RAPID STIMULATION, BY VASOPRESSIN AND ADRENALINE, OF INORGANIC PHOSPHATE INCORPORATION INTO PHOSPHATIDYL INOSITOL IN ISOLATED HEPATOCYTES

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1. Introduction

In recent years there has been considerable interest in the enhanced metabolism of phosphatidyl inositol (PI) which may be observed in a wide variety of tissues in response to physiological stimuli [1]. Such enhanced metabolism is often first detected as an increase in the rate of incorporation of inorganic phosphate into PI, which is frequently accompanied by a similar effect upon phosphatidic acid. It has been suggested that these results reflect enhanced turnover of PI, with the stimulus acting to enhance the breakdown of PI to 1,2-diacylglycerol within the plasma membrane [1]. The ubiquity of enhanced PI turnover in response to various stimuli suggests that the phenomenon may be fundamentally implicated in the cellular responses to such stimuli. For example, recent evidence suggests that enhanced PI breakdown may be intrinsic to the mechanism of the muscarinic cholinergic receptor [2].

As part of a study concerning the short-term influences of hormones upon hepatic metabolism, this paper reports the effects of vasopressin, adrenaline and glucagon upon the incorporation of inorganic phosphate into PI. These three hormones exert similar effects upon hepatic metabolism, enhancing glycogenolysis and gluconeogenesis in the perfused liver [3,4] and glycogenolysis in isolated hepatocytes [5–7].

There is general agreement that glucagon and β-adrenergic agonists exert their metabolic effects upon the liver by elevating the intracellular concentration of adenosine 3′,5′-cyclic monophosphate [3,8]. Neither α-adrenergic agents nor vasopressin elevate hepatic cyclic AMP [8,9], and their mechanisms of action remain obscure. However recent evidence suggests that Ca^{2+} may be involved [6,7]. The possibility that enhanced PI metabolism may have a role in the control of hepatic metabolism by these hormones merits consideration. The experiments described in this paper were designed as a first approach to testing this hypothesis.

2. Materials and methods

2.1. *Preparation and incubation of hepatocytes*

Isolated hepatocytes were prepared from fed male Wistar rats (200 g) by perfusion of the liver with collagenase [10,11]. The original method was modified in the following ways:

(i) Hyaluronidase was omitted from the Ca^{2+}-free perfusion medium, which contained collagenase (0.5 mg/ml).

(ii) After perfusion, the step which involved further shaking in a flask was omitted.

The yield of hepatocytes was 3–5 g from livers that normally weighed about 9 g. The viability of the hepatocyte suspensions was monitored in several ways, including microscopic examination to check that most cells had intact plasma membranes and did not form clusters. Trypan blue was excluded by about 95% of the cells in all suspensions used in the present study. The hepatocyte preparations exhibited glycogen concentrations similar to those observed in vivo (200–300 μmol glucose/g wet liver). Also, vasopressin induced glycogenolysis at lower concentrations of hormone

than are effective in the perfused liver (Whitton and Hems, unpublished results).

Hepatocytes were incubated in plastic scintillation vials at 37°C in a shaking water bath. Each vial contained 10–15 mg dry cells in 2 ml bicarbonate-buffered saline [12] containing 2% w/v bovine serum albumin (fraction V) and 30–500 μCi [^{32}P]-phosphate. Hormones and dihydroergotamine were added to the vials in 0.01 ml water, and the solutions were freshly prepared each day. Pre-warmed hepatocyte suspensions were added at zero time to vials containing [^{32}P]phosphate and hormones at 37°C. Incubations were terminated with the addition of 0.2 mls 80% (w/v) trichloracetic acid (TCA). For each cell preparation, the dry wt cells/ml incubation medium was determined in duplicate.

2.2. *Analytical techniques*

At the end of the incubation period, the TCA precipitate was collected by centrifugation and extracted for 2 h with 10 ml chloroform:methanol:HCl (66:33:0.25) at 37°C in a shaking water bath. The extract was filtered under vacuum and the residue washed once with 10 ml chloroform:methanol:HCl (66:33:0.25). This lipid extract was then evaporated to dryness in a heated sand bath under a stream of N_2.

Phospholipids were separated by two dimensional thin-layer chromatography (t.l.c.) on 0.5 mm plates of silica gel G prepared in 0.01 M Na_2CO_3 [13]. The developing solvents were chloroform:methanol:acetic acid:water (125:75:19:3) followed by chloroform:methanol:4 M NH_4OH (75:37:7). The plates were left to dry in a stream of air for 30 mins between solvents. Triacylglycerols have an R_F of nearly 1.0 in both solvent systems, and consequently did not interfere with the separation of the major hepatic phospholipids. These phospholipids were identified with the help of commercially available pure standard samples. PI, for use as a chromatographic standard, was prepared by solvent fractionation of a crude extract of ovine cephalin [14].

