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Phospholipid-dependence of rat liver plasma membrane protein kinase activities – a new approach

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The influence of the phospholipid composition and fluidity on protein kinase A and protein kinase C activities in rat liver plasma membranes was studied. We observed that enrichment of membranes with phosphatidylglycerol, phosphatidylserine, phosphatidylethanolamine and dioleoylphosphatidylcholine caused activation of both protein kinases. Phosphatidylglycerol was found to be most effective activator. The enrichment of plasma membranes with dipalmitoylphosphatidylcholine and sphingomyelin led to decrease in protein kinase A and C activities. The stimulatory effect of phosphatidylglycerol was confirmed in plasma membranes pretreated with exogenous phospholipases A_2, C and D, and subsequently enriched with phosphatidylglycerol. We suggest that besides the specific presence of definite phospholipids protein kinases A and C require a more fluid membrane lipid bilayer to display an optimal activity.

Introduction

Protein kinases are known to be major mediators in the response of cells and tissues to hormonal influence as well as in the control of cell growth and development [1]. Many protein kinases either span the plasma membranes or are associated with the inner lipid monolayer [2]. Protein kinase C is a Ca^{2+}- and phospholipid-dependent enzyme, which is activated by diacylglycerols [2–4]. Protein kinase C is considered to be active when associated with the lipid bilayer [5]. Wolf et al. [6] have shown that the intracellular calcium concentration regulates the process of association-dissociation of the enzyme to and from the lipid bilayer. The mechanisms underlying the phospholipid-induced activation of protein kinase C have recently been extensively studied [7–10]. Of the various phospholipids tested, phosphatidylserine (PS) appeared to be indispensable whereas other anionic phospholipids such as phosphatidic acid (PA), phosphatidylglycerol (PG), phosphatidylinositol (PI) and diphosphatidylglycerol (DPG) were less effective [8,9]. These findings indicate that the membrane phospholipids and their asymmetric membrane distribution might play a significant role in protein kinase C regulation and that each phospholipid could display a specific effect [11].

The major receptor for cAMP in eukaryotic cells is the regulatory subunit of cAMP-dependent protein kinase (protein kinase A) [12]. Some cAMP-dependent protein kinases and phosphate-acceptor proteins are integral components of plasma membranes, sarcoplasmic reticulum, nuclei and ribosomes [12]. Therefore it is not unlikely that the membrane lipid composition and physical state are of certain importance for their activities.

Recently, Klemm et al. [13] reported that PG is a strong mediator of another protein kinase-protein kinase P.

There is a lot of evidence concerning the role of the membrane phospholipid composition and physicochemical properties in regulation of the activities of many membrane-bound enzymes such as adenylatecyclase [14], Na^+/K^+-ATPase [15], phospholipase A_2 [16], neutral sphingomyelinase [17], 5′-nucleotidase [18] etc.

The purpose of our experiments was to study the effect of different phospholipids on protein kinase A and protein kinase C activities in rat liver plasma membranes.

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Materials and Methods

Animals

Male Wistar rats weighing about 200 g and fed standard laboratory diet have been used in all experiments.

Reagents

Phospholipase A_2 (from *Vipera russeli*), phospholipase C (from *Clostridium perfringens*), phospholipase D (from Cabbage), 3-*sn*-phosphatidylethanolamine (PE), 3-*sn*-phosphatidyl-DL-glycerol (PG), sphingomyelin (SM), 3-*sn*-phosphatidyl-L-serine (PS), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dioleoyl-*sn*-glycerol, ATP, Histone II A, Histone III S, cAMP, theophylline, leupeptin were purchased from Sigma Chemical Co., St. Louis, MO) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,5-diphenyl-1,3,5-hexatriene (Fluka Chemie AG, Buch, Switzerland).

[γ-^{32}P]ATP (3000 Ci/mmol) was purchased from Amersham International.

Isolation of rat liver plasma membranes

Liver plasma membranes have been isolated by the procedure described by Wisher and Evans [19]. This method includes membrane flotation through discontinuous sucrose gradient in a Sorvall OTD-50 ultracentrifuge, rotor AH-627 at $96000 \times g$ for 3 h. The purity of the membrane fraction was assessed by the specific activities of marker enzymes (5'-nucleotidase, glucose-6-phosphatase, succinate–cytochrome-*c* reductase and acid phosphatase) as well as by electron microscopy. The membranes obtained were used for investigations without being frozen.

Enrichment of the plasma membranes with phospholipids [20]

Incubations were performed for 1 or 2 h at 37°C in the presence of partially purified lipid transfer proteins (LTP). The incubation medium contained LTP/membrane protein/phospholipid liposomes at a ratio 1:1:0.4 (w/w/w). LTP of different origin were used for enrichment of the membrane fractions as follows:

for enrichment with sphingomyelin (SM)-LTP from chicken liver,

for enrichment with DPPC and PG-LTP from rat lung,

for enrichment with DOPC, PE, PS-LTP from rat liver.

The enriched membranes were washed twice with 10 mM Tris-HCl buffer (pH 7.4) and used for further investigations. Electron microscopy study showed no liposomes attached to the plasma membranes after double washing and that membrane integrity was preserved.

Partial purification of LTP

The postmitochondrial $105000 \times g$ supernatant was adjusted to pH 5.1 with 3 M HCl. After 1 h, the precipitate was sedimented by centrifugation for 15 min at $14000 \times g$ under refrigeration and discarded. The pH of the supernatