Phosphatidylinositol metabolism in rat hepatocytes stimulated by vasopressin

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In isolated rat hepatocytes, vasopressin evoked a large increase in the incorporation of [^{32}P]P_i into phosphatidylinositol, accompanied by smaller increases in the incorporation of [1- ^{14}C]oleate and [U- ^{14}C]glycerol. Incorporation of these precursors into the other major phospholipids was unchanged during vasopressin treatment. Vasopressin also promoted phosphatidylinositol breakdown in hepatocytes. Half-maximum effects on phosphatidylinositol breakdown and on phosphatidylinositol labelling occurred at about 5 nM-vasopressin, a concentration at which approximately half of the hepatic vasopressin receptors are occupied but which is much greater than is needed to produce half-maximal activation of glycogen phosphorylase. Insulin did not change the incorporation of [^{32}P]P_i into the phospholipids of hepatocytes and it had no effect on the response to vasopressin. Although the incorporation of [^{32}P]P_i into hepatocyte lipids was decreased when cells were incubated in a Ca²⁺-free medium, vasopressin still provoked a substantial stimulation of phosphatidylinositol labelling under these conditions. Studies with the antagonist [1-(β -mercapto- β , β -cyclopentamethylenepropionic acid),8-arginine]vasopressin indicated that the hepatic vasopressin receptors that control phosphatidylinositol metabolism are similar to those that mediate the vasopressor response *in vivo*. When prelabelled hepatocytes were stimulated for 5 min and then subjected to subcellular fractionation, the decrease in [^3H]phosphatidylinositol content in each cell fraction was approximately in proportion to its original phosphatidylinositol content. This may be a consequence of phosphatidylinositol breakdown at a single site, followed by rapid phosphatidylinositol exchange between membranes leading to re-establishment of an equilibrium distribution.

Recent work has established that vasopressin can exert control over several facets of hepatic metabolism; it stimulates gluconeogenesis and glycogenolysis, and it inhibits the synthesis of glycogen, fatty acids and ketone bodies (Hems & Whitton, 1973; Ma & Hems, 1975; Williamson *et al.*, 1980). At present, vasopressin is the most potent known hormonal activator of glycogen phosphorylase (Hems *et al.*, 1978a). Cyclic nucleotides do not appear to be implicated in these hepatic actions of vasopressin (Kirk & Hems, 1974; Hems *et al.*, 1978b) and recent studies have indicated that a rise in cytosolic Ca²⁺ concentration may be the essential intracellular mediator of these responses. In particular, hepatic responses to vasopressin require the

presence of extracellular Ca²⁺ (Stubbs *et al.*, 1976; Whitton *et al.*, 1977; Keppens *et al.*, 1977; Garrison *et al.*, 1979) and they are accompanied by Ca²⁺ fluxes into and out of the stimulated cells (Keppens *et al.*, 1977; Blackmore *et al.*, 1978; Chen *et al.*, 1978). Two other hormones that seem to control the metabolism of rat liver through mechanisms similar to, though possibly not identical with, that of vasopressin are adrenaline acting at α -receptors and angiotensin (Keppens *et al.*, 1977; Assimacopoulos-Jeannet *et al.*, 1977; Garrison *et al.*, 1979).

Associated with the effects of vasopressin, angiotensin II and α -adrenergic stimuli on the central metabolic pathways of the liver there is a marked stimulation of the metabolism of phosphatidylinositol (Kirk *et al.*, 1977, 1978, 1979, 1980; Billah & Michell, 1978, 1979; Tolbert *et al.*, 1980). Similar

* Deceased.

responses, consisting of enhanced phosphatidylinositol breakdown followed by its resynthesis, occur in a variety of cells treated with Ca^{2+} -mobilizing hormones (Michell, 1979). It has been suggested that such responses are involved in the mechanism(s) whereby these hormones provoke intracellular Ca^{2+} mobilization (Michell, 1975, 1979).

In the present paper, we describe some detailed studies of the effects of vasopressin on the metabolism of phosphatidylinositol in rat hepatocytes.

Materials and methods

Preparation and incubation of hepatocytes

For the experiments described in Fig. 1 and Table 1, hepatocytes were isolated from fed male Wistar rats (200–250 g) by collagenase perfusion as described previously (Kirk *et al.*, 1977). For all other experiments, hepatocytes were prepared by a modification of this method in which: (1) Ca^{2+} was added to the perfusion medium (final concentration 2.5 mM) together with collagenase (0.04%, w/v) after perfusion for 10 min in the absence of Ca^{2+} (Seglen, 1976); and (2) the final resuspension medium in which experiments were performed did not contain bovine serum albumin. Hepatocytes were resuspended to a final concentration of about 12 mg dry wt./ml, as determined in duplicate for each cell preparation. Cell viability was judged to be satisfactory by the criteria described previously (Hems *et al.*, 1978a).

