Volume 156, number 2 FEBS 0454 June 1983

Norepinephrine-induced loss of phosphatidylinositol from isolated rat liver plasma membrane

Effects of divalent cations

Michael A. Wallace*, Josiane Poggioli, Françoise Giraud+ and Michel Claret

Laboratoire de Physiologie Comparée, LA 89, Bat 443, Université Paris Sud, 91405 Orsay, Cedex and
+*Laboratoire de Physiologie de la Nutrition, U 231, ERA 415, Bat 447, Université Paris-Sud, Orsay Cedex, France*

Received 21 April 1983

Norepinephrine at 5 μM induces a rapid (60 s) and specific loss of phosphatidylinositol (PtdIns) when added to isolated rat liver plasma membranes. The hormone action is inhibited by the α-adrenergic antagonist phentolamine (20 μM). Depletion of Mg^{2+} and Ca^{2+} singly or in combination from the incubation buffer mimicks the hormone effect on PtdIns breakdown. No further effect on PtdIns degradation could be measured when norepinephrine was added to the cation-depleted buffers. Addition of the Ca^{2+} ionophore A23187 to the isolated membranes has no effect. It is concluded that PtdIns degradation can be provoked in isolated rat liver plasma membrane through α-adrenergic receptor activation and that this effect is dependent on divalent cations in the sense that loss of cations from the membrane allows degradation to commence.

Noradrenaline *Phosphatidylinositol* *Ca^{2+}* *Mg^{2+}*
Isolated plasma membranes

1. INTRODUCTION

Many hormones exert their effects on cellular metabolism through mobilization of Ca^{2+} [1]. These same hormones almost invariably cause changes in phosphoinositide metabolism [2]. Numerous studies with intact cells have attempted to demonstrate that hormone-promoted changes in phosphatidylinositol (PtdIns) turnover were either all causes or all effects of Ca^{2+} mobilization. A consideration of the effects of Ca^{2+} on PtdIns synthesis has led to the description of primary *vs* secondary changes in PtdIns metabolism [3]. Secondary changes are considered those which are clearly affected by changes in cytosolic [Ca^{2+}], while primary changes are those promoted by hormone and independent of transmembrane Ca^{2+} flux. From dose–response studies using hepatocytes, it was argued [4] that PtdIns degradation is closely linked to hormone–receptor occupation (i.e., a primary response). In [5] a loss of [^{3}H]PtdIns was reported specifically from the plasma membranes of hepatocytes which were treated with vasopressin.

In [6], vasopressin and epinephrine induced a specific loss of PtdIns from isolated membranes; the vasopressin response was seen in the presence of deoxycholate [6], but subsequently the deoxycholate requirement was not found [7]. Norepinephrine (NE) could initiate loss of PtdIns from plasma membranes in the presence but not absence of cytosol [8]. NE worked through α-adrenergic receptors. These reports favor the

* Present address: Division of Biology and Medicine, Brown University, Providence, RI 02912, USA

Abbreviations: PtdIns, phosphatidylinositol; NE, l-norepinephrine; EGTA, ethyleneglycol-bis (β-aminoethyl ether)-*N,N'*-tetraacetic acid

Published by Elsevier Science Publishers B.V.
00145793/83/$3.00

hypothesis that PtdIns degradation in membranes is linked closely to hormone-receptor occupancy. In [9], NE working through α-adrenergic receptors was found to cause a change in membrane fluidity in isolated liver plasma membranes incubated only in the presence of 5 mM Mg^{2+} and traces of Ca^{2+}. If plasma membranes were incubated with $^{45}Ca^{2+}$ the radioactive cation bound to the membrane and subsequently could be released by treatment with NE or angiotensin II. Thus, the membrane preparations in [9] must have preserved the functional coupling between hormones, receptors, and whatever else constitutes the transmembrane signal for α_1-adrenergic hormones. It was important then to examine the relationship of PtdIns to these hormone actions. We demonstrate here, that, in identical membrane preparations, 5 μM NE induced a specific loss of PtdIns in a Ca^{2+}- and Mg^{2+}-dependent way.

2. METHODS AND MATERIALS

Plasma membranes were prepared by the method in [10] as described [9]. The preparation involves Dounce homogenization of rat liver in hypotonic media and isolation of plasma membranes on a discontinuous sucrose gradient. The incubation buffer, unless otherwise stated, contained 50 mM Tris (pH 7.4), 0.1 mM EGTA, 5 mM $MgCl_2$ and 5 μM 1-propranolol. The membranes were resuspended to 1.5–3.0 mg protein/ml. All experiments used freshly prepared membranes.

