 1,3,4-P_3 ($P < 0.001$), but not so for Ins 1-P ($P < 0.02$), Ins 1,4,5-P_3 (NS) and Ins 1,3,4,5-P_4 (NS) (Figure 2).

Tissue deacylated lipid extracts

Following chromatographic separation of deacylated tissue extracts four inositol-containing peaks were detected; two of these eluted with the predicted retention times of GroP-Ins and glycerophosphoinositol 4-phosphate (GroP-Ins 4-P). Two peaks were located in the region of the eluting gradient associated with the expected retention time of glycerophosphoinositol 4,5-bisphosphate (GroP-Ins 4,5-P_2) (Figure 3a).

The possible existence of two structural isomers of phosphatidylinositol bisphosphate (Ptd-Ins P_2) has recently been investigated in rat parotid glands by Hawkins *et al.* (1986) and Downes *et al.* (1986). These authors identified only three inositol-containing compounds present in lipid extracts and these were identified as phosphatidylinositol (Ptd-Ins), phosphatidylinositol 4-phosphate (Ptd-Ins 4-P) and phosphatidylinositol 4,5-bisphosphate (Ptd-Ins 4,5-P_2). Using an identical lipid deacylation procedure we revealed the existence of a possible Ptd-Ins P_2 structural isomer present in rat resistance arterioles (Figure 3a). In order to identify further peaks associated with Ptd-Ins P_2 glycerol ester, a single lipid extract was treated with sodium periodate and 1,1-dimethylhydrazine to remove the glycerol moieties (Irvine *et al.*, 1985). Identification of the resulting inositol head group should then have been possible on subsequent chromatographic analysis. Following this treatment three of the four inositol-containing peaks, on chromatography co-eluted with Ins 1-P, Ins 1,4-P_2 and Ins 1,4,5-P_3 (Figure 3b). However, the peak which had eluted in fraction 58 remained intact following treatment suggesting that this compound probably was not a glycerol ester (Figure 3b), and so did not appear to represent the postulated deacylated isomer of Ptd-Ins-P_2. Identification of the elution position of glycerophosphoinositol 4,5-bisphosphate (GroP-Ins 4,5-P_2) was then possible and this was found to be associated with the peak eluting in fraction 65 (Figure 3b).

Following exposure to noradrenaline radioactivity associated with all four inositol-containing lipid extract components was reduced, and this was most

