Effects of glucagon and Ca^{2+} on the metabolism of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate in isolated rat hepatocytes and plasma membranes

David E. WHIPPS, Annie E. ARMSTON, Hilary J. PRYOR and Andrew P. HALESTRAP

Department of Biochemistry, University of Bristol, Bristol BS8 1TD, U.K.

1. Rat hepatocytes whose phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2) had been labelled for 60 min with ^{32}P were treated with glucagon for 10 min or phenylephrine for 2 min. 2. Glucagon caused a 20% increase in PIP but no change in PIP_2 whereas phenylephrine caused a similar increase in PIP but a 15% decrease in PIP_2 . Addition of both hormones together for 10 min produced a 40% increase in PIP. 3. A crude liver mitochondrial fraction incubated with [^{32}P]PI and ADP incorporated label into PIP, PIP_2 and phosphatidic acid. The PIP_2 was shown to be in contaminating plasma membranes and PIP in both lysosomal and plasma-membrane contamination. 4. A minor but definitely mitochondrial phospholipid, more polar than PIP_2 , was shown to be labelled with ^{32}P both *in vitro* and in hepatocytes. 5. The rate of ^{32}P incorporation into PIP was faster in mitochondrial/plasma-membrane preparations from rats treated with glucagon or if $3\text{ }\mu\text{M-Ca}^{2+}$ and Ruthenium Red were present in the incubation buffer. 6. Loss of ^{32}P from membranes labelled *in vitro* was shown to be accompanied by formation of inositol 1,4,5-trisphosphate (IP_3) and inositol 1,4-bisphosphate, and was faster in preparations from glucagon-treated rats or in the presence of $3\text{ }\mu\text{M-Ca}^{2+}$. It is concluded that glucagon stimulates both PIP_2 phosphodiesterase and phosphatidylinositol kinase activities, as does the presence of $3\text{ }\mu\text{M-Ca}^{2+}$. The resulting formation of IP_3 may be responsible for the observed release of intracellular Ca^{2+} stores. 8. The roles of a guanine nucleotide regulatory protein and phosphorylation in mediating these effects are discussed.

INTRODUCTION

It is now well established that Ca^{2+} -mobilizing hormones such as phenylephrine, vasopressin and angiotensin stimulate the breakdown of PIP_2 in the plasma membrane of liver cells, and that the released IP_3 is responsible for mobilization of Ca^{2+} from the endoplasmic reticulum (see Michell *et al.*, 1981; Berridge, 1984; Exton, 1985; Williamson *et al.*, 1985). These responses are extremely rapid and can be detected within a few seconds of hormone addition. Glucagon and exogenous cyclic AMP are also known to cause Ca^{2+} mobilization from the same intracellular stores, but the mechanism of this release is not established (Charest *et al.*, 1983; Sistare *et al.*, 1985; Mauger & Claret, 1986; Poggioli *et al.*, 1986). Wakelam *et al.* (1986) have reported that concentrations of glucagon below 10 nM can increase IP_3 by a cyclic AMP-independent mechanism, whereas higher concentrations of the hormone are ineffective. This confirms the observations made in other laboratories that no effects on either IP_3 or its parent phospholipid are apparent after 30 s or 1 min of glucagon (> 10 nM) treatment (Creba *et al.*, 1983; Charest *et al.*, 1985; Poggioli *et al.*, 1986). Paradoxically it has been reported that glucagon treatment leads to a stimulation of Ca^{2+} uptake into a liver microsomal preparation by a cyclic AMP-dependent mechanism, and this would appear to work against a glucagon-induced

release from this store (Taylor *et al.*, 1979, 1980; Andia-Waltenbaugh *et al.*, 1980).

During work in our laboratory on the effects of hormone treatment of rats on liver mitochondrial metabolism (for reviews see Halestrap, 1981, 1986; Halestrap *et al.*, 1983, 1985) we began to investigate possible changes in phospholipid composition of the mitochondrial membrane induced by hormones (Quinlan *et al.*, 1983; Halestrap *et al.*, 1983; Armston & Halestrap, 1984). In the course of these studies it became apparent that, when a normal liver mitochondrial preparation was incubated with respiratory substrate, ADP and [^{32}P]PI, there was incorporation of ^{32}P into endogenous phosphatidic acid, PIP and PIP_2 (Halestrap *et al.*, 1983). Furthermore, glucagon treatment of the rats from which the mitochondrial fraction was derived was shown to influence this labelling process and stimulate subsequent breakdown of PIP and PIP_2 . Early work on the metabolism of these phospholipids suggested that they might be located in the mitochondrial membranes themselves, but it was later shown that they were synthesized in contaminating plasma membranes (Galliard & Hawthorne, 1963; Michell & Hawthorne, 1965; Galliard *et al.*, 1965; Hajra *et al.*, 1965). In the present paper we confirm that it is the contaminating plasma membranes of our crude liver mitochondrial preparation that incorporate ^{32}P into PIP and PIP_2 and that this preparation is suitable for studying the

Abbreviations used: PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2 , phosphatidylinositol 4,5-bisphosphate; IP_3 , *myo*-inositol 1,4,5-trisphosphate.