Incubation of plasma membranes was initiated by addition of 0.5 ml above suspension to 0.5 ml buffer. The samples were shaken for 10 min at 37°C at which time hormone, ionophore, excess EGTA, or a combination of these were added and the incubations continued for 1 min. When present, 20 μM phentolamine was included throughout the entire 11 min incubation, and for some experiments $MgCl_2$ was not present.

Reactions were stopped by the addition of 3.75 ml $CHCl_3$:MeOH (1:2, v/v) with 1% HCl. $CHCl_3$ (1.25 ml) was added followed by 1.25 ml H_2O. Extractions were performed overnight at 4°C. After centrifugation of the samples, the upper aqueous and interface were removed by aspiration and the entire organic phase dried under a vacuum. Phospholipids were resuspended in $CHCl_3$ for chromatography.

The chromatography of phospholipids was performed on commercial silica gel plates which were first soaked at least 2 h in H_2O to remove extraneous phosphorus and then dried and reactivated by heating at ~100°C. The solvent system was as in [11] containing $CHCl_3$:MeOH:methylamine (94.5:45:15). Chromatography plates were run in this solvent for 1 h, about ~17 cm. This chromatography system allowed excellent separation of phosphatidylinositol, phosphatidylcholine, and phosphatidylethanolamine from other lipids. Phosphatidylserine was not resolved from phosphatidic acid. Polyphosphoinositides could not be identified (or P content assayed) from samples containing up to 1.5 mg plasma membrane protein/ml. Phospholipids were visualized under white and/or UV light after I_2 vapour staining. Lipid phosphorus was then determined on silica gel scrapings as in [12]. 5′-Nucleotidase was assayed as in [13]; glucose 6-phosphatase measured as in [14]; cholesterol extracted as in [15] and then assayed as in [16].

NE was purchased from Sigma (St Louis MO) as was 1-propranolol. Phentolamine (regitine) was secured through Ciba-Geigy (Basel). A23187 was bought from Boehringer (Mannheim). Thin-layer silica gel chromatography plates containing *luminescer* came from Schleicher and Schull (Dassel). All other chemicals (e.g., $CHCl_3$) were reagent grade.

3. RESULTS

The plasma membranes prepared for this study were enriched 13-fold over the original homogenate for the plasma membrane marker enzyme, 5′-nucleotidase. In contrast, no enrichment was found for the endoplasmic reticulum marker enzyme, glucose 6-phosphatase. Since cholesterol is primarily localized in the plasma membrane, we measured the cholesterol:phospholipid ratio as an index of purity for plasma membrane preparation. A value of 0.66 ± 0.02 (mean ± SEM of 9 preparations) was obtained. Thus, considering this ratio and the 5′-nucleotidase enrichment, these plasma membrane preparations were contaminated to ~8% with a combination of other subcellular

Table 1

Norepinephrine-induced loss of PtdIns from isolated rat liver plasma membranes

Phospholipid	Δ% Change from control		
	+ Mg^{2+}		− Mg^{2+}
	+ NE	NE + phentolamine	+ NE
Phosphatidylcholine	+0.6 ± 2.3	+0.6 ± 3.0	+0.8 ± 1.3
Phosphatidylethanolamine	−0.9 ± 3.4	−4.2 ± 3.3	−1.3 ± 1.5
Phosphatidylinositol	−7.7 ± 3.4[a]	−1.1 ± 2.7	+3.5 ± 3.7

[a] Different from control at $p \leqslant 0.05$

Rat liver plasma membranes (~1 mg protein/ml) were incubated in 1 ml aliquots in 50 mM Tris (pH 7.4), 0.1 mM EGTA, 5 mM $MgCl_2$ and 5 μM 1-propranolol at 37°C for 10 min; 5 μM (−)norepinephrine was added and incubation continued for 1 min. When added, phentolamine (20 μM) was present throughout the entire incubation. Results are the average % change from control values ± SEM based on 11–12 determinations

phospholipids and enzymes.

The mol% distribution of phospholipid phosphorus in these membranes (average control values for 27 determinations ± SEM) was phosphatidylcholine 40.8 ± 0.7, phosphatidylethanolamine 26.4 ± 0.6, phosphatidylinositol 7.9 ± 0.2, and all others, including phosphatidylserine, sphingomyelin, and phosphatidic acid 24.9.

Incubation of plasma membranes with 5 μM NE for 1 min at 37°C caused a specific loss of PtdIns (table 1). All incubations included (−)propranolol to block any β-adrenergic effects of NE. No phospholipid except PtdIns was apparently affected. NE induced about a 7–8% loss of PtdIns, representing about 0.7% of the total membrane phospholipid. The effect of 5 μM NE was blocked by 20 μM phentolamine (table 1) showing that the hormone was probably working through α-adrenergic receptors.