Portions of the hepatocyte suspension were incubated in plastic scintillation vials in a shaking water bath at 37°C. The vials were gassed with O_2/CO_2 (19:1) at the beginning of the incubation period, and every 20 min thereafter. In the early labelling experiments, the results of which are described in Fig. 1 and Table 1, incubations were commenced with the addition of 1 ml of a concentrated cell suspension to plastic vials containing radioactive precursors and vasopressin in 1 ml of bicarbonate-buffered saline (Krebs & Henseleit, 1932). In subsequent labelling experiments, hepatocytes were pre-incubated in bulk with [^{32}P]orthophosphate for 20 min and experimental incubations were commenced with the addition of 1 ml of the pre-incubated suspension to vials already containing hormones in buffer (10–30 μl). Basal phosphatidylinositol labelling in these experiments was determined in samples of hepatocyte suspensions extracted immediately after the pre-incubation period. The influence of vasopressin on total hepatic phosphatidylinositol concentration was determined by measuring phosphatidylinositol lipid phosphorus or by measuring the disappearance of the lipid that had been pre-labelled with [$2\text{-}^3\text{H}$]myo-inositol. In the latter case, hepatocytes were prepared from rats that were injected with 30 μCi of [$2\text{-}^3\text{H}$]myo-inositol 20 h

previously. In all experiments concerning phosphatidylinositol degradation, 1 ml portions of cell suspensions were added to plastic vials containing hormone additions in 10–30 μl of buffer.

Lipid extraction and isolation

In early experiments where there was no pre-incubation period (Fig. 1, Table 1), incubations were terminated by the addition of 0.1 vol. of trichloroacetic acid (80%, v/v), and lipids were extracted (Kirk *et al.*, 1979) and separated by t.l.c. (Kirk *et al.*, 1977) as described previously. All other incubations were terminated with the addition of 3.75 vol. of chloroform/methanol (1:2, v/v), the lipids extracted by the method of Lapetina & Michell (1972) and phosphatidylinositol separated from other lipids by chromatography on formaldehyde-treated paper (Kirk *et al.*, 1979).

The radioactivity in ^{32}P -labelled phospholipids separated by t.l.c. was determined by liquid-scintillation spectrometry of the silica-gel-bound lipids. Radioactivity in ^{14}C - or ^3H -labelled phospholipids was determined by liquid-scintillation spectrometry after they were eluted from the silica gel with chloroform/methanol/ NH_3 (sp.gr. 0.88) (50:50:1, by vol.). Radioactivity in [^{32}P]phosphatidylinositol separated by paper chromatography was determined by Čerenkov counting after the wet-ashing of lipid samples in HClO_4 (Jones *et al.*, 1979). Initial experiments revealed that greater than 98% of the radioactivity of phospholipids extracted from hepatocytes labelled with [$2\text{-}^3\text{H}$]myo-inositol *in vivo* was located in phosphatidylinositol. Consequently, phosphatidylinositol degradation was assessed by measuring the loss of radioactivity from the total cell lipid by liquid-scintillation spectrometry.

Subcellular fractionation

All manipulations were performed at 0–4°C. Hepatocyte suspensions were sedimented by centrifugation at 500 g for 1 min to yield 2.5–3 ml of packed cells and resuspended in 8 vol. (20–24 ml) of homogenization medium containing 0.3 M-sucrose and 1 mM-EDTA. The pH of this medium, which was used for all subsequent manipulations including resuspension of sedimented fractions, was adjusted to 7.0 with NaHCO_3 . The hepatocytes were homogenized with 60–70 strokes of a tight-fitting Dounce homogenizer (Blaessig Glass, Rochester, NY, U.S.A.), when more than 90% of the cells were disrupted as observed by phase-contrast microscopy.

A portion of the homogenate was stored at –20°C for analysis and the remainder was centrifuged at 1000 g for 10 min. The pellet thus sedimented was washed twice by resuspension in 5 ml of medium followed by centrifugation at 1000 g for 10 min. The final pellets were resuspended to 5 ml

('nuclear' fraction). The combined supernatants were centrifuged at 5000 *g* for 10 min, and the pellets were resuspended and sedimented twice more from 3 ml of medium at 5000 *g* for 10 min. The final pellets were resuspended to 5 ml ('mitochondrial' fraction). The combined supernatants were centrifuged at 12000 *g* for 20 min. The pellets were washed once by resuspension in 3 ml of medium and sedimented at 12000 *g* for 20 min. The final pellets were resuspended in 5 ml ('light-mitochondrial' or 'lysosomal' fraction). The combined supernatants were centrifuged at 144000 *g* for 40 min to yield a 'particle-free supernatant' and a sedimented 'microsomal' pellet. The latter pellet was resuspended to a volume of 5 ml. Samples of these fractions were immediately subjected to lipid extraction, and the remainder was used for other analyses either immediately or after storage at -20°C .

Enzyme assays

These were performed in duplicate, usually on fractions that had been stored at -20°C and thawed only once.

Succinate dehydrogenase (EC 1.3.99.1) and alkaline phosphodiesterase I (EC 3.1.4.1) were measured as described by Lloyd-Davies *et al.* (1972). 5'-Nucleotidase (EC 3.1.3.5) was measured essentially by the method of Michell & Hawthorne (1965), but the P_i liberated was determined by the method of Baginski *et al.* (1967). β -Glucuronidase (EC 3.2.1.31) and arylesterase (EC 3.1.1.2) were assayed as described by Shephard & Hübscher (1969).

Adenylate cyclase (EC 4.6.1.1) was assayed in incubations lasting 10 min at 30°C by a modification of the method of Houslay *et al.* (1976) in which residual ATP was removed from the assay mixture by the addition of 0.045 g of neutral alumina (dry solid) to each tube followed by vortex-mixing and centrifugation. Glucagon-stimulated adenylate cyclase activity was determined in the presence of $1\mu\text{M}$ -glucagon. Cyclic AMP formed in the adenylate cyclase assays was determined in heat-inactivated assay medium as described previously (Kirk & Hems, 1974).

Chemical methods

Protein and DNA were determined as described by Lloyd-Davies *et al.* (1972). P_i was measured as described by Jones & Michell (1974).

Materials

Bovine serum albumin (fraction V) was supplied by Miles Laboratories (U.K.). Collagenase, vasopressin (grade VI), phospholipid standards, neutral alumina (WN3) and substrates for the determination of enzyme activities were from Sigma Chemical Co.

$[^{32}\text{P}]\text{P}_i$, $[2\text{-}^3\text{H}]\text{myo}$ -inositol, $[\text{U-}^{14}\text{C}]\text{glycerol}$,

$[1\text{-}^{14}\text{C}]\text{oleic acid}$ and $[8\text{-}^3\text{H}]\text{adenosine } 3\text{:}5\text{-cyclic monophosphate}$ were obtained from The Radiochemical Centre.

$[1 - (\beta\text{-Mercapto} - \beta, \beta\text{-cyclopentamethylenepro-pionic acid}), 8\text{-arginine}]\text{vasopressin}$ ($[1 - \{\alpha\text{-(1-mercaptopcyclohexyl)acetic acid}\}, 8\text{-arginine}]\text{vasopressin}$) was a generous gift from Professor M. Manning, Medical College of Ohio, Toledo, OH, U.S.A.

Results

Influence of vasopressin on the incorporation of precursors into hepatocyte phospholipids

The incorporation of P_i into major hepatic phospholipids followed a rising time course over 60 min incubation (Fig. 1) and a constant rate of $[^{32}\text{P}]\text{P}_i$ labelling was only achieved after about 20 min. Hence, in subsequent labelling experiments, hormone treatment was commenced after 20 min pre-incubation with $[^{32}\text{P}]\text{P}_i$. Vasopressin significantly stimulated the incorporation of P_i into phosphatidylinositol at all times (Fig. 1; $P < 0.01$). The hormone did not significantly change the labelling of five other hepatic phospholipids. The apparent stimulations of the low rates of P_i incorporation into sphingomyelin and lysophosphatidylcholine were not statistically significant and probably resulted from cross contamination with phosphatidylinositol during t.l.c.

Vasopressin also enhanced the incorporation of $[\text{U-}^{14}\text{C}]\text{glycerol}$ and of albumin-bound $[1\text{-}^{14}\text{C}]\text{oleic acid}$ into phosphatidylinositol, but not into phosphatidylcholine or phosphatidylethanolamine (Table 1). The rates at which these two precursors were incorporated into hepatocyte phospholipids in the absence of vasopressin were similar to those observed in previous studies (Ontko, 1972; Groener & Van Golde, 1977).

Influence of vasopressin on phosphatidylinositol breakdown

Vasopressin caused a small, but rapid, breakdown of phosphatidylinositol in hepatocytes. The time course of this breakdown, which was similar whether detected as a decrease in the concentration of phosphatidylinositol or as a loss of radioactivity from the phosphatidylinositol of hepatocytes pre-labelled with $[2\text{-}^3\text{H}]\text{myo}$ -inositol *in vivo*, is shown in Fig. 2. A statistically significant breakdown of hepatic phosphatidylinositol was discernible within 5 min of hormone treatment, and maximal depletion of about 10% of the lipid occurred 10 min later.