Table 1. Enzyme marker distribution of various cell fractions

Preparation of plasma membranes from hepatocytes and Percoll or digitonin treatment of mitochondrial fractions isolated from whole liver were performed as described in the Experimental section. Results are given as means $\pm$ S.E.M. of n observations and represent the enzyme specific activity of the fraction as a percentage of that of the starting material (cells or mitochondria).

Enzyme	Enzyme specific activity as percentage of that in:			
	Hepatocytes	Isolated liver mitochondria		
	Plasma membranes	Percoll	Digitonin	Percoll + digitonin
	$n =$			
Citrate synthase	3	4	4	1
5'-Nucleotidase	—	116 $\pm$ 6.7	137 $\pm$ 12.0	146
Glucose-6-phosphatase	214 $\pm$ 1.1	18 $\pm$ 1.1	64 $\pm$ 11.4	6
Acid phosphatase	117 $\pm$ 4.3	68 $\pm$ 9.8	60 $\pm$ 10.0	32
Succinate dehydrogenase	98 $\pm$ 15.0	88 $\pm$ 5.5	42 $\pm$ 14.8	21
	75 $\pm$ 13.4	—	—	—

metabolism of these phospholipids *in vitro*. We confirm that plasma membranes derived from glucagon-treated rats are able to hydrolyse PIP and PIP₂ faster than those from control rats, and provide evidence that this also occurs *in situ* when hepatocytes are treated with glucagon. In addition we present data from experiments with isolated liver cells and plasma-membrane preparations that suggest that both Ca²⁺ and glucagon may cause a stimulation of PI kinase.

EXPERIMENTAL

Materials

[1-¹⁴C]Arachidonic acid was from Amersham International, Amersham, Bucks., U.K., and was stored in 5 μ Ci batches under N₂. Neomycin sulphate was from Sigma, Poole, Dorset, U.K. All other chemicals, biochemicals and radiochemicals were from the sources cited previously (Quinlan *et al.*, 1983).

Liver cells were isolated from male Wistar rats (250–300 g body wt.) starved for 24 h before use, and liver mitochondria from fed female Wistar rats (225–260 g body wt.), by standard methods described previously (Halestrap, 1978; Quinlan *et al.*, 1983). Where required, rats were first injected (intraperitoneally) with glucagon (1 mg/kg body wt.) 15 min before death (Halestrap, 1978).

Liver-cell incubations

For the labelling of the phospholipids with ³²P, liver cells were incubated at 10 mg of protein/ml in low-phosphate (0.575 mM) bicarbonate-buffered saline medium (Krebs & Henseleit, 1932) gassed with O₂/CO₂ (19:1) and containing [³²P]P_i (0.1 mCi/ml), bovine serum albumin (20 mg/ml), 10 mM-L-lactate and 1 mM-pyruvate as described previously (Vargas *et al.*, 1982; Quinlan *et al.*, 1983). After preincubation for 1 h, at which time it was confirmed that the labelling of the 4- and 5-phosphate groups of PIP and PIP₂ had reached equilibrium (see also Creba *et al.*, 1983; Thomas *et al.*, 1983), 100 nM-glucagon, 25 nM-vasopressin or 20 μ M-phenylephrine was added as required. Incubation was continued for 10 min (glucagon) or 2 min (phenylephrine and vasopressin) before centrifugation of 1 ml samples of

the cell suspension for 3 s in an Eppendorf 5414 centrifuge. The supernatant was then discarded and the pellet resuspended in either 1 ml of ice-cold extraction buffer (300 mM-sucrose, 20 mM-Tris/HCl, 2 mM-EGTA and 0.2 mM-neomycin sulphate) for the preparation of mitochondrial and plasma-membrane fractions (see below) or 250 μ l of water for the measurement of whole-cell [³²P]phospholipids. Where incorporation of [1-¹⁴C]arachidonic acid was to be studied, the protocol was identical except that [³²P]P_i was replaced by 25 μ M-[1-¹⁴C]arachidonic acid (1 μ Ci/ml) and preincubation was for 90 min (Thomas *et al.*, 1983).

Fractionation of liver cells

Liver cells were disrupted by sonication of the resuspended cell pellet as described previously (Vargas, 1982; Vargas *et al.*, 1982; Quinlan *et al.*, 1983). Mitochondria were purified by density-gradient centrifugation by using Percoll (Vargas *et al.*, 1982) and resuspended in 250 μ l of water for extraction of phospholipids. A plasma-membrane fraction was prepared by addition of Percoll (final concn. 17.5%, v/v) to the sonicated cell homogenate and centrifuging for 5 min at 10000 g and 5 °C. A 200 μ l fraction was removed from the top of the Percoll gradient and mixed with 1 ml of sucrose extraction medium and centrifuged as before. The bottom 200 μ l of the resulting gradient was taken for the plasma-membrane fraction.