The separated phospholipids were visualised in I_2 vapour and their positions were marked on the plate. When the I_2 on the plate had resublimed, the spots were scraped into scintillation vials containing 10 ml scintillant (8 g/litre butyl PBD in toluene). The radioactivity of the separated phospholipids was determined together with that of samples of incubation medium in an LKB 1210 Ultrobeta liquid scintillation counter. The efficiency of counting was calculated by means of a channels ratio quench correction technique. In some experiments, the radioactivity of total lipid phosphorus was determined in the chloroform:methanol:HCl extracts prior to separation of individual phospholipids by t.l.c. The results of these experiments indicated that there was an 85% recovery of total lipid phosphorus following the t.l.c. procedure described above.

2.3. *Chemicals*

L-Adrenaline bitartrate, [8-arginine] vasopressin, dihydroergotamine tartrate and phospholipid standards were from Sigma Chemical Co., and glucagon was kindly donated by Eli-Lilly Ltd. Collagenase (Grade II) was from C. F. Boehringer Corp. Ltd and bovine serum albumin (fraction V) was supplied by Miles Lab. Ltd. [^{32}P]Orthophosphate was from the Radiochemical Centre, Amersham. All other chemicals were of the highest grade commercially available.

3. Results

3.1. *The effects of hormones upon PI metabolism*

The incorporation of inorganic phosphate into PI was stimulated in hepatocyte suspensions exposed for 20 min to vasopressin (10 mU/ml) or adrenaline (2×10^{-5} M), but not in suspensions exposed to glucagon (10^{-6} M) table 1). Vasopressin treatment resulted in a 7-fold stimulation of phosphate incorporation into PI, whilst the effect of adrenaline treatment was less than 2-fold. The effects of these two hormones were also tested in the presence of dihydroergotamine (2.5×10^{-5} M), when adrenaline failed to influence phosphate incorporation into PI, but the stimulation by vasopressin of this process was undiminished. Hepatocyte suspensions treated with dihydroergotamine (2.5×10^{-5} M) alone exhibited rates of phosphate incorporation into PI similar to those in control suspensions. The basal rates of phosphate incorporation into both PI and total hepatic phospholipids were similar to those observed in vivo [15] and in hepatocyte suspensions [16].

Table 1
The effects of various hormones upon the incorporation of inorganic phosphate into hepatic PI

Hormone addition	Incorporation of phosphate into PI (ng atom/g dry wt/20 min)
None	6.6 ± 1.2 (5)
Vasopressin (10 mU/ml)	47.8 ± 5.3 (7)[a]
Adrenaline (2×10^{-5} M)	12.2 ± 1.0 (6)[a]
Glucagon (10^{-6} M)	5.0 ± 0.6 (4)
Dihydroergotamine (2.5×10^{-5} M)	3.6 ± 0.5 (5)
Adrenaline (2×10^{-5} M) + Dihydroergotamine (2.5×10^{-5} M)	3.6 ± 0.7 (5)
Vasopressin (10 mU/ml) + Dihydroergotamine (2.5×10^{-5} M)	69.2 ± 7.7 (3)[a]

[a] p versus hormone-free controls, < 0.01

Hepatocytes were prepared and incubated for 20 min, in the presence of 5 mM glucose, as described in the text. Results are expressed as means ± SEM of the number of observations in parentheses, which were from three separate hepatocyte preparations

3.2. *The time course of basal and vasopressin-stimulated phosphate incorporation into hepatic phospholipids*

The rate of incorporation of [^{32}P]phosphate into both PI and total phospholipids in the absence of exogenous hormones increased over a period of 60 min (fig.1). This increase may reflect a gradual rise in the specific radioactivity of inorganic phosphate within the cell. The vasopressin-induced stimulation of phosphate incorporation into PI was about 4–6-fold at all times tested, while that into total phospholipids was 2–3-fold. The vasopressin-stimulated rates of phosphate incorporation into PI remained significantly greater than the control rates at all times ($p < 0.01$).

The results of experiments designed to elucidate the effect of vasopressin upon the incorporation of