Concentration dependence of vasopressin-stimulated phosphatidylinositol breakdown and resynthesis, and the effects of Ca^{2+} depletion and insulin

The concentration curve describing vasopressin-stimulated P_i incorporation into hepatocyte

Fig. 1. Time course of P_i incorporation into hepatic phospholipids

Hepatocytes were prepared and incubated, with (●) or without (○) 23 nM-vasopressin, as described in the text. Where cells were treated with vasopressin, the hormone was added simultaneously with [32 P] P_i . Incubations were terminated at the times indicated with 0.1 vol. of 80% (w/v) trichloroacetic acid, and lipids were extracted as described in the text. Results are means $\pm$ S.E.M. of the results from three to five separate hepatocyte preparations.

phosphatidylinositol is shown in Fig. 3(a). At saturating concentrations, vasopressin stimulated phosphatidylinositol labelling about 9-fold. The concentration dependence of this phenomenon was not significantly influenced when hepatocytes were incubated in the presence of insulin (100 m-units/ml).

When extracellular Ca^{2+} was chelated with excess EGTA, both basal and vasopressin-stimulated P_i incorporation into phosphatidylinositol were diminished ($P < 0.01$ at all concentration). Nevertheless, in these conditions, saturating vasopressin concentrations still increased phosphatidylinositol labelling 5-fold.

Table 1. Influence of vasopressin on the incorporation of glycerol and fatty acids into hepatic phospholipids

Hepatocytes were prepared as described in the text and incubated for 20 min with radioactive precursors at the concentration indicated. Lipids were extracted and isolated by thin-layer (phosphatidylcholine, phosphatidylethanolamine) or paper (phosphatidylinositol) chromatography. Results are expressed as means ± s.e.m. from three separate hepatocyte preparations. *P (versus hormone-free controls) < 0.01.

[Vasopressin] (M)	¹⁴ C-labelled precursor	Incorporation of precursor (nmol/g dry wt./20 min) into:		
		Phosphatidyl- choline	Phosphatidyl- ethanolamine	Phosphatidyl- inositol
0	Glycerol (2 mM)	144 ± 27	174 ± 40	68 ± 11
2.3 × 10 ⁻⁸	Glycerol (2 mM)	116 ± 16	184 ± 51	156 ± 5*
0	Oleic acid (1 mM)	375 ± 22	567 ± 187	470 ± 36
2.3 × 10 ⁻⁸	Oleic acid (1 mM)	401 ± 55	477 ± 169	758 ± 55*

Fig. 3. Concentration dependence of vasopressin-stimulated phosphatidylinositol metabolism: influence of Ca²⁺ depletion and insulin

Hepatocytes were preincubated for 20 min in the presence of [³²P]P_i before hormone additions were made (Fig. 3a). Incubations were terminated 20 min later. Cells were incubated in standard medium containing Ca²⁺ (2.5 mM) with (■) or without (●) insulin (100 m-units/ml) or in Ca²⁺-free medium to which 2 mM-EGTA was added at the beginning of the pre-incubation period (○). Hepatocytes prepared from rats injected with 30 μCi of [2-³H]myo-inositol 20 h previously were incubated for 15 min with various concentrations of hormone (Fig. 3b). Phosphatidylinositol degradation in vasopressin-stimulated hepatocytes is expressed as percentage diminution of lipid-soluble ³H (d.p.m.) compared with hormone-free controls. Results are means ± s.e.m. for the results from three to seven separate hepatocyte preparations for each point.

Hepatocytes were prepared from normal or [2-³H]myo-inositol-injected rats as described in the text and incubated with (●) or without (○) vasopressin (0.23 μM) for the periods indicated. P_i was assayed in HClO₄ digests of separated phosphatidylinositol from non-radioactive hepatocyte suspensions (Fig. 2a). In hepatocytes prepared from [2-³H]inositol-pretreated rats, ³H (d.p.m.) in phosphatidylinositol was assayed as described in the text (Fig. 2b). In each experiment using [2-³H]myo-inositol-injected rats, phosphatidylinositol content (i.e. radioactivity in phosphatidylinositol) at a given time was calculated as a percentage of the mean phosphatidylinositol content of the controls for all the time periods tested. Results are means ± s.e.m. for three to five separate hepatocyte preparations. P (versus hormone-free controls): * < 0.01, † < 0.02.

The concentration dependence of vasopressin-induced phosphatidylinositol breakdown (Fig. 3b) was very similar to that of the enhanced labelling of the lipid with [32 P]P_i. Hence the P_i-labelling response in hepatocytes seems to be a sensitive and approximately quantitative reflection of vasopressin-stimulated phosphatidylinositol breakdown.