³²P-labelling of plasma membrane phospholipids *in vitro*

Time courses of the incorporation of ³²P from [γ -³²P]ATP into the phospholipids of plasma membranes in crude mitochondrial preparations were performed at 37 °C in a medium containing 125 mM-KCl, 10 mM-Mops, 7 mM-Tris, 1 mM-EGTA, 0.25 mM-[³²P]P_i (0.1 mCi/ml), 5 mM-succinate, 0.1 mM-ADP and rotenone (1 μ g/ml), adjusted to pH 7.25 with KOH. Buffer (2 ml) was incubated in sealed plastic vials (10 ml capacity) for 5 min at 37 °C in a shaking water bath before the crude mitochondrial preparation (8 mg of protein/ml) was added and rapidly vortex-mixed. At the required times 0.25 ml samples were removed and rapidly mixed with 0.6 ml of chloroform/methanol/12 M-HCl (80:40:1, by vol.) containing 0.1 mg of 2,6-dibutyl-p-cresol/ml (Dawson & Eichberg, 1965) in a 1.5 ml

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Separation of diacylglycerols, inositol phospholipids and inositol phosphates

Inositol-containing phospholipids were separated by t.l.c. on Merck 5735 silica-coated plastic sheets pretreated by wetting with acetone, soaking in potassium oxalate (1%, w/v) for 5 min, activating at 110 °C for 10 min and then developed in chloroform/acetone/methanol/acetic acid/water (40:15:13:12:8, by vol.) as described by Jolles *et al.* (1979). For the separation of neutral lipids, oxalate treatment was omitted and the solvent system was benzene/diethyl ether/ethanol/ammonia (500:400:200:1, by vol.) as described by Rittenhouse-Simmons (1979). Spots were detected by radioautography (Vargas *et al.*, 1982), and the radioactivity in each was determined. This was performed either by liquid-scintillation counting after cutting out the relevant spot or by densitometric scan of the film with a Joyce-Loebl Chromoscan 3 interfaced to a Hewlett Packard 9845s computer. Known amounts of ^{32}P were spotted before radioautography for standardization. When both techniques were performed on the same t.l.c. plate, the results agreed within 10% provided that the optical density of a spot on the radioautogram did not exceed 1. No attempt was made to quantify incorporation of label in absolute terms, but rather incorporation relative to control was estimated.

Inositol phosphates produced during incubation of the mitochondria/plasma membranes with $^{32}\text{P}]\text{P}_i$ were measured in the supernatant after precipitation of protein with 2% (w/v) HClO_4 . After neutralization with 1 M-KOH containing 0.2 M-ethanolamine, a fraction containing the inositol phosphates was partially purified by formic acid/ammonium formate elution from Dowex 1 as described by Downes & Michell (1981). A 1 ml portion of neutralized extracts was applied to a 1.5 ml column of Dowex-1 (formate form), and the column was washed with 30 ml of 0.1 M-formic acid/0.2 M-ammonium formate. The fraction eluted with 15 ml of 0.1 M-formic acid/1.0 M-ammonium formate was passed through a 50 ml column of Dowex 50W-X8 (acid form) and freeze-dried, before redissolution in water and separation of inositol phosphates by high-voltage electrophoresis by using a procedure modified from Agranoff *et al.* (1983). Electrophoresis was for 20 min at 1000 V in 60 mM-oxalic acid (pH 1.8), on thin-layer cellulose plates (Eastman-Kodak). Samples of ^{32}P -labelled IP_2 and IP_3 were purified from erythrocytes as described by Downes *et al.* (1982) and used as standards. Spots were detected by radioautography as described above.

RESULTS

Effects of hormones on polyphosphoinositide metabolism by hepatocytes

Table 2 presents data on the effects of hormones on the ^{32}P content of phosphatidic acid, PI, PIP and PIP_2 of hepatocytes and a plasma-membrane fraction derived from them. The enrichment of this fraction with plasma membranes was only 2–3-fold, as shown in Table 1. However, it could be produced with sufficient speed to minimize breakdown of PIP and PIP_2 ; 200 μM -neomycin was also present throughout for this purpose. The cells were pre-equilibrated with ^{32}P for 1 h before addition of 10 nM-glucagon for 10 min or 20 μM -phenylephrine and 25 nM-vasopressin for 2 min. In agreement with the work

Fig. 1. Time course of ^{32}P incorporation into liver plasma-membrane/mitochondrial fractions derived from control and glucagon-treated rats

Liver mitochondrial plasma-membrane preparations were incubated at 10 mg of protein/ml and 37 °C in buffer containing $^{32}\text{P}]\text{P}_i$ and ADP as described in the Experimental section. At the times shown, samples were removed and the ^{32}P -labelled phospholipids extracted, separated by t.l.c. and counted for radioactivity. Incorporation of ^{32}P into PIP (●, ○), PIP_2 (■, □) and phosphatidic acid (▲, △) was expressed as a percentage of the ^{32}P incorporation into total phospholipids (the sum of radioactivity in all radioactive spots) at 15 min. In (b) the ratio of PIP to PIP_2 at each time is plotted. Values are given as means $\pm$ S.E.M. (error bars) of experiments on 10 separate mitochondrial preparations from livers of both control (●, ■, ▲, ▼) or glucagon-treated (○, □, △, ▽) rats, except for the 30 s time point, where only three experiments were performed. The statistical significance of the difference between control mitochondrial preparations and those prepared on the same day from glucagon-treated animals was determined by paired Student's *t* test: **P* < 0.05; ***P* < 0.02; ****P* < 0.01).

