Biochemical Pharmacology, Vol. 29, pp. 2521–2523
0006-2952/80/0915-2521 $02.00/0

Stimulation of phosphatidylcholine breakdown by isoproterenol in rat liver plasma membranes

(*Received* 27 *December* 1979; *accepted* 24 *April* 1980)

Hormones are the common means of carrying messages from the environment to the cells [1]. The first step in the sequence of these events is their combining with the appropriate receptor. One of the most important structures transmitting the information carried by the hormones is the catecholamine stimulated adenylate cyclase–cAMP system [1, 2], the activity of which has been found to be dependent also on the presence of a specific phospholipid milieu [3, 4]. The catecholamines also affect the phospholipid metabolism of the cells: they increase the turnover of phosphatidic acid and phosphatidylinositol [5–8]. Besides catecholamines, acetylcholine [9, 10], insulin [11] and thyroid stimulating hormone [12] have also been shown to stimulate the incorporation of ^{32}P into phosphatidic acid and phosphatidylinositol in a variety of tissues.

Our knowledge about the structural changes taking place in membranes, upon formation of the hormone–receptor complex, is rather poor. It can be accepted, as a working hypothesis, that the hormones locally disturb the membrane structure and both the 'phospholipid effect' and the stimulation of adenylate cyclase can be traced back to this event. Phospholipase C, similarly to phospholipase D of plants [13], can be activated by disrupting membrane integrity [14]. In this paper we demonstrate that isoproterenol triggers an intensive decomposition of phosphatidylcholine in rat liver plasma membrane and by treating plasma membranes with exogenous phospholipase C the 'phospholipid effect' of this catecholamine can be mimicked.

To check this hypothetical effect of catecholamines on phospholipase C, rat liver plasma membranes were used. The plasma membranes (250 μg), obtained from male rats (body wt 120 g) by the method of Neville [15], were incubated in 200 μl 40 mM Tris–HCl buffer, pH 7.5, containing [1, 2-^{14}C]choline chloride (New England Nuclear, Boston, MA, U.S.A. sp. act. 55.6 μCi. μmol^{-1}, 0.25 μCi. mg protein^{-1}) at 25° for 10 min to label phosphatidylcholine. The unreacted label was removed by repeated centrifugation at +4° in Tris–HCl buffer, pH 7.5. The sediment was resuspended in the same buffer and protein content determined by the method of Lowry *et al.* [16]. Isoproterenol, in varying concentrations (10^{-4}, 10^{-5}, 10^{-6} M), was then added to the membrane preparation and incubations were carried out for various lengths of times at 25°. A typical reaction mixture consisted of 150 μg membrane protein suspended in 200 μl 40 mM Tris–HCl buffer, pH 7.5. $CaCl_2$ was absent from this incubation system. The reaction was terminated by adding 2 ml chloroform–methanol (2:1). In some other experiments the membranes, suspended in 40 mM Tris–HCl buffer pH 7.5, were treated with phospholipase C (Sigma Chem. Co., St. Louis, MO, U.S.A., from *Clostridium welchii*, 0.1 mg. mg protein^{-1}) in a reaction mixture containing also 0.5 mM $CaCl_2$ and [^{32}P]orthophosphate, 1 μCi.mg protein^{-1}, at 25°. After 10 min of incubation 1 mM EDTA, 2 mM $MgCl_2$ in 25 mM Tris–HCl buffer, pH 7.5, was added to stop the reaction. The effect of isoproterenol on incorporation of ^{32}P into phospholipids was investigated in a reaction mixture devoid of $CaCl_2$. [^{32}P]Orthophosphate was present also in this case in a ratio of 1 μCi.mg protein^{-1}. Phospholipids were extracted by the conventional Folch technique [17] and separated by two dimensional t.l.c. [18]. Spots, made visible by brief exposure of the plates to iodine vapours, were removed in scintillation vials and counted from toluene based scintillation cocktail to 5 per cent counting error using a Tri Carb Liquid Scintillation Spectrometer. Counts were corrected for quenching and counting efficiency.

About 0.1–0.3 per cent of the label of the applied [1, 2-^{14}C]choline chloride appeared in phosphatidylcholine under our experimental conditions. This value is one order of magnitude less than that obtained by Jungalwala and Dawson [19] using rat liver microsomes. Evidently, in our case a passive exchange of polar head groups might have taken place instead of active biosynthesis of phosphatidylcholine occurring in microsomes.