Competitive inhibition of vasopressin-induced phosphatidylinositol labelling by [1-(β -mercapto- β , β -cyclopentamethylenepropionic acid),8-arginine]-vasopressin

Vasopressin-induced phosphatidylinositol labelling was competitively inhibited by [1-(β -mercapto- β , β -cyclopentamethylenepropionic acid),8-arginine]-vasopressin (Fig. 4). The data in Fig. 4 were replotted by the method of Schild (1947) and the pA₂ [$-\log$ (antagonist concentration that doubles the concentration of agonist required to evoke a half-maximal response)] for this antagonism was found to be 9.0 (Fig. 4, inset).

The subcellular distribution of vasopressin-induced phosphatidylinositol depletion

The distribution of [3 H]phosphatidylinositol, vasopressin-induced phosphatidylinositol depletion and various subcellular markers amongst primary fractions prepared from vasopressin-treated and control hepatocytes is shown in Table 2. Relative specific activities for these parameters were calculated by the methods of De Duve *et al.* (1955) and these are presented as distribution diagrams in Fig. 5. Control experiments showed that the [3 H]phosphatidylinositol content of homogenates did not change if they were maintained on ice during the entire period of the subcellular fractionation.

A substantial separation of the various subcellular components of the hepatocytes was achieved, but it was not possible to determine the distribution of the relatively small phosphatidylinositol depletion provoked by vasopressin treatment for 5 min with the same precision as the distributions of the other markers in Table 2. Nevertheless it is clear from Table 2 and Fig. 5 that

Fig. 4. *Inhibition of vasopressin-stimulated phosphatidylinositol labelling by [1-(β -mercapto- β , β -cyclopentamethylenepropionic acid),Arg⁸]vasopressin*

Hepatocytes were prepared and incubated in the presence of [Arg⁸]vasopressin as described in Fig. 3(a). The antagonist, [1-(β -mercapto- β , β -cyclopentamethylenepropionic acid),Arg⁸]vasopressin was added after 15 min pre-incubation with [32 P]P_i (5 min before the addition of [Arg⁸]vasopressin) to give the following concentrations: 24 nM (Δ), 80 nM ($\square$), 0.24 μ M ($\blacktriangle$), 0.8 μ M ($\blacksquare$). Results in the absence of antagonist (O) are taken from Fig. 3(a). Other results are means $\pm$ S.E.M. from three separate hepatocyte preparations. When analysed by the method of Schild (1947), these results yield a pA₂ of 9.0 for the inhibition of vasopressin-stimulated phosphatidylinositol labelling by this antagonist (see the inset).

Table 2. *Distribution of phosphatidylinositol, vasopressin-induced phosphatidylinositol depletion and subcellular markers amongst primary fractions of rat hepatocytes* Hepatocytes were prepared from [$2\text{-}^3\text{H}$]myo-inositol-injected rats, incubated for 5 min with or without vasopressin ($0.23\text{ }\mu\text{M}$), homogenized and fractionated as described in the text. The distribution of [^3H]phosphatidylinositol, DNA and marker enzymes was measured in fractions prepared from control and vasopressin-treated hepatocytes from the same animal. Distributions are expressed as percentage of total recovered activity. Protein content is expressed as mg/ml of homogenate. Specific activities of marker enzymes were determined in samples of unfractionated homogenates, and are expressed as μmol of substrate converted/mg of protein per h. Phosphatidylinositol degradation was calculated from the diminution of lipid-soluble ^3H (d.p.m.) in vasopressin-treated fractions compared with control fractions from the same animal. Results are means $\pm$ s.e.m. for the numbers of separate hepatocyte preparations indicated in parentheses. Abbreviation used: N.D., not detectable.