Fig. 2. Radioautographs of t.l.c. separations of ^{32}P -labelled phospholipids in crude mitochondrial fraction before and after Percoll and digitonin treatment

Phospholipids in the mitochondrial/plasma-membrane fraction were either labelled with ^{32}P *in vitro* for 10 min as described in the legend to Fig. 1 (part a, and part b, tracks 1–4), or *in situ* with isolated hepatocytes (part b, tracks 5–9) as described by Quinlan *et al.* (1983). Treatment of the mitochondrial fraction with Percoll and digitonin was performed as described in the Experimental section before extraction of the phospholipids, separation by t.l.c. and radioautography. The location of phosphatidic acid (PA), phosphatidylethanolamine (PE), phosphatidylcholine (PC), PI, PIP and PIP_2 were determined with standards. In addition, the location of an unidentified phospholipid (?), solvent front (F) and the origin (O) are marked.

of many others (see Exton, 1981; Creba *et al.*, 1983; Thomas *et al.*, 1983), both phenylephrine and vasopressin produced a decrease in PIP_2 and an increase in phosphatidic acid and PI labelling. Glucagon was without significant effect on any of these phospholipids.

However, in contrast with previous reports (Creba *et al.*, 1983; Poggioli *et al.*, 1986), we also detected a significant increase in PIP in response to either glucagon or phenylephrine and an additive effect of both hormones together. In a separate set of experiments the incorporation of [^{14}C]arachidonic acid into diacylglycerol was measured as described by Thomas *et al.* (1983). The documented increased incorporation of label was observed with phenylephrine and vasopressin, but with glucagon the slight increase observed was not statistically significant.

Time course of ^{32}P incorporation into PIP and PIP_2 of a mitochondrial/plasma-membrane fraction

The time courses of ^{32}P incorporation from [$\gamma\text{-}^{32}\text{P}$]ATP into phosphatidic acid, PIP and PIP_2 in crude mitochondrial/plasma-membrane fractions prepared from control and glucagon-treated rats are shown in Fig. 1(a). Results are given as the means $\pm$ S.E.M. of ten separate experiments. As expected, the labelling of PIP precedes that of PIP_2 and reaches a plateau at about 4 min, compared with the PIP_2 which reaches a plateau at about 10 min. This is reflected in the steadily decreasing ratio of PIP to PIP_2 at each successive time point (Fig. 1b). It was confirmed that the specific radioactivity of [$\gamma\text{-}^{32}\text{P}$]ATP, which is formed from [^{32}P]P_i and ADP by the mitochondria in the assay, was maximal in less than 30 s. From the known rates of oxidative phosphorylation of the mitochondria, the labelling of the γ -phosphate of ATP would reach equilibrium within 5 s or less. Several conclusions can be drawn from the data of Fig. 1. The incorporation of ^{32}P into PIP_2 at equilibrium was less in the mitochondria from glucagon-treated rats than in those from control animals, whereas that into phosphatidic acid was greater. There was no change in the equilibrium labelling of PIP. The ratio of PIP to PIP_2 is the most sensitive indicator of changes in the metabolism of these two phospholipids since any variations in recovery and loading of the t.l.c. plate cancel out. This ratio is shown in Fig. 1(b) to be significantly elevated in the mitochondrial/plasma-membrane preparation from glucagon-treated rats. At 15 min in 18 separate preparations, the increase in this ratio was $27.5 \pm 5.6\%$ ($P < 0.001$ by paired Student's *t* test). There is also a small increase in labelling of PIP at 1 min. In 17 separate preparations from both control and glucagon-treated rats the incorporation of ^{32}P at 1 min was $17.3 \pm 7.4\%$ higher in the hormone-treated preparation ($P < 0.05$ by paired Student's *t* test).

In Fig. 2(a) the effect of removing lysosomal and plasma-membrane contamination from the crude mitochondrial fraction on the labelling of PIP and PIP_2 is shown. Removal of plasma membranes by Percoll-density-gradient centrifugation greatly decreased the amount of PIP_2 concomitantly with the loss of most of the 5'-nucleotidase (Table 1). Some PIP remained, but this was largely removed by digitonin treatment to destroy lysosomes, as shown by the loss of acid phosphatase (Table 1). Lysosomes are known to possess a PI kinase but not a PIP kinase, which is consistent with these observations (Collins & Wells, 1983; Seyfred & Wells, 1984; Cockcroft *et al.*, 1985). Taken together, these results imply that the major incorporation of ^{32}P into the PIP and PIP_2 observed *in vitro* in the crude mitochondrial fraction is into plasma membranes, as was concluded in earlier studies (Michell & Hawthorne, 1965). However, it

Fig. 3. Effects of Ca^{2+} on the incorporation of ^{32}P into phospholipids of the liver mitochondrial/plasma-membrane fraction