Phospholipase C is known to hydrolyse phosphatidylcholine to phosphorylcholine and 1,2 diglyceride. When the prelabelled membranes were incubated in Tris–HCl buffer in the absence of $CaCl_2$, for various lengths of times, only a few counts disappeared from the phosphatidylcholine, indicating a very low level of endogenous activity of phospholipase C. On the other hand, including also isoproterenol in the incubation medium, an increased hydrolysis of phosphatidylcholine occurred, probably due to activation of phospholipase C. The response of membranes to the hormone was very rapid and seemed also to be concentration dependent (Fig. 1). Thirty seconds of incubation were sufficient to bring about a maximal response using medium hormone concentrations. Isoproterenol has

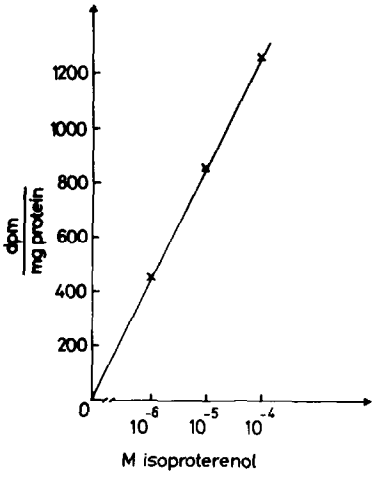

Fig. 1. Stimulation by l-isoproterenol of rat liver plasma membrane phospholipase C. Plasma membranes were incubated in the presence of [1,2-^{14}C]choline chloride to label phosphatidylcholine prior to the assay. A typical reaction mixture consisted of 150 μg of labeled membrane protein suspended in 200 μl 40 mM Tris–HCl buffer, pH 7.5, containing the hormone as indicated. The control membranes incorporated 2000 ± 20 dpm choline chloride. mg protein^{-1} in phosphatidylcholine. The figures on the ordinate represent the counts disappeared from phosphatidylcholine during the first minute of the incubation.

Table 1. Effect of adrenergic blocking agents on stimulation by isoproterenol of phospholipase C in rat liver plasma membrane*

Experiment	I		II	
	dpm in PC	% Stimulation	dpm in PC	% Stimulation
Control	270	0	280	0
Isoproterenol, 10^{-5}M	135	50	140	50
Propranolol, 10^{-5}M	220	19	250	11
Propranolol + Isoproterenol	150	45	165	41
Phentolamine 10^{-5}M	230	15	270	4
Phentolamine + Isoproterenol	160	41	192	31

* Plasma membranes were preincubated with [1,2-^{14}C]choline chloride to label phosphatidycholine prior to the experiment. Labeling of phosphatidylcholine took place by adding 2.2–2.3 × 10^5 dpm [1,2-^{14}C]choline to 250 μg membrane protein suspended in 200 μl 40 mM Tris–HCl buffer, pH 7.5, at 25° for 10 min. The disappearance of the label from phosphatidylcholine was followed. Each experiment was run in duplicate. PC = phosphatidylcholine.

recently been shown also to stimulate phospholipase A in canine cardiac sarcolemma [20].

To understand the rapid activation of phospholipase C by isoproterenol we can have at least three different possibilities: (1) this enzyme is masked in the membrane by some proteins; (2) the polar head groups of phosphatidylcholines are protected from the enzyme attack by some proteins; (3) the same protein masks phospholipase C and protects the polar head groups. Whichever is the case, as soon as the hormone was bound to the membrance an intensive decomposition of phosphatidylcholine was triggered off. So, if any of the above considerations are correct, the most likely explanation as to the reaction mechanisms is that phospholipase C activation could be a result of perturbation of membrane integrity by the hormone.

Isoproternol is a β-agonist and in general β-receptors are among the structures known to bind catecholamines on the membrane surface. It seemed logical to check whether adrenergic blocking agents prevent this effect of the investigated catecholamine.

Table 1 indicates that the β-adrenergic blocking agent, propranolol, did not exert any significant stimulatory effect on decomposition of phosphatidylcholine when included in the incubation medium. It was, however, unable to prevent isoproterenol from activating phospholipase C. Phentolamine, an α-adrenergic blocking agent, also failed to counteract isoproterenol. This experiment seems to indicate that the hormone achieved its effect on phospholipase C in a rather aspecific way and neither α nor β-receptors are involved in the response.