	[Vasopressin] (M)	Values for homogenate	Distribution (% of total recovered)					Recovery (% of total in homogenate)
			Nuclear	Mitochondrial	Lysosomal	Microsomal	Particle-free supernatant	
Protein	0	14.3 ± 0.3	29.3 ± 0.7	15.5 ± 1.5	5.2 ± 0.3	15.3 ± 0.9	34.8 ± 2.3	93.8 ± 2.2 (4)
[^3H]Phosphatidyl- inositol distribution	2.3×10^{-7}	14.5 ± 0.4	29.0 ± 0.6	15.6 ± 1.4	5.3 ± 0.4	15.4 ± 1.0	34.9 ± 4.3	93.2 ± 3.6 (4)
[^3H]Phosphatidyl- inositol depletion	0	—	23.1 ± 1.2	17.1 ± 1.8	13.1 ± 0.9	42.8 ± 1.2	4.0 ± 0.7	92.0 ± 1.5 (4)
Alkaline phospho- diesterase	2.3×10^{-7}	—	23.5 ± 1.1	17.5 ± 1.5	13.2 ± 1.1	41.9 ± 1.4	4.0 ± 0.8	91.7 ± 1.7 (4)
5'-Nucleotidase	2.3×10^{-7}	—	18 ± 5	16 ± 4	14 ± 4	51 ± 4	3 ± 2	104 ± 13 (4)
Basal adenylate cyclase	0	6.9 ± 0.7	16.2 ± 3.3	24.8 ± 3.1	18.9 ± 3.6	36.4 ± 2.0	3.9 ± 0.9	81.7 ± 7.5 (3)
Glucagon-stimulated adenylate cyclase	2.3×10^{-7}	7.9 ± 0.6	17.0 ± 3.6	24.7 ± 3.5	19.2 ± 3.8	34.9 ± 2.7	4.1 ± 0.8	77.8 ± 8.6 (3)
Arylesterase	0	3.0 ± 0.5	16.6 ± 1.3	19.6 ± 2.0	19.9 ± 3.8	35.3 ± 4.5	8.7 ± 2.9	94.0 ± 5.1 (4)
β -Glucuronidase	2.3×10^{-7}	2.7 ± 0.5	16.5 ± 0.7	21.7 ± 1.9	18.9 ± 3.7	35.1 ± 5.1	7.8 ± 2.2	103.5 ± 6.7 (4)
Succinate dehydrogenase	0	$(2.7 \pm 0.7) \times 10^{-4}$	19.1 ± 4.1	21.7 ± 1.7	17.3 ± 4.3	21.0 ± 4.7	20.9 ± 3.8	96.8 ± 9.9 (3)
DNA	2.3×10^{-7}	$(2.6 \pm 0.5) \times 10^{-4}$	19.1 ± 2.0	20.6 ± 2.4	18.5 ± 4.3	21.8 ± 4.0	20.2 ± 3.1	92.7 ± 3.4 (3)
	0	$(1.4 \pm 0.2) \times 10^{-3}$	20.5 ± 5.9	26.3 ± 3.8	20.1 ± 5.6	19.8 ± 2.0	12.6 ± 1.4	83.8 ± 7.2 (3)
	2.3×10^{-7}	$(1.4 \pm 0.1) \times 10^{-3}$	20.1 ± 2.1	28.9 ± 0.7	19.3 ± 5.4	17.2 ± 1.0	14.6 ± 3.2	86.7 ± 6.8 (3)
	0	91.2 ± 12.0	13.3 ± 2.3	12.0 ± 1.9	14.2 ± 1.3	54.2 ± 1.9	6.2 ± 0.6	86.8 ± 1.9 (3)
	2.3×10^{-7}	90.6 ± 18.6	12.6 ± 1.9	12.7 ± 1.8	13.6 ± 0.9	54.2 ± 1.5	7.1 ± 0.8	91.9 ± 6.4 (3)
	0	$(5.1 \pm 0.4) \times 10^{-1}$	13.9 ± 1.1	32.3 ± 1.2	17.0 ± 0.9	26.3 ± 0.4	10.6 ± 0.4	86.5 ± 1.8 (3)
	2.3×10^{-7}	$(4.9 \pm 0.2) \times 10^{-1}$	14.0 ± 0.3	30.6 ± 2.6	19.1 ± 2.8	25.7 ± 0.3	10.5 ± 0.6	83.9 ± 0.4 (3)
	0	4.2 ± 0.1	62.5 ± 1.7	36.5 ± 2.0	2.5 ± 0.3	1.9 ± 0.3	N.D.	80.9 ± 5.1 (3)
	2.3×10^{-7}	3.9 ± 0.4	60.7 ± 1.8	34.8 ± 1.8	2.7 ± 0.6	1.8 ± 0.3	N.D.	85.9 ± 6.2 (3)
	0	5.6 ± 0.6	62.6 ± 8.6	7.4 ± 0.7	5.5 ± 1.1	8.8 ± 0.7	17.1 ± 6.4	100.2 ± 6.2 (3)
	2.3×10^{-7}	5.0 ± 0.9	65.7 ± 5.9	7.9 ± 0.2	5.1 ± 0.5	7.3 ± 0.2	15.7 ± 3.9	104.1 ± 7.0 (3)

Fig. 5. Distribution of phosphatidylinositol, vasopressin-induced phosphatidylinositol depletion and subcellular markers amongst primary fractions of rat hepatocytes

Hepatocytes were prepared, incubated and fractionated as described in Table 2. The relative specific activities (De Duve *et al.*, 1955) for distributions of phosphatidylinositol, DNA and various marker enzymes are expressed as the means of the values obtained for control and vasopressin-treated cells (from Table 2). Fractions are represented in the order of their isolation, from left to right: nuclear (N), mitochondrial (Mt), lysosomal (L), microsomal (Mc), particle-free supernatant (PFS). Phosphatidylinositol depletion was calculated from the diminution of lipid-soluble ^3H (d.p.m.) in vasopressin-treated fractions compared with control fractions from the same animal. The parameters depicted in the Figure are (a) phosphatidylinositol degradation, (b) phosphatidylinositol distribution, (c) 5'-nucleotidase activity, (d) alkaline phosphodiesterase activity, (e) basal adenylate cyclase activity, (f) glucagon-stimulated adenylate cyclase activity, (g) arylesterase activity, (h) succinate dehydrogenase activity, (i) β -glucuronidase activity and (j) DNA. Results are from three or four separate hepatocyte preparations. Other details are as described in Table 2.