The experimental protocol was the same as for Fig. 1. Incubations were performed in the presence of $3 \mu\text{g}$ of Ruthenium Red/ml with (○, □, △, ▽) or without (●, ■, ▲, ▼) 0.95 mM Ca^{2+} . At the concentration of EGTA in the buffer (1 mM), this was shown by the use of Arsenazo III to give a free $[\text{Ca}^{2+}]$ of about $3 \mu\text{M}$. The values given are the means $\pm$ S.E.M. of six separate experiments. Explanations of the symbols used are given in the legend to Fig. 1.

should be noted that a minor ^{32}P -containing phospholipid running just below the PIP₂ is consistently seen in the purified mitochondrial extract and, as shown in Fig. 2(b), it is also present in the mitochondrial fraction purified from hepatocytes labelled with ^{32}P . Before Percoll treatment this mitochondrial fraction (labelled

Fig. 4. Effect of glucagon treatment on polyphosphoinositide breakdown in the mitochondrial/plasma-membrane preparation

PIP (●, ○) and PIP₂ (■, □) were labelled with ^{32}P for 6 min under the conditions of Fig. 1 before resuspension of the membrane preparation in fresh buffer and incubation at 37°C for the times shown. Details are given in the experimental section. Values shown are means $\pm$ S.E.M. (error bars) of observations on three different membrane preparations from control (●, ■, ▲) or glucagon-treated (○, □, △) rats. The ratio of PIP to PIP₂ (▲, △) is given on a different scale and without error bars for clarity. Additional values for the 20 min time point from several experiments are given in Table 3.

in situ) also contained a considerable quantity of PIP₂ and is known to be contaminated with plasma membranes (Vargas, 1982). However, after the Percoll treatment most of the PIP₂ and plasma membranes were lost, but the novel phospholipid remained just as was apparent in the mitochondrial fraction labelled *in vitro*. The identity of this phospholipid is unknown, but it does appear to be truly mitochondrial rather than contamination by another membrane fraction.

In Fig. 3(a) we show the effects of Ca^{2+} on the time course of ^{32}P incorporation into the inositol phospholipids. Ruthenium Red was present to avoid mitochondrial damage by Ca^{2+} accumulation, and sufficient Ca^{2+} was added to give a $[\text{Ca}^{2+}]$, measured with Arsenazo III, of $3 \mu\text{M}$ (Halestrap *et al.*, 1986). This concentration of Ca^{2+} slightly increased the rate of PIP synthesis, the incorporation at 1 min being greater by $22.8 \pm 9.5\%$ (mean $\pm$ S.E.M. of 15 separate experiments) in the incubations with Ca^{2+} ($P < 0.05$ by paired Student's *t* test). At later time points the ^{32}P incorporation into PIP₂ was significantly decreased, giving rise to an increase of $41.9 \pm 9.9\%$ ($n = 6$; $P < 0.01$ by paired Student's *t* test) in the ratio of PIP to PIP₂ at 15 min, as shown in Fig 3(b). Labelling of phosphatidic acid was significantly decreased in the presence of $3 \mu\text{M}$ Ca^{2+} at times less than 6 min, but this difference was lost at 15 min. We have no explanation for this observation.

Loss of ^{32}P from pre-labelled PIP and PIP₂ of plasma membranes

The time courses of the loss of ^{32}P from PIP and PIP₂ of the crude mitochondrial/plasma-membrane fraction

Table 3. Effects of Ca²⁺ and neomycin on the loss of [³²P]P_i from ³²P-labelled phospholipids in a liver plasma-membrane fraction derived from control and glucagon-treated rats

Plasma-membrane/mitochondrial preparations from the livers of control and glucagon-treated rats were labelled with ³²P by 6 min incubation with [³²P]P_i and ADP as described in the legend to Fig. 1. After sedimentation by centrifugation, the mitochondrial preparations were resuspended in 0.25 ml of fresh medium without [³²P]P_i for 20 min before the reaction was stopped with 0.6 ml of acidified chloroform/methanol. Extraction of phospholipids and separation by t.l.c. were performed as described in the Experimental section. Values are given as means ± s.e.m. for *n* mitochondrial preparations. Statistical significance of the difference between glucagon-treated and control were measured by unpaired Student's *t* test (†*P* < 0.01), whereas a paired *t* test was used for assessing the effects of additions to the incubation (***P* < 0.01; **P* < 0.05).

		Percentage of initial phospholipid remaining after 20 min incubation					
		PIP ₂		PIP		Phosphatidic acid	
	<i>n</i>	Control	Glucagon	Control	Glucagon	Control	Glucagon
Control	17	79.1 ± 2.10	67.9 ± 2.44†	81.3 ± 2.92	70.6 ± 4.63	78 ± 2.4	76.7 ± 4.59
+ 3 μM-Ca ²⁺	17	49.0 ± 3.31**	47.2 ± 3.37**	61.0 ± 3.76**	60.8 ± 4.32	68.8 ± 4.46	78.3 ± 4.59
+ 250 μM-Neomycin	13	87.2 ± 3.58*	79.4 ± 2.82**	85.2 ± 2.51	77.0 ± 4.68	75.6 ± 3.79	85.8 ± 4.13
+ Ca ²⁺ and neomycin	13	58.8 ± 3.98**	58.7 ± 5.50**	63.8 ± 4.35**	60.3 ± 5.21	68.5 ± 4.6	76.3 ± 5.9