There is, however, some similarity between the so-called 'phospholipid effect' of isoproterenol and phospholipase C. Both catecholamines [5–8] and phospholipase C [21–23] have been shown to stimulate the incorporation of ^{32}P into phosphatidic acid and phosphatidylinositol under various experimental conditions. Table 2 reveals that isoproterenol and exogenous phospholipase C exerted similar effect on the incorporation of [^{32}P]orthophosphate in phosphatidic acid and phosphatidylinositol of rat liver plasma membrane. The other phospholipids also picked up label from ^{32}P as it is evident from their increased specific radioactivities (Table 2). More detailed investigations using different enzyme and hormone concentrations are necessary to see whether the observed differences in specific radioactivities reflect real differences between isoproterenol and phospholipase C. Propranolol, as shown in an earlier study [8], failed to counteract isoproterenol on the incorporation of ^{32}P in phosphatidylinositol and phosphatidic acid of rat heart slices. Experiments are being carried out to see whether α- and β-adrenergic blocking agents modify the pattern of labeling of phospholipids in rat liver plasma membranes. These observations, however, permit the suggestion that the first step is to disturb the integrity of the outer membrane lamella by the hormone followed by

Table 2. Stimulation by isoproterenol and phospholipase C of incorporation of ^{32}P into rat liver plasma membrane phospholipids*

	Control		Isoproterenol (10^{-5}M)		Phospholipase C (0.1 mg.membrane protein^{-1})	
	dpm	dpm.μg^{-1}	dpm	dpm.μg^{-1}	dpm	dpm.μg^{-1}
Phosphatidic acid	385 ± 22	550 ± 31	581 ± 40	1162 ± 80	550 ± 35	917 ± 58
Phosphatidylinositol	550 ± 22	50 ± 4	1066 ± 45	100 ± 6	642 ± 25	87 ± 4
Phosphatidylserine	485 ± 30	57 ± 3	728 ± 30	91 ± 6	625 ± 20	75 ± 2.5
Phosphatidylcholine	1010 ± 35	24 ± 1	1656 ± 40	41 ± 1.3	546 ± 30	33 ± 2.5
Phosphatidylethanolamine	678 ± 15	22 ± 0.5	1073 ± 25	35 ± 15	779 ± 25	38 ± 1.0

* The plasma membranes were incubated in the presence of ^{32}P orthophosphate, 1 μCi. mg protein^{-1}, and various additives at 25° for 10 min. The phospholipase C treatment was performed in a reaction mixture of 250 μg membrane protein, 25 μg phospholipase C, 0.5 mM $CaCl_2$, suspended in 200 μl 40 mM Tris–HCl buffer, pH 7.5. The results are the means ± S.E. of triplicate determinations from a single experiment.

activation of phospholipase C and phosphorylation of the liberated diglycerides in the inner membrane layer. Rapid transbilayer diffusion of 1,2 diacylglycerol was demonstrated for phospholipase C-treated red blood cells by Allan *et al.* [23].

Removing part of the phosphatidylcholine from the outer membrane layer may result also in structural alterations. The major consequence of reduction of this phospholipid is a relative increase in sphingomyelin, cholesterol and phosphatidylethanolamine. Cholesterol is known to increase the ordering of the membranes [24]. Moreover, a relative increase in phosphatidylethanolamine might contract the membranes locally due to more frequent formation of H bonds between N^+H groups of this phospholipid and the $P{\rightarrow}O^-$ groups of the neighbouring phospholipid [25]. As a result of such alterations concave areas might develop on the outer surface of the membrane, the presence of which has been suggested by Allan *et al.* [23] and also by freeze etch electron microscopy of phospholipase C-treated membranes [26]. The local increase in microviscosity in the outer membrane layer may be accompanied by a local increase of fluidity in the attaching inner membrane leaflet, due partly also to the presence of diglycerides and phosphatidic acid [23].

Although the 'phospholipid effect' of catecholamines and their stimulation of adenylate cyclase appear to be independent events the question still arises whether the above structural alterations of membrane favour the coupling of β-adrenergic receptor situated on the outer surface of the membrane with the catalytic subunit located in the interior membrane surface. Consistent with this is the observation that binding of β-adrenergic agonists results in a decrease of the entropy of the (outer) membranes [27] on the one hand and that coupling of β-adrenergic receptor with the catalytic subunit is a diffusion controlled process and is facilitated by a more fluid milieu [28], on the other. Thus, because catecholamines bring about structural alterations in the membrane they may render it thermodynamically more favourable for the activation of adenylate cyclase.

In summary, incubation of rat liver plasma membranes in the presence of isoproterenol resulted in a rapid decomposition of phosphatidylcholine. Phentolamine, an α-, and propranolol, a β-adrenergic blocking agent, were unable to prevent this action of the hormone. Both isoproterenol and phospholipase C treatment resulted in an enhanced labeling by ^{32}P of phospholipids present in the membrane. It was suggested that morphological changes of the membrane architecture by hormone may also facilitate the coupling of β-adrenergic receptor with the catalytic subunit of the adenylate cyclase.

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