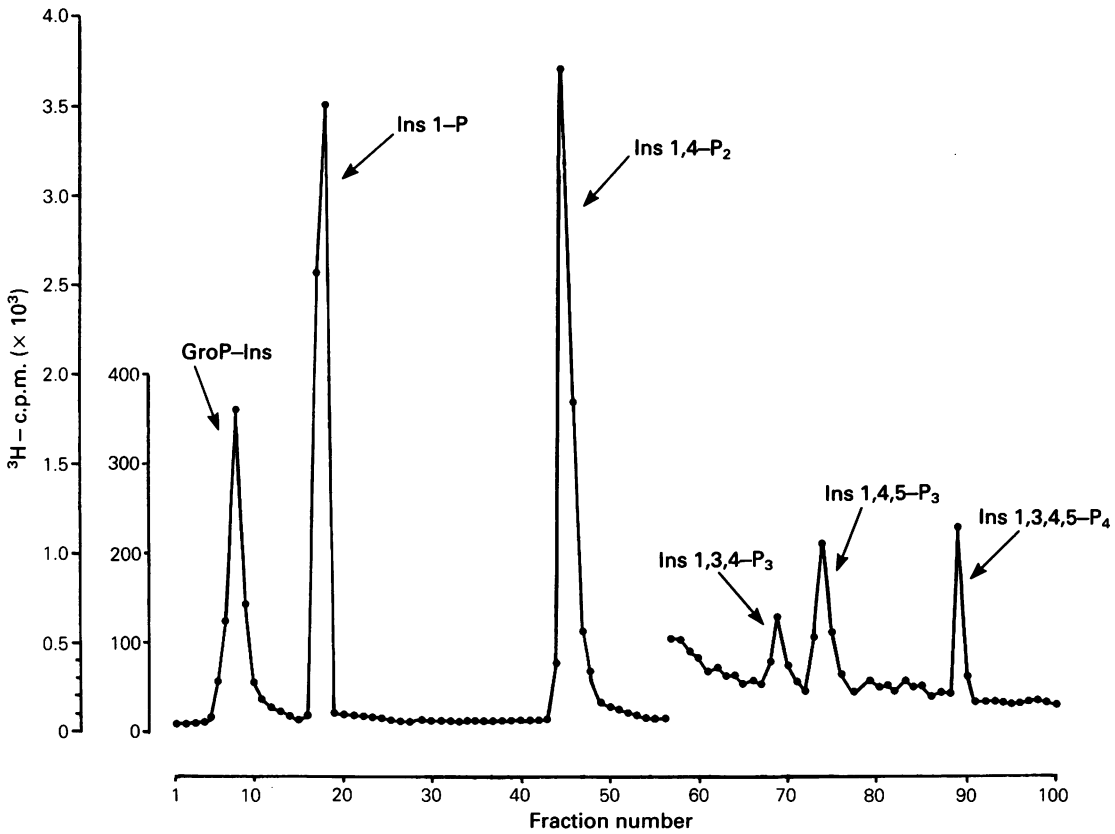

Figure 1 H.p.l.c. chromatogram showing the resolution of 6 inositol-containing peaks in a resistance arteriole aqueous extract following pre-labelling with [^{3}H]-myoinositol. Beyond fraction 56 the peaks were smaller in comparison to those eluted earlier. Therefore the scale has been altered (0–400 ^{3}H-c.p.m.) for clarity and consequently included in the figure. For abbreviations used in this and subsequent figures see Results section.

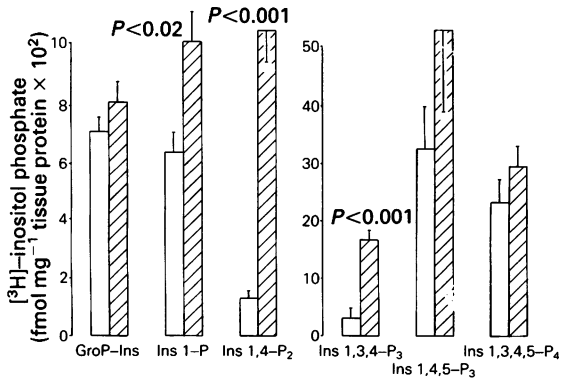

Figure 2 The changes in the liberation of inositol phosphates following exposure of labelled resistance arterioles to vehicle (open columns) or 1 mM noradrenaline (hatched columns) for 20 s. Data are represented as means with vertical bars indicating s.e. mean.

evident for the polyphosphoinositide glycerol esters glycerophosphoinositol 4-phosphate (GroP-Ins 4-P) ($P < 0.02$) and GroP-Ins 4,5-P_2 ($P < 0.02$) (Figure 4). Radioactivity in the unknown peak (X) eluted in fraction 58 was also reduced by 40% following agonist exposure although this did not attain statistical significance (Figure 4). The net loss of 768 fmol mg^{-1} tissue protein of labelled Ptd-Ins 4-P, Ptd-Ins 4,5-P_2 and an unknown inositol-containing hydrophobic compound on stimulation, was associated with the net gain of 773 fmol mg^{-1} tissue protein of labelled inositol bis-, tris-, and tetrakisphosphates. By far the greatest increase in inositol phosphate on stimulation of resistance arterioles was found in the Ins 1,4-P_2 peak. However, reductions in the amount of labelled polyphosphoinositide were similar for both Ptd-Ins 4-P and Ptd-Ins 4,5-P_2. The reduction in radioactivity found in the peak corresponding to Ptd-Ins was not significant, but it was greater than the increase in tissue Ins 1-P.