Fig. 5. Separation of ³²P-labelled inositol phosphates by high-voltage electrophoresis

Details of the preparation and running of samples are given in the Experimental section. Radioautograms are shown of two separate experiments. In (a) the membrane preparation was from a glucagon-treated rat and was incubated in the absence (1) or presence of 250 μM-neomycin (2), 3 μM-Ca²⁺ (3) or both (4). Track 5 represents a rather impure preparation of IP₃ from human erythrocytes. In (b) membrane preparations were from control (1) or glucagon-treated (2) rats, with track 3 representing another IP₃ preparation from erythrocytes. The positions of ATP and P_i were determined with commercial standards, pyrophosphate (PP_i) by susceptibility to pyrophosphatase (Sigma), and IP₂ with a standard prepared from erythrocytes. The position of IP (IP?) is conjectured from the behaviour of impurities in the IP₂ standard and of samples of glycerol 3-phosphate and glucose 6-phosphate prepared by using [γ-³²P]ATP and glycerol kinase or hexokinase. The spot denoted (?) is of unknown identity; O indicates the origin.

pre-labelled with ³²P for 6 min are shown in Fig. 4. Mean data from three separate experiments with preparations from both control and glucagon-treated rats are shown, and in Table 3 combined data from 17 experiments are given for the breakdown of PIP and PIP₂ at 20 min. Both PIP and PIP₂ were lost during the incubation, and breakdown was significantly greater in the preparation

from glucagon-treated rats. Table 3 also includes data demonstrating that 3 μM-Ca²⁺ stimulated and 250 μM-neomycin inhibited breakdown. Loss of ³²P could occur by either monoesterase or diesterase (phospholipase C) activity (Downes & Michell, 1981; Irvine, 1982; Irvine *et al.*, 1984). The former enzyme would convert PIP₂ into PIP and then PI. Thus a lag in the disappearance of PIP

Fig. 6. Endogenous NAD(P)H fluorescence of hepatocytes treated with hormones

Hepatocytes were incubated at 4 mg of protein/ml in a fluorimeter under the conditions outlined in the Experimental section. Data were sampled 10 times/s and averaged to one data point/s. The vertical dotted line indicates the time of addition of phenylephrine (Phen), vasopressin (Vaso) or glucagon (Gluc) at the concentrations shown. Statistical analysis of the lag time between hormone addition and fluorescence response is given in the text.

would be expected. This was not observed, as shown by the decrease in the ratio of PIP to PIP_2 over the first 7 min (Fig. 4). In some experiments the ^{32}P -labelled PIP and PIP_2 remaining after only 1 min incubation were measured, but were not found to be significantly different from the zero-time values. The ratio of PIP to PIP_2 after 1 min incubation decreased by $7.6 \pm 4.0\%$ ($n = 6$). These data are best explained by a phosphodiesterase activity being responsible for breakdown of PIP and PIP_2 . The inhibition of breakdown by 250 μM -neomycin supports this conclusion (Downes & Michell, 1981).

Such a phosphodiesterase would produce IP_3 and IP_2 , which it should be possible to detect in the supernatant after deproteinizing samples from incubations in the ^{32}P -labelling experiments. The usual method for measuring these water-soluble products involves incubating cells with ^3H inositol and separation of the ^3H inositol phosphates on ion exchange columns. The incorporation of ^3H into peaks eluted at increasing ionic strength is used to calculate amounts of the individual inositol phosphates. However, ^{32}P is incorporated into many different water-soluble components, some of which are inseparable from the inositol phosphates on ion-exchange columns. Thus, to measure ^{32}P incorporation into the inositol phosphates, we were forced to use a combination of ion-exchange chromatography and electrophoresis. The HClO_4 supernatant was neutralized and partially purified on Dowex 1 by using stepped elution with formic acid/ammonium formate before concentration by freeze-drying and running on high-voltage electrophoresis at pH 1.8. Details are given in the Experimental section. Typical radioautograms of such electrophoretic separa-

tions are shown in Fig. 5. Use of ^{32}P -labelled standards prepared from human erythrocytes (Downes *et al.*, 1982) confirmed the presence of IP_3 and IP_2 . There was evidence that both of these increased when 3 μM - Ca^{2+} was present in the incubation and decreased in the presence of 250 μM -neomycin (Fig. 5a). In addition there was more IP_3 present in the incubations of the membrane preparations from glucagon-treated animals (Fig. 5b). However, we found it impossible to obtain quantitative data with these techniques, since the recovery of inositol phosphates from day to day was too variable, as seen by comparison of Figs. 5(a) and 5(b). This may be explained partially by loss of the inositol phosphates during their isolation and separation, but is more likely due to a variable degree of hydrolysis in the incubation. In addition to the presence of radioactive spots corresponding to IP_3 and IP_2 and those of ATP, pyrophosphate and phosphate, there was a major radioactive spot in the position where inositol monophosphate and other sugar phosphates such as glycerol 3-phosphate and glucose 6-phosphate were shown to run. It is possible that at least some of this is inositol 4-monophosphate, since it changes in parallel with IP_2 and IP_3 and would be produced by the presence of IP_2 monoesterase in the incubation. This enzyme would be active at the concentration of Mg^{2+} required in these experiments to maintain mitochondrial stability (Storey *et al.*, 1984; Seyfred *et al.*, 1984; Joseph & Williams, 1985) and is likely to be present in variable activities from one preparation to another. Thus it could cause the variable recovery of IP_2 and IP_3 observed. Nevertheless the qualitative changes in the inositol phosphates observed in response to glucagon treatment and Ca^{2+} complement the quantitative effects observed on the parent phospholipids.