the distribution of phosphatidylinositol depletion in the separated fractions was quite different from all other measured distributions except those of total [^3H]phosphatidylinositol and the endoplasmic-reticulum marker arylesterase. At the precision with which our measurements were made, the distribution of phosphatidylinositol depletion did not differ significantly from either of these parameters.

Discussion

Of the major phospholipids, only phosphatidylinositol exhibits significantly enhanced P_i incorporation after vasopressin treatment in hepatocytes (see also Kirk *et al.*, 1977). Hence the effect of vasopressin is specific to phosphatidylinositol metabolism and not a more general metabolic effect such

as on the labelling of intracellular ATP. As in other tissues (for references, see Jones *et al.*, 1979), enhanced phosphatidylinositol labelling in hepatocytes is accompanied by a rapid diminution in the cellular concentration of the lipid. Thus the primary effect of vasopressin is to promote degradation of hepatic phosphatidylinositol rather than its synthesis *de novo* (see also Billah & Michell, 1979).

Unlike some other ligands, which promote only the incorporation of [^{32}P] P_i into phosphatidylinositol in their target tissues (for review, see Michell, 1975), vasopressin also enhances phosphatidylinositol labelling with [$\text{U-}^{14}\text{C}$]glycerol and [$1\text{-}^{14}\text{C}$]oleic acid, but to a lesser extent (2-fold) than is observed with [^{32}P] P_i (9-fold). The ineffectiveness of other stimuli in enhancing glycerol or fatty-acid incorporation into phosphatidylinositol has been cited as

evidence for a closed cycle of phosphatidylinositol breakdown and re-synthesis via diacylglycerol and phosphatidic acid (for review, see Michell, 1975). However, there have now been reports of ligand-stimulated glycerol and fatty-acid incorporation into phosphatidylinositol in an appreciable number of tissues (see Table II of Michell, 1975; Shohet, 1970; Calderon *et al.*, 1979). Such reports are not inconsistent with the cycle of degradation and resynthesis referred to above if newly synthesized phosphatide is able to enter the same pool as is generated from the diacylglycerol liberated during phosphatidylinositol degradation. The liver is an organ that supports a high rate of phosphatide synthesis *de novo*, and the present results suggest that vasopressin provokes a small increase in the proportion of this newly synthesized phosphatide that is converted into phosphatidylinositol.

A common feature of receptors that mediate enhanced phosphatidylinositol metabolism in a variety of tissues is their ability to provoke other physiological responses by increasing cytosolic Ca^{2+} concentration (Michell, 1979). Indeed, such responses are frequently dependent on the presence of extracellular Ca^{2+} . In the present study, Ca^{2+} depletion decreased basal or hormone-stimulated P_i incorporation into phosphatidylinositol. Nevertheless, under conditions of Ca^{2+} chelation that completely suppress the hormone's activation of glycogen phosphorylase (Whitton *et al.*, 1977; Blackmore *et al.*, 1978), vasopressin still enhanced phosphatidylinositol labelling over the same concentration range as was effective in the presence of 2.5 mM-Ca^{2+} . This observation is in agreement with the findings of Billah & Michell (1979), who also observed that ionophore A23187, which elevates cytosolic Ca^{2+} concentration and activates glycogen phosphorylase in hepatocytes (Blackmore *et al.*, 1978), did not influence basal or vasopressin-stimulated phosphatidylinositol breakdown (the hormone-stimulated event) or the subsequent labelling response. Taken together, these results indicate that vasopressin-stimulated phosphatidylinositol breakdown in hepatocytes, like that provoked by ligands in several other tissues (see Billah & Michell, 1979, for references), is not a consequence of Ca^{2+} mobilization in the cytosol. It should be noted here that recent work has revealed a subgroup of cell types, mainly derived from stem-cells in the bone-marrow (platelets, polymorphonuclear leucocytes and mast cells), in which phosphatidylinositol breakdown is, at least in part, a response to a rise in cytosol Ca^{2+} concentration (Lapetina & Cuatrecasas, 1979; Bell & Majerus, 1980; Cockcroft *et al.*, 1980); the significance of this is not yet clear.