When added to hepatocytes, the Ca^{2+} ionophore A23187 can promote activation of glycogen phosphorylase by causing redistribution of Ca^{2+}. Just as with vasopressin [18], the ionophore can transiently activate phosphorylase in the absence of extracellular Ca^{2+} (unpublished). Thus, A23187 can probably mobilize Ca^{2+} bound to the plasma membrane. A23187 at 10 μM caused no changes in any of the membrane phospholipids during the standard 1 min incubations (not shown).

Depletion of $MgCl_2$ from the incubation buffer (remaining contaminating Mg^{2+} = 2.5 μM) resulted in a specific loss of PtdIns (table 2). These

Table 2

Effect of $MgCl_2$ and Ca^{2+} depletion on liver membrane-PtdIns

Phospholipid	Δ% Change	
	Due to omission of Mg^{2+}	Due to removal of Ca^{2+}
Phosphatidyl-choline	−0.8 ± 1.6	−0.2 ± 1.0
Phosphatidyl-ethanolamine	−2.0 ± 2.1	−2.4 ± 1.3
Phosphatidyl-inositol	−11.0 ± 4.0[a]	−8.5 ± 2.8[a]

[a] Significantly different at $p \leqslant 0.01$

Rat liver plasma membranes were incubated for 11 min with or without 5 mM $MgCl_2$ in the standard buffer, or in this buffer with $MgCl_2$ for 10 min, at which time 2 mM EGTA was added (reducing free $[Ca^{2+}]$ to <0.5 nM) and incubation continued for 1 min. Results are the average % change from controls (+ Mg^{2+}) ± SEM for 11–17 determinations

results led us to investigate whether reducing free Ca^{2+} in the incubation buffer could also affect PtdIns. The free Ca^{2+} in the normal buffer containing 0.1 mM EGTA is estimated to be ~10 nM [9]. Raising the EGTA content to 2.1 mM reduces free Ca^{2+} to <0.5 nM, and more of the Ca^{2+} bound originally to the plasma membrane may be stripped away. Increasing [EGTA] from 0.1–2.1 mM during the last minute of incubation provoked a specific loss of PtdIns (table 2). If $MgCl_2$ was absent, raising the EGTA concentration had no further effect on PtdIns than did simple $MgCl_2$ depletion alone (not shown). Further, 5 μM NE had no effect in the absence of $MgCl_2$ (table 1), suggesting that hormone addition or divalent cations depletion promoted loss of PtdIns through some common mechanisms.

4. DISCUSSION

The present study indicates that a hormone-induced loss of PtdIns occurs in isolated rat liver plasma membranes. This is in accord with the hypothesis that PtdIns degradation is a primary event closely linked to hormone binding to receptor. No study with isolated plasma membranes has characterized the products of PtdIns degradation, however, so it is premature to say that it occurs through action of phospholipase C as is generally thought. Blocking of NE action by phentolamine, but not propranolol, demonstrates α-adrenergic specificity in accord with the results in [8] and with the studies on intact hepatocytes [17].

The relationship of PtdIns loss to Ca^{2+} mobilization in intact hepatocytes is not understood. The present data demonstrate a loss of PtdIns which is induced by lowering free Ca^{2+} (or Mg^{2+}) while probably removing Ca^{2+} from the plasma membrane. This is quite different from any hypothesis which suggests that raising cytosolic Ca^{2+} levels induces PtdIns turnover by stimulating phospholipase C. Rather, it may be the hormone-induced loss of Ca^{2+} from the membrane [9] which allows degradation to proceed.

From these data, it can be calculated that hormone-stimulated PtdIns degradation must have occurred at >1 nmol.min^{-1}.mg $protein^{-1}$. Estimates of the number of plasma membrane α_1-adrenergic receptors are on the order of 1 pmol/mg protein [19]. Thus, many moles of PtdIns are lost per bound mole of NE. This rules out any hypothesis that the degradation of PtdIns is restricted to a few phospholipid molecules intimately associated with the receptor. Once NE is bound a general stimulation of PtdIns degradation occurs. This finding is in accord with [8], but no added cytosol was necessary to demonstrate NE effects on PtdIns. We do not know the reason for this difference. Our plasma membranes may be sufficiently contaminated with cytosol to allow the phenomena to occur. On the other hand, our preparations may be preserving a 'normal' association between hormone-receptors and lipases which act on plasma membrane phospholipids. At any rate, lipases whose substrates are the phospholipids must of necessity have affinity for the plasma membrane and so their designation as 'membrane-bound' or 'cytosolic' is partly semantic. That is, whether or not a phospholipase is an integral membrane protein, it must somehow be bound to the membrane to act. Both studies suggest that many moles of PtdIns throughout the membrane are exposed to degradative enzymes per mole of receptor occupied.