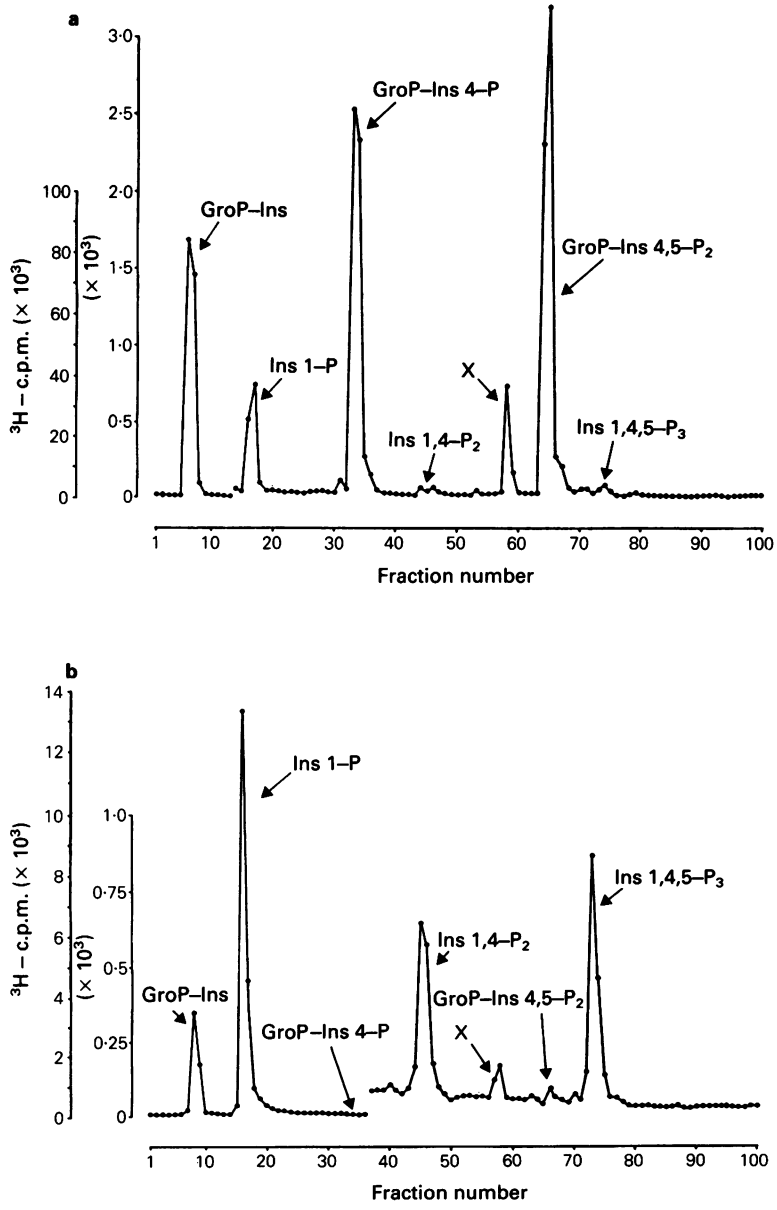

Figure 3 (a) H.p.l.c. chromatogram showing the resolution of inositol-containing components in a resistance arteriole deacylated lipid extract following pre-labelling with [^{3}H]-myoinositol. At fraction 14 the scale has been altered (0–3 × 10^3 ^{3}H-c.p.m.) for clarity. (b) Chromatogram showing a resistance arteriole deacylated lipid extract following removal of glycerol moieties. At fraction 37 the scale has been altered (0–1 × 10^3 ^{3}H-c.p.m.) for clarity. Esterified glycerol was removed by sodium periodate and 1,1-dimethylhydrazine treatment, the sample was then diluted four fold before chromatography.

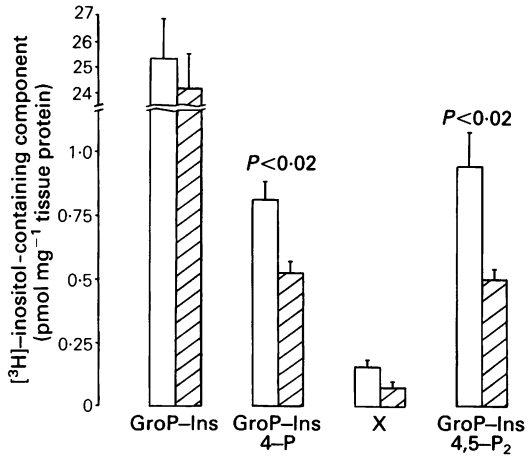

Figure 4 Noradrenaline-induced changes in resistance arteriole inositol-containing lipid extract components following deacylation. Data are represented as means for control (open columns) and agonist-stimulated (hatched columns) incubations. Vertical bars indicate s.e. mean.

Discussion

The results of this study indicate that the methods described are sensitive enough to demonstrate that stimulation of the phosphoinositide signalling system in resistance arterioles results in the production of inositol monophosphate, bisphosphate, 1,3,4 and 1,4,5 structural isomers of inositol trisphosphate and the highly phosphorylated Ins 1,3,4,5-P_4. This is probably the result of the stimulation of phospholipase C mediated hydrolysis of phosphoinositide as the production of inositol phosphate was associated with the concomitant reduction in radiolabelling of phosphoinositides.