The concentration of vasopressin required to provoke half-maximal phosphatidylinositol breakdown (4.8 nM ; Fig. 3) is approx. 16-fold greater than

that required to cause half-maximal activation of glycogen phosphorylase in hepatocytes (0.3 nM ; Kirk *et al.*, 1979). Current techniques do not allow us to detect significant phosphatidylinositol depletion at this latter concentration of vasopressin. However, studies in two laboratories suggest that the concentration of vasopressin that provokes half-maximal phosphatidylinositol breakdown is very similar to the K_a for vasopressin binding to hepatic receptors (Cantau *et al.*, 1980; Knowles, 1981). These data suggest that only a small proportion of hepatic vasopressin receptors need be occupied to activate glycogen phosphorylase, but that the degree of activation of phosphatidylinositol breakdown is approximately proportional to the extent of receptor occupation by the agonist. These observations are consistent with the suggestion that phosphatidylinositol degradation is a direct consequence of vasopressin-receptor interaction, which might evoke the mobilization of Ca^{2+} in the cytosol that is responsible for physiological effects such as glycogenolysis (Kirk *et al.*, 1977, 1979, 1980; Michell *et al.*, 1979; Billah & Michell, 1979).

Insulin is capable of diminishing the extent of vasopressin-induced glucose release from the perfused rat liver (Kirk & Hems, 1979). However, the present results indicate that insulin does not influence vasopressin-induced phosphatidylinositol turnover (Fig. 3). If the stimulation of phosphatidylinositol turnover is intrinsic to the mechanism whereby vasopressin mobilizes intracellular Ca^{2+} and activates glycogen phosphorylase in liver, then the suppression by insulin of hepatic glucose release, but not of phosphatidylinositol turnover, may indicate that insulin acts directly on the enzymes of glycogen metabolism rather than by inhibition of stimulus-response coupling at the vasopressin receptor.

We and others have previously reported that the ligand selectivity of the hepatic vasopressin receptor is very similar to that of the receptors responsible for mediating the pressor response to the hormone *in vivo* (Kirk *et al.*, 1978; Keppens & De Wulf, 1979). The present observation that [1-(β -mercapto- β , β -cyclopentamethylenepropionic acid),8-arginine] vasopressin antagonizes vasopressin-stimulated phosphatidylinositol labelling with a pA_2 of 9.0 provides further evidence of the similarity between hepatic and pressor receptors for vasopressin, since this analogue antagonizes the pressor response to vasopressin *in vivo* with a pA_2 of 8.35 (Kruszynski *et al.*, 1980). Recent experiments have also indicated that this antagonist inhibits vasopressin-stimulated phosphatidylinositol labelling in vascular smooth muscle with a pA_2 of 8.1 (Takhar & Kirk, 1981), and the similarity between the apparent affinities of the antagonist in these three assay systems supports our previous suggestion that a

single class of vasopressin receptors (V_1 -receptors) is responsible for both the hepatic and pressor actions of vasopressin (Kirk *et al.*, 1979; Michell *et al.*, 1979).

The suggestion that vasopressin-stimulated phosphatidylinositol degradation is involved in the coupling of receptor occupation to the mobilization of Ca^{2+} in the cytosol (Kirk *et al.*, 1979, 1980) leads to the prediction that phosphatidylinositol breakdown should be localized at the plasma membrane. The present results do not allow a conclusion on this point, in that a constant proportion of the initial phosphatidylinositol complement was lost from all of the subcellular fractions derived from cells stimulated with vasopressin for 5 min. The relatively close resemblance between the subcellular distributions of phosphatidylinositol depletion, of the total phosphatidylinositol of the cell, and of arylesterase, and enzyme localized in the endoplasmic reticulum, presumably reflect the fact that the endoplasmic reticulum contains the majority of the cellular presumably reflects the fact that the endoplasmic explanation of these observations would be that phosphatidylinositol breakdown occurs only at the endoplasmic reticulum, in which case an intracellular messenger other than Ca^{2+} would have to carry the vasopressin message from the plasma membrane to endoplasmic reticulum. A second alternative, that vasopressin might induce rapid phosphatidylinositol breakdown at all membranes throughout the cell, would also invoke some unknown intracellular messenger. The latter alternative also seems unlikely because individual enzyme-catalysed reactions do not usually occur at all intracellular sites at precisely equal rates. Thirdly, and maybe most likely, it is possible that phosphatidylinositol breakdown initially occurs at some single cellular locus and that the phosphatidylinositol exchange proteins of the cytosol (Zborowski & Wojtczak, 1975; Zborowski, 1979) then rapidly re-equilibrate the remaining phosphatidylinositol amongst all cellular compartments. If this ultimately proves to be the case, then the information reported here will constitute a convincing demonstration of the rapidity with which such phospholipid exchange occurs within the intact cell. It would also mean that the identification of the cellular site at which phosphatidylinositol breakdown occurs might not be amenable to study by subcellular fractionation techniques such as those described above.

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