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THE EFFECT OF ISOPROTERENOL ON THE METABOLISM OF PHOSPHATIDYLINOSITOL BY RAT HEART *IN VITRO*

Z. KISS and T. FARKAS

Institute of Biochemistry, Biological Research Center of the Hungarian Academy of Sciences, Szeged, Hungary

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Abstract—The effect of epinephrine and isoproterenol (10^{-4} M) on the incorporation of ^{32}P orthophosphate and [1-^{14}C] palmitic acid into rat heart slices was investigated in pulse and chase experiments. Epinephrine stimulated the uptake of ^{32}P into phosphatidic acid and phosphatidylinositol. Isoproterenol stimulated the labelling of phosphatidic acid but had no effect on phosphatidylinositol metabolism in the first 60 min of the incubation. Propranolol stimulated the incorporation of ^{32}P and [1-^{14}C] palmitic acid into phospholipids in the first 20–30 min of the incubation but counteracted isoproterenol thereafter. These results indicate that the main effect of isoproterenol on phospholipid metabolism in rat heart is to increase the turnover of phosphatidic acid and to slow down the conversion of phosphatidic acid into phosphatidylinositol.

Phospholipids are integral constituents of cell membranes and as such they may be involved in binding biologically active substances to these structures. For instance phosphatidyl serine has been shown to participate in binding epinephrine to phospholipid vesicles [1]. The binding of these substances to their receptors may bring about changes in membrane conformation which in turn can affect the processes connected to these structures or even the biosynthesis of phospholipids inside the cell.

Several hormones such as epinephrine and norepinephrine [2–12], acetylcholine [13–17], insulin [18] and thyroid stimulating hormone [19–22] have been shown to increase the incorporation of ^{32}P into phosphatidyl inositol and phosphatidic acid in a variety of tissues. How these compounds stimulate the metabolism of phospholipids is not clear as yet but at least in the case of catecholamines it is probable that it is mediated via alpha receptors. Studying the phospholipid metabolism of rat heart, an organ rich in beta receptors we found evidence for the possible involvement of beta receptors in increased turnover of phosphatidic acid but not of phosphatidyl inositol. This paper gives an account of these experiments.

EXPERIMENTAL

Animals and incubation conditions. Female Wistar rats weighing 150–200 g were used in the experiments. The animals were killed by decapitation, their hearts were quickly excised and washed with ice-cold physiological saline. Hand-cut tissue slices (90–100 mg/ml) were shaken in Krebs–Ringer bicarbonate buffer containing 5 mM Tris–HCl, pH 7·4 at 37° in the presence or absence of drugs and labelled materials. Usually 60–100 μCi/ml ^{32}P or 0·38–0·40 μCi/ml [1-^{14}C] palmitic acid was present in the incubation medium. Palmitic acid was complexed with albumin; the concentration of albumin was 2·5%. In chase experiments the tissues were thoroughly rinsed with Krebs–Ringer buffer and reincubated in cold medium.

Extraction of lipids. The reactions were stopped by rapid homogenization of the tissues in ice-cold chloroform–methanol (2:1, v/v) and the total lipids were extracted by the method of Folch *et al.* [23]. The phase separation was achieved by adding 0·2 vol of 0·1 M KCl to the filtered extracts. After removing the upper, aqueous layer the organic phase was washed eight times with Folch theoretical upper phase containing 0·1 M K_2HPO_4 in the ^{32}P experiments; ^{14}C labelled lipids were washed only twice. The lower phase was then adjusted to 10 ml and aliquots were removed for determination of total phospholipid content and radioactivity as well as for separation of lipid classes.

Chromatography. Lipid class separation was performed on silicagel G plates using petroleum ether–diethyl ether–acetic acid (85:15:1, by vol) as solvent. Phospholipids were separated by two dimensional thin-layer chromatography according to Rouser *et al.* [24]. The spots were visualised by brief exposure to iodine vapor in the experiments with [1-^{14}C] palmitic acid, or charred with 50% sulphuric acid at 180° for 60 min when labelled with ^{32}P phosphate.

Determination of radioactivity. The spots were transferred to counting vials and counted in the presence of silicagel. [1-^{14}C]palmitic acid was counted in toluene scintillation cocktail (4% PPO and 0·2% POPOP) while ^{32}P was counted on the basis of its Cherenkov radiation [25] in distilled water. The counts were corrected for quenching and counting efficiency. An Isocap 300 Nuclear Chicago liquid scintillation spectrometer was used for radioactivity measurements.

Determination of specific activities. For determination of ^{32}P specific activities aliquots were removed from the counting vials after counting and the phosphorus contents were determined according to Kahovcova and Odavic [26]. In the ^{14}C experiments parallel plates were run for radioactivity measurements and phosphorus determination.