Data obtained in isolated smooth muscle cells and other tissues indicate that during stimulation the initial hydrolysis of phosphoinositide is followed by a period of re-synthesis (Litosch *et al.*, 1983; Griendling *et al.*, 1986) and it is becoming evident that during the early phase of stimulation phospholipase C activity may be directed against Ptd-Ins 4-P and Ptd-Ins in addition to Ptd-Ins 4,5-P_2 (Imai & Gershengorn, 1986). This may be secondary to the specific hydrolysis of Ptd-Ins 4,5-P_2 and the rapid Ins 1,4,5-P_3 mediated increase in cellular calcium concentration (Majerus *et al.*, 1986). It has been suggested that the probable key to prolonged stimulation of this signalling system is the hydrolysis of Ptd-Ins and Ptd-Ins 4-P. Together these two lipids represent approximately 95% or more of tissue phosphoinositide and so could provide a constant supply of 1,2-DAG necessary for the continued activation of protein kinase C. Although it is not possible in the present study to state to what extent the hydrolysis of Ptd-Ins 4-P directly contributes to the very large increase in tissue Ins 1,4-P_2 observed, which may also be the result of dephosphorylation of inositol phosphates formed following hydrolysis of Ptd-Ins 4,5-P_2. However, the highly significant increase in tissue Ins 1,3,4-P_3 and the very modest rise in Ins 1,3,4,5-P_4, which occur following the short period of stimulation, provide evidence of the rapid formation and subsequent dephosphorylation of Ins 1,3,4,5-P_4. In addition these data indicate the loss of Ptd-Ins on stimulation, although this was not statistically significant, to be approximately three times greater than that gained by Ins 1-P. However, this may be a reflection of the rapid dephosphorylation of Ins 1-P to inositol catalyzed by the lithium-sensitive inositol 1-phosphatase.

Because the period of stimulation in these experiments, carried out in the presence of labelled inositol, was apparently terminated before a period of phosphoinositide re-synthesis, it is unlikely that possible changes in isotopic specific activity associated with the various pools of inositol-containing compounds would have any significant influence upon the observed agonist-induced changes. Further work is required to determine the relative contribution of individual inositol phospholipid substrate to the agonist-induced liberation of 1,2-DAG.

The detection of two inositol-containing compounds in the region of the h.p.l.c. gradient corresponding to GroP-Ins 4,5-P_2 was exciting and unexpected but perhaps in accord with findings in other tissues (Downes *et al.*, 1986; Hawkins *et al.*, 1986). These two compounds were found probably not to represent esters of Ptd-Ins P_2 structural isomers but authentic Ptd-Ins 4,5-P_2 glycerol ester and an unknown inositol-containing component eluting after GroP-Ins 4-P and just prior to GroP-Ins 4,5-P_2. This novel compound, which was resistant to periodate and hydrazine treatment, possibly indicating the absence of esterified glycerol, was shown to be sensitive to noradrenaline stimulation suggesting a possible role in phosphoinositide signalling. The period of pre-labelling was comparatively short in these experiments and it is unlikely that the existence of such an unidentified compound could be accounted for by the incorporation of radioactivity into a non inositol-containing compound. Upon hydrolysis one might expect the novel lipid soluble compound to release an inositol-containing water-soluble component. All radioactive peaks eluting in aqueous tissue extracts were identified as inositol phosphates which could originate from the successive dephosphorylation of Ins 1,3,4,5-P_4. Therefore, it is possible that the novel hydrophilic product of hydrolysis is inositol or may co-elute with inositol

phosphate. Alternatively, this component may be more polar than Ins 1,3,4,5-P_4 and was undetected because the h.p.l.c. gradient of ammonium formate used did not exceed 1.7 M.

More than two decades ago analysis of mammalian tissue lipid soluble inositol-containing compounds revealed the presence of at least three phosphoinositides, including one or more compounds with a suggested molecular structure closely resembling inositol-containing lipid found in yeasts and the seeds of higher plants (Klenk & Hendricks, 1961; Ellis *et al.*, 1963). More recently the presence of a novel inositol-containing lipid has been found in cultured myocytes and in liver cell membranes by Saltiel *et al.* (1986). These workers have further shown this novel lipid to be rich in glucosamine and to be hydrolysed on exposure of tissue to hormone, releasing a hydrophilic component capable of modulating the activity of cyclic AMP phosphodiesterase. It is becoming clear that interactions exist between cell second messenger systems (Yoshimasa *et al.*, 1987). The novel compound found in rat resistance arterioles in the present study may represent such an interactive compound, more polar than the compound reported by Saltiel's group but which nevertheless appears to be agonist-sensitive. It is of interest that despite an intense search, two groups of workers have not detected such a compound to be present in lipid extracts prepared from rat parotid glands (Hawkins *et al.*, 1986; Downes *et al.*, 1986). We ourselves have been unable to find such a compound in rat brain lipid extracts (unpublished observations) and this may suggest a degree of tissue specificity. The novel compound detected here in resistance arterioles now requires further characterization of structure and function.

The precise functions of individual inositol phosphates generated upon agonist stimulation of arterioles remain unclear. However, the methods described here provide the means to investigate this further and indeed to extend studies into disease states such as hypertension, where a fundamental defect of this second messenger system may contribute to the increased vascular resistance observed in this condition.

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