DISCUSSION

Effects of glucagon treatment on polyphosphoinositide metabolism

The data presented in Table 2 show that glucagon can increase the amount of PIP in the plasma membrane of hepatocytes without affecting the amount of PIP_2 . These results contrast with those of Creba *et al.* (1983) and Poggioli *et al.* (1986), who observed no effect of glucagon on PIP, but their studies used only a 1 min or 30 s exposure to glucagon, as opposed to 10 min used in the present studies. The effects that we observe could be caused by either an increase in PI kinase activity or an increase in the monoesterase that removes the 5-phosphate from PIP_2 . The slight but significant increase ($17.3 \pm 7.4\%$; $n = 17$) in the rate of incorporation of ^{32}P into PIP in the liver mitochondrial preparation containing plasma membranes of glucagon-treated rats suggests that perhaps the kinase is activated. Effects of cyclic AMP on the synthesis of PIP have been described in the adrenal cortex, lymphocytes, platelets and granulocytes (Farese *et al.*, 1980; Sarkadi *et al.*, 1983; Enyedi *et al.*, 1983; Farkas *et al.*, 1984; de Chaffoy de Courcelles *et al.*, 1986), and the activity of PI kinase towards endogenous substrate is increased in extracts of adrenal cortex under conditions of increased cyclic AMP (Farese *et al.*, 1980).

In addition to an effect of glucagon treatment on the PI kinase activity, the data of Fig. 3 and Table 3 show that this hormone also causes a stable increase in the

phosphodiesterase activity responsible for cleaving PIP_2 to IP_3 and diacylglycerol. The former water-soluble product was detected by high-voltage electrophoresis, but appeared to be rapidly broken down by monoesterases present in the membrane fraction and contaminating cytoplasm (Storey *et al.*, 1984; Seyfred *et al.*, 1984; Joseph & Williams, 1985). The diacylglycerol produced is probably the precursor of the phosphatidic acid that accumulates during the incubation. The time course of its production supports such a sequence of events. The increase in the formation of PIP might stimulate the synthesis of PIP_2 , but a concomitant increase in the breakdown of PIP_2 could explain why the increase in the labelling of PIP seen in hepatocytes treated with glucagon is not accompanied by a change in PIP_2 . It could also explain the increase in the ratio of the labelling of PIP to PIP_2 seen at equilibrium when the crude mitochondrial/plasma-membrane fraction from glucagon-treated rats is incubated with $^{32}\text{P}\text{PI}$. A consequence of the stimulation of both enzyme activities would be an increase in the production of IP_3 and a subsequent release of intracellular Ca^{2+} from the endoplasmic reticulum. Such an increase in Ca^{2+} has been observed by using quin2 fluorescence (Charest *et al.*, 1983; Sistare *et al.*, 1985) and appears to be due to the release of Ca^{2+} from the same intracellular stores as those involved in the release of Ca^{2+} induced by agonists known to increase IP_3 (Sistare *et al.*, 1985; Mauger & Claret, 1986). However, no significant increase in IP_3 has been observed after treatment with glucagon at concentrations of 10 nM or greater (Charest *et al.*, 1985; Poggioli *et al.*, 1986; Wakelam *et al.*, 1986). This does not mean that a small increase in concentration does not occur, however, since such a small rise would be hard to detect (Williamson *et al.*, 1985). The effect of glucagon on cytoplasmic $[\text{Ca}^{2+}]$ is less than that caused by phenylephrine, and changes in the concentration of IP_3 caused by this agonist are small (Charest *et al.*, 1985; Williamson *et al.*, 1985). There is some suggestion of an increase in IP_3 at 100 nM-glucagon in the experiments of Wakelam *et al.* (1986), but it is not significant.

The data presented in Table 2 show that, 10 min after addition, glucagon and phenylephrine have an additive effect on the increase in PIP . The combination of these two hormones is also known to have more than an additive effect on cellular Ca^{2+} metabolism (Morgan *et al.*, 1983, 1984; Mauger *et al.*, 1985; McCormack, 1985b; Quinlan & Halestrap, 1986), but there is no evidence that this combination of hormones increases the production of IP_3 in the hepatocyte within the first 1 min of their addition (Poggioli *et al.*, 1986). Rather, the available evidence suggests that the synergistic effect of the addition of both hormones is a consequence of altered Ca^{2+} flux across the plasma membrane (Mauger *et al.*, 1985; Poggioli *et al.*, 1986).