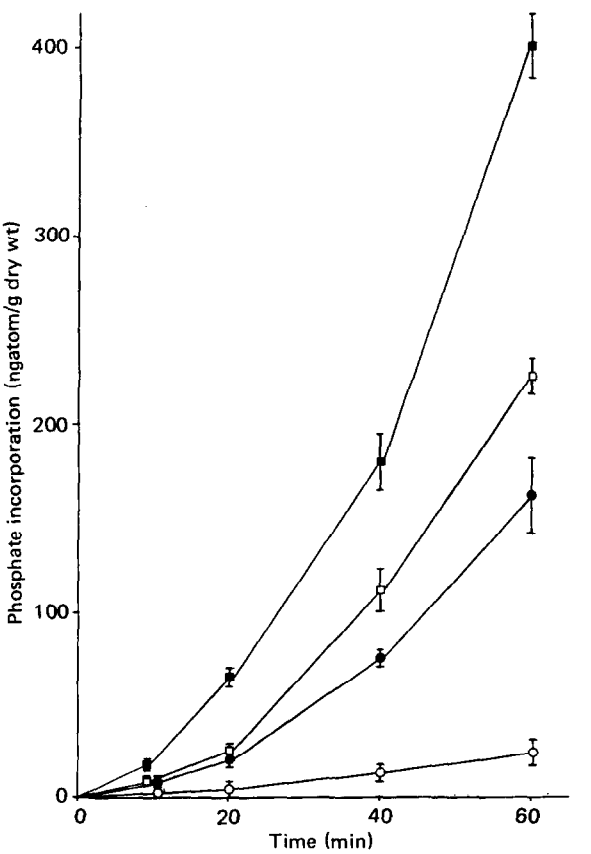

Fig.1. Time course of phosphate incorporation into hepatic phospholipids. Hepatocytes were prepared without added glucose, and incubated as described in the text. Incubations were performed in the presence of vasopressin (10 mU/ml, closed symbols) and in hormone-free conditions (open symbols). The radioactivity was determined, and phosphate incorporation calculated in total phospholipids (■ □) and in PI isolated by t.l.c. (● ○). Each point represents the mean ± SEM of at least five determinations from three separate hepatocyte preparations.

Table 2
The early effects of vasopressin upon phosphate incorporation into hepatic PI

Incubation time (min)	Vasopressin concentration (mU/ml)	Incorporation of phosphate into PI (pg atom/g dry wt)
2	0	41 ± 8
2	10	376 ± 133[a]
5	0	447 ± 92
5	10	2172 ± 354[a]
10	0	1249 ± 447
10	10	5310 ± 1729[a]

[a] p versus hormone-free controls, < 0.01

Hepatocytes were prepared without added glucose and incubated as described in the text. Hormones and [^{32}P]-phosphate were present throughout the incubation period. Results are means ± SEM five determinations from these separate hepatocyte preparations

inorganic phosphate into PI after brief periods of exposure to the hormone are shown in table 2. After 2 min exposure to the hormone, the incorporation of inorganic phosphate into PI was 9 times that observed in hormone-free suspensions. After 5 min and 10 min exposure, the hormone exerted 5- and 4-fold effects, respectively. In all cases, the hormone-stimulated rate of phosphate incorporation was significantly greater than that in hormone-free conditions ($p < 0.01$).

4. Discussion

It is evident from these results that the hormones vasopressin and adrenaline exert a rapid and profound effect upon the rate at which inorganic phosphate is incorporated into hepatic PI. The observation that adrenaline but not glucagon enhances phosphate incorporation into PI is in agreement with the results of an earlier study using liver slices [17], which also reported that this effect of adrenaline was abolished in the presence of dihydroergotamine. The dihydroergotamine sensitivity of the adrenaline-stimulated process, which was also demonstrated in the present study, suggests that the enhanced incorporation of phosphate into PI observed in the presence of adrenaline may be classified as an α-adrenergic response. The vasopressin-stimulated process remained insensitive to dihydroergotamine, indicating that these two hormones stimulate PI synthesis via different receptors.

PI constitutes only about 9% of total hepatic phospholipids [18] and the basal incorporation of exogenous [^{32}P]phosphate into PI was also about 9% of that into total lipids (fig.1). However, under vasopressin-stimulated conditions, the incorporation of [^{32}P]phosphate into PI accounted for about 50% of that incorporated into total phospholipids. In fact, the total increment in phosphate incorporation into all hepatic phospholipids following vasopressin treatment could be accounted for by the enhanced incorporation into PI. Further evidence in favour of this conclusion emerged in the same experiments as those described in fig.1, in which vasopressin did not significantly influence phosphate incorporation into phosphatidyl choline, lysophosphatidyl choline, sphingomyelin, phosphatidyl ethanolamine or phosphatidyl serine (Kirk, C. J. and Verrinder, T. R. unpublished results). Thus the stimulation by vasopressin of phosphate incorporation into PI cannot merely be the result of enhanced inorganic phosphate transfer across the plasma membrane, or of increased incorporation of the isotope into intermediates of phospholipid synthesis, such as ATP.

The stimulation of inorganic phosphate incorporation into PI was greatest after the briefest period of exposure to the hormone which was tested (2 min, table 2). In this respect the stimulation of PI synthesis by vasopressin is similar to the stimulation of hepatic cyclic AMP production by glucagon, in that a maximal effect occurs within a very short time of exposure to the hormone [3]. The observation that vasopressin enhances PI synthesis within 2 min establishes this effect as one of the most rapid upon PI metabolism which has yet been reported. This maximal effect of vasopressin in the present study coincides with the maximal stimulation of glycogen phosphorylase activity and with the rapid onset of Ca^{2+} uptake in vasopressin-treated hepatocytes [7]. The fact that these responses to the hormone occur simultaneously could imply the existence of a functional relationship between them.

It has been suggested that PI turnover plays a role in the coupling between receptor activation and enhanced membrane permeability to Ca^{2+} in smooth muscle [2,19,20]. It is probable that the results of

the present study reflect enhanced turnover of PI (although the data do not exclude net synthesis). The cleavage of the glyceryl-phosphate bond in PI is catalysed by an enzyme in the plasma membrane, (at least in the brain: [20,21]). Thus this reaction is a suitable candidate for direct stimulation by receptor-agonist interaction in tissue plasma membrane. The influence of vasopressin and adrenaline upon this reaction remains to be established.

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