It has been proposed that PtdIns degradation closes Ca^{2+} gates [20] or that the stimulation of PtdIns breakdown leads to phosphatidic acid formation which in turn acts as a natural ionophore to gate in Ca^{2+} [21]. Neither proposition is excluded by these data.

The losses of PtdIns seen here could not be due to hormone stimulation of the phosphorylation of PtdIns to form phosphatidylinositol-4phosphate and/or phosphatidyl-4,5bis-phosphate as suggested [22], since there was no ATP present to support their synthesis.

Thus, we have found that PtdIns degradation is stimulated when NE is added to hepatic plasma membranes and that this effect is mimicked by Mg^{2+} and/or Ca^{2+} depletion. Much more study is needed, however, to elucidate the role of PtdIns degradation in overall metabolic regulation. The isolated plasma membrane system will constitute a powerful tool to investigate α_1-adrenergic action and its interaction with other hormones because at least 3 functions can now be stimulated by addition of Ca^{2+}-mobilizing hormones to isolated rat liver plasma membranes (i.e., Ca^{2+} release [9]), increases in membrane fluidity [9] and PtdIns degradation ([6–8]; these results).

ACKNOWLEDGEMENTS

The authors wish to thank J.N. Fain for his enthusiastic support of this project. M.A.W. received traveling fellowships from NATO and from the Phillipe Foundation, Inc., New York. Supported in part by a United States Public Health Services grant (AM 21470) to John N. Fain, Division of Biology and Medicine, Brown University, Providence RI.

REFERENCES

[1] Rasmussen, H. (1981) Calcium and cAMP as Synarchic Messengers, Wiley, New York.
[2] Michell, R.H. (1975) Biochim. Biophys. Acta 415, 81–147.
[3] Fain, J.N. (1981) in: Horizons in Biochemistry (Palmieri, F. ed) vol.6, Academic Press, New York.
[4] Kirk, C.J., Creba, J.A., Downes, C.P. and Michell, R.H. (1981) Biol. Soc. Trans. 9, 377–379.
[5] Lin, S.-H. and Fain, J.N. (1981) Life Sci. 19, 1905–1912.
[6] Wallace, M.A., Randazzo, P., Li, S.-Y. and Fain, J. (1982) Endocrinology 3, 341–343.
[7] Fain, J.N., Lin, S.-H., Randazzo, P., Robinson, S. and Wallace, M.A. (1983) in: Isolation, Characterization and Use of Hepatocytes (Harris, R.A. and Cornell, N.W. eds) Academic Press, New York, in press.
[8] Harrington, C.A. and Eichberg, J. (1983) J. Biol. Chem. 258, 2087–2090.
[9] Burgess, G.M., Giraud, F., Poggioli, J. and Claret, M. (1983) Biochim. Biophys. Acta, in press.
[10] Song, C.S., Rubin, W., Rifkind, A.B. and Kappas, A. (1969) J. Cell. Biol. 41, 124–132.
[11] Harrington, C.A., Fenimore, D.C. and Eichberg, J. (1980) Anal. Biochem. 106, 307–313.
[12] Rouser, G., Fleischer, S. and Yamamoto, A. (1970) Lipids 5, 494–496.
[13] Bodansky, O. and Schwartz, M.K. (1963) J. Biol. Chem. 238, 3420–3427.
[14] Harper, A.E. (1965) in: Methods of Enzymatic Analysis (Bergmeyer, H.U. ed) pp.788–792, Academic Press, London, New York.
[15] Rose, H.G. and Ocklander, M. (1965) J. Lipid Res. 6, 428–431.
[16] Rudel, L.L. and Morris, M.D. (1973) J. Lipid Res. 14, 364–366.
[17] Tolbert, M.E.M., White, A.C., Aspry, K., Cutts, J. and Fain, J.N. (1980) J. Biol. Chem. 235, 1938–1944.
[18] Blackmore, P.F., Hughes, B.P., Shuman, E.A. and Exton, J.A. (1982) J. Biol. Chem. 257, 190–197.
[19] Goodhardt, M., Ferry, N., Geynet, P. and Hanoune, J. (1982) J. Biol. Chem. 257, 11577–11583.
[20] Berridge, M.S. and Fain, J.N. (1979) Biochem. J. 178, 59–69.
[21] Putney, J.W. (1981) Life Sci. 29, 1183–1194.
[22] Michell, R.H., Kirk, C.J., Jones, L.M., Downes, C.P. and Creba, J.A. (1981) Phil. Trans. R. Soc. Lond. B 296, 123–137.