The stability of the effect of glucagon treatment of animals that is observed in the crude plasma-membrane/mitochondrial fraction suggests a covalent modification. The effects of glucagon on cytoplasmic $[\text{Ca}^{2+}]$ are only seen after a delay of a few seconds, and can also be elicited by addition of cyclic AMP or forskolin, suggesting the involvement of cyclic AMP-dependent protein kinase (Charest *et al.*, 1983; Sistare *et al.*, 1985). In Fig 6 we show data obtained by using the endogenous NAD(P)H fluorescence to follow changes in cytoplasmic $[\text{Ca}^{2+}]$. The validity of this approach has been argued

in detail elsewhere (Quinlan & Halestrap, 1986) and relies on the Ca^{2+} activation of the mitochondrial dehydrogenases in the liver (McCormack, 1985a). On addition of concentrations of phenylephrine or vasopressin to give maximal fluorescence responses, there was no observable lag time in the rise in fluorescence, whereas with glucagon a lag time of 5.5 ± 0.33 s (mean $\pm$ S.E.M. of 14 observations on separate cell preparations) was detected. This agrees with the data obtained with quin2 by others (Charest *et al.*, 1983; Sistare *et al.*, 1985). As is apparent in Fig. 6 and from the work of Thomas *et al.* (1984), at submaximal concentrations of phenylephrine a lag time is barely detectable, whereas at submaximal concentrations of glucagon the lag time increases considerably. These results would be hard to reconcile with a direct interaction of a glucagon receptor with the phospholipase C responsible for the breakdown of PIP_2 . However, they would be compatible with a mechanism involving the cyclic AMP-dependent phosphorylation of some component involved in the activity of this enzyme, perhaps one of the GTP-binding proteins involved in hormone signal transduction.

Effects of Ca^{2+} on polyphosphoinositide metabolism

The data of Fig. 2 show that the presence of $3 \mu\text{M-Ca}^{2+}$ in the buffer used for labelling PIP and PIP_2 in the crude mitochondria/plasma-membrane fraction caused an increased rate of PIP synthesis of a similar order to that seen in membranes from glucagon-treated animals. In these experiments the equilibrium concentration of PIP_2 was decreased by the presence of Ca^{2+} , and consequently the ratio of the two phospholipids was also changed in the same direction as with glucagon treatment. The decrease in PIP_2 can be explained by the increase in its breakdown induced by Ca^{2+} , as shown in Table 3. Such an effect of Ca^{2+} on the phosphodiesterase activity is well established, although the exact sensitivity depends on many factors (Irvine *et al.*, 1984; Wallace & Fain, 1985; Melin *et al.*, 1986; Uhing *et al.*, 1986). In Table 2 data were presented to show that phenylephrine, like glucagon, caused an increase in the concentration of PIP concomitant with a decrease in PIP_2 . Creba *et al.* (1983) and Poggioli *et al.* (1986) showed no change in the concentration of PIP 30 s or 1 min after addition of adrenaline, but a decrease in PIP_2 similar to that observed here. Time courses of the effects of vasopressin and angiotensin show that the concentrations of both phospholipids fall rapidly in the first few seconds, and then PIP returns to control values over the next 5 min, whereas PIP_2 concentrations are only partially restored (Creba *et al.*, 1983; Thomas *et al.*, 1983). However, the concentrations of the inositol phosphates stay elevated throughout this period (Thomas *et al.*, 1984). Taken together, these observations suggest that, coincident with an agonist-induced breakdown of PIP_2 , there is an increased synthesis. Release of a restraint on PI kinase imposed by PIP_2 has been proposed as a mechanism for this effect (Van Rooijen *et al.*, 1985), but this would not account for the restoration of PIP preceding that of PIP_2 . There are also reports of protein kinase C-dependent stimulation of PI kinase activity in platelets (de Chaffoy de Courcelles *et al.*, 1984; Halenda & Feinstein, 1984), which might also occur in liver, although such a mechanism would oppose the down-regulation of the α_1 -adrenergic receptor induced by protein kinase C

phosphorylation of the receptor protein (Cooper *et al.*, 1985; Lynch *et al.*, 1985; Corvera *et al.*, 1986).

The data in the present paper would support an alternative mechanism for the stimulation of PIP formation seen after the initial agonist-mediated breakdown. Our studies *in vitro* with crude plasma-membrane/mitochondrial preparations exhibited PIP₂ phosphodiesterase activity that was enhanced by the presence of micromolar [Ca²⁺] and by glucagon treatment of the rats from which they were derived. Both procedures also enhanced the synthesis of PIP in the incubations *in vitro*. Thus it is possible that the activity of the phosphodiesterase is in some way functionally related to that of the PI kinase. For glucagon treatment it is suggested that cyclic AMP-dependent phosphorylation of the guanine nucleotide regulatory protein linking the receptors to the phosphodiesterase may activate both processes. The effect of Ca²⁺ may not be physiological, but rather may mimic the activity of an agonist which activates the phosphodiesterase through the mediation of the GTP-binding protein. Such a mechanism would account for the observed sensitization of the α_1 -adrenergic receptor towards its agonists by glucagon and cyclic AMP (Morgan *et al.*, 1984). It is also compatible with the observed additive effect of glucagon and phenylephrine on PIP production (Table 1).

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