Reagents. Carrier-free ^{32}P orthophosphate was bought from the Isotope Institute of the Hungarian Academy of Sciences, Budapest; [1-^{14}C] palmitic

Table 1. Phospholipid composition of the rat heart

Phosphatidylcholine	39·1 ± 1·4
Phosphatidylethanolamine	34·1 ± 1·9
Cardiolipin	12·9 ± 1·4
Sphingomyelin	3·4 ± 0·4
Phosphatidylinositol	3·1 ± 0·4
Phosphatidylserine	2·9 ± 0·6
Phosphatidic acid	1·1 ± 0·5
Lysophosphatidylcholine	0·9 ± 0·3
Lysophosphatidylethanolamine	0·6 ± 0·2
Phosphatidylglycerol	0·8 ± 0·2

Phospholipids are expressed as % of total phospholipid. Results are means ± S.E. of 8–12 determinations.

acid (59·2 mCi/m-mole) was from New England Nuclear. Epinephrine, isopropylnorepinephrine and propranolol were obtained from Sigma, St. Louis, Mo. All the other chemicals were analytical grade.

RESULTS

Composition of phospholipids in the rat heart. The major phospholipids are phosphatidylcholine and phosphatidylethanolamine followed by cardiolipin, sphingomyelin, phosphatidylinositol and phosphatidylserine (Table 1). The figures obtained in this laboratory agree well with those given by Simon and Rouser [27] and Soula *et al.* [28] for the same organ with the exception of phosphatidic acid (1·10% against 0·3 and 0·7%, respectively).

Incorporation of ^{32}P and [1-^{14}C] palmitic acid into the phospholipids. Control heart slices incorporated ^{32}P slowly in the first 30 min of the incubation. The ^{32}P uptake became linear between 30 and 120 min. [1-^{14}C] Palmitic acid uptake followed an S-shaped curve. The specific activities of total phospholipids, phosphatidyl inositol and phosphatidic acid are given in Fig. 2.

Effect of catecholamines. The presence of epinephrine or isoproterenol (10^{-4} M) did not change significantly the incorporation of ^{32}P into total phospholipids during the first 50 or 80 min of the incubation (Fig. 1 and Fig. 2A, respectively). When the tissues were incubated in the presence of isoproterenol for a longer period of time, however, a slight stimulation was obtained (Table 2). On the other hand both epinephrine and isoproterenol stimulated the incorporation of ^{32}P into phosphatidic acid (Figs. 1 and 2C). Phosphatidyl inositol labelling was stimulated by about 30% by epinephrine. Isoproterenol had no effect during the first 60 min; measurable stimulation was observed only at the end after 120 min incubation (Table 2). Phosphatidylcholine and phosphatidyl ethanolamine did not respond to the hormone but the labelling of phosphatidylserine was stimulated also after 120 min. It is worth mentioning that the specific activity of phosphatidylserine is much higher than that of the phosphatidylethanolamine. We have shown that the bulk of phosphatidylserine is not formed from phosphatidylethanolamine in the rat heart (manuscript in preparation).

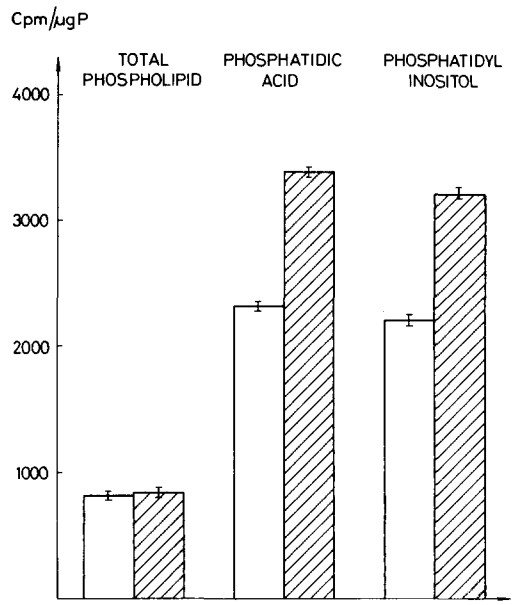

Fig. 1. Effect of epinephrine on ^{32}P incorporation into phospholipids. Heart slices were incubated in the presence of ^{32}P orthophosphate (60 μCi/ml) for 50 min. The concentration of epinephrine was 10^{-4} M. □, control; ▨, epinephrine. Results are expressed as means ± S.E. of three incubations.

The incorporation of [1-^{14}C] palmitic acid into total phospholipids and almost all glycerophospholipids was stimulated by isoproterenol by about 70–80% (Fig. 2). Phosphatidylinositol was the least affected compound, being stimulated by only 10% at 80 min.

To obtain more detailed information about the effect of isoproterenol on the metabolism of phospholipids in rat heart prelabelled control and hormone-treated slices were incubated for another 80

Table 2. Effect of isoproterenol and propranolol on ^{32}P incorporation into phospholipids

	Specific radioactivity (cpm/μg of P)			
	Control	Isoproterenol	Propranolol	Propranolol + isoproterenol
Total phospholipid	1712 ± 78	1968 ± 64	1475 ± 92	1392 ± 122
Phosphatidylcholine	2952 ± 202	2926 ± 118	2147 ± 107	1909 ± 303
Phosphatidylethanolamine	119 ± 21	154 ± 18	148 ± 26	206 ± 23
Phosphatidylinositol	5706 ± 388	9487 ± 465	5543 ± 414	7098 ± 615
Phosphatidylserine	1158 ± 62	1515 ± 181	836 ± 117	602 ± 32
Sphingomyelin	563 ± 36	696 ± 94	574 ± 39	592 ± 68
Phosphatidic acid	6453 ± 377	15733 ± 1845	9854 ± 618	8416 ± 834

Heart slices were incubated with isoproterenol, 10^{-4} M, and with propranolol, 10^{-4} M for 2 hr. The incubation medium contained ^{32}P (130 μCi/ml). Results are expressed as means ± S.E. of six incubations.

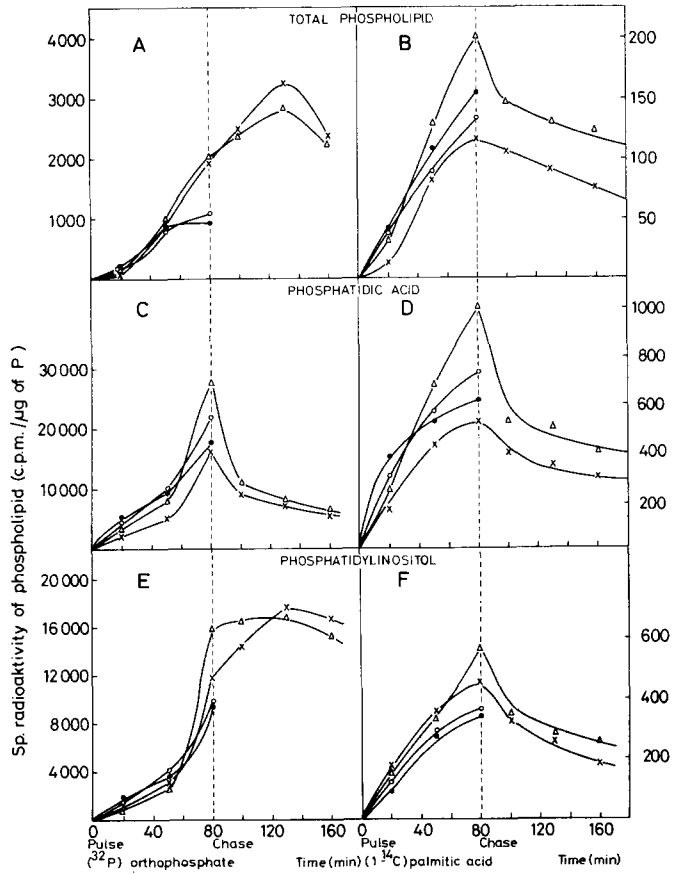

Fig. 2. Kinetics of ^{32}P and [1-^{14}C]palmitic acid incorporation into phospholipids of rat heart. Tissue slices were incubated in the presence of ^{32}P orthophosphate (100 μCi/ml) and [1-^{14}C]palmitic acid (0·40 μCi/ml) for 80 min. The concentration of isopropylnorepinephrine and propranolol was 10^{-4} M. Further details in the text. ×, control; △, isoproterenol; ○, propranolol; ●, isoproterenol + propranolol. Each point represents the average of three incubations.

min in the absence of radioactive precursors and hormones and the decay of the radioactivity in different phospholipids was determined.

In the chase period total phospholipids continued to incorporate ^{32}P, but not [1-^{14}C] palmitate, for about 50–60 min (Fig. 2A,B). The drop in accumulating palmitate in the chase period is due to the correct precursor concentration employed rather than phospholipase activity. At higher palmitate concentrations total phospholipids continued to incorporate label in the chase period. Phosphatidic acid, labelled with ^{32}P or [1-^{14}C] palmitate began to lose label immediately after putting the slices into the inactive medium (Fig. 2C,D). The loss of label occurred in two kinetically distinct phases. It was very rapid in the first 20 min of the chase then became slower. This phenomenon was very pronounced in slices incubated in the presence of isoproterenol in the pulse period. The specific activity of phosphatidic acid fell to the control level within the first 20 min of the chase. The ^{32}P specific activity of phosphatidylinositol in control slices continued to increase in the chase period and after 50 min it reached the activity of the isoproterenol treated slices. In slices incubated in the presence of isoproterenol in the pulse period the incorporation of label into phosphatidylinositol molecules was not increased in the chase period because the phosphatidylinositol molecules were close to the isotopic equilibrium at the end of the pulse period.

In contrast to this [1-^{14}C] palmitic acid was lost rapidly from phosphatidylinositol. The decay of [^{14}C] radioactivity was biphasic especially in hormone treated slices (Fig. 2E,F).

Effect of propranolol. Propranolol, a potent beta adrenergic blocking agent failed to counteract the isoproterenol-stimulated uptake of ^{32}P into phosphatidic acid and of [1-^{14}C] palmitate into total phospholipids and phosphatidic acid in the first 60 min of the incubation but was stimulatory in the first 20–30 min of the incubation (Fig. 2). This effect was even more pronounced when glucose was included in the incubation medium (data not given). It was found to be inhibitory only in the second half of the incubation (Fig. 2, Table 2). In the case of phosphatidyl choline and phosphatidyl serine there was even a decreased incorporation in the presence of propranolol or propranolol plus isoproterenol.

DISCUSSION

In agreement with Gaut and Huggins [12] we found that epinephrine stimulated the uptake of ^{32}P into phosphatidylinositol of the rat heart. We found also an increased incorporation into phosphatidic acid. However under our experimental conditions we

were unable to show any significant increase in the specific activity of the total phospholipids. As Table 1 shows phosphatidylinositol and phosphatidic acid make up only 4·2% of the total phospholipids; it is not surprising, therefore, that the increased specific activity of these compounds was not reflected in the specific activity of the total phospholipids.

Epinephrine and norepinephrine have been shown to stimulate the incorporation of ^{32}P into the phospholipids of a variety of tissues *in vivo* and *in vitro* [2–12]. As the hormones can be antagonized by alpha receptor blocking agents [3–6, 8, 29] it is generally accepted that these structures are involved in the so called 'phospholipid effect' of the hormones. In our experiments isoproterenol, a beta receptor stimulating agent increased the incorporation of ^{32}P only into phosphatidic acid while its effect on the labelling of phosphatidylinositol was very weak and appeared only after a long delay. Isoproterenol, in contrast to epinephrine, does not stimulate the incorporation of ^{32}P into parotid slices [4, 5] or rat pineal cultures [6]. This suggests that epinephrine and norepinephrine have an effect different from that of isoproterenol on the metabolism of phospholipids in the different tissues.

The mechanism of action of isoproterenol is not yet understood. In the *de novo* pathway of phosphatidylinositol biosynthesis, phosphatidic acid is first converted to CDP-diglyceride, the acceptor of inositol [29–31]. In this case the phosphorus moiety of phosphatidic acid is conserved in the newly synthesized phosphatidylinositol. Although, in our experiments isoproterenol increased the incorporation of both ^{32}P and [^{14}C]palmitic acid into phosphatidic acid there was no increase but rather a small decrease in the specific activity of phosphatidylinositol at least in the first 60 min of the incubation. The only explanation we can offer for this is that isoproterenol slows down the formation of phosphatidylinositol from phosphatidic acid. To our knowledge, this is the first example of an extracellular stimulus decreasing the turnover of phosphatidylinositol. Thus it seems that the enhancement of phosphatidylinositol turnover is not a generalised response to any extracellular stimulus mediated through cell-surface receptors.

Chase experiments with both ^{32}P and [1-^{14}C] palmitic acid suggest that there are at least two different metabolic pools of phosphatidic acid in the rat heart with half-lives of about 20 min and a few hours, respectively. As phosphatidic acid in isoproterenol-stimulated slices lost [1-^{14}C] palmitate in the chase period more rapidly than in the unstimulated ones, we assume that a rapidly metabolizing pool may be involved in the response of the tissue to the hormones. However, the effect of isoproterenol on phosphatidic acid metabolism is probably not connected directly to its effect on the beta receptor. The activation of the beta receptor is a very rapid process while the increase of the turnover of phosphatidic acid is slow even if the rapidly metabolizing pool is involved. The metabolic change responsible for increased turnover of phosphatidic acid remains to be investigated.

Propranolol effects the metabolism of phospholipids in a complex manner. A stimulatory effect on ^{32}P incorporation was observed by some authors [6–8, 22, 32]. In our experiments it also stimulated [^{14}C] labelled palmitic acid incorporation especially in the first 30–40 min of the incubation. We feel, however, that this effect of propranolol is due to its local anaesthetic-like effect and not to its beta receptor antagonist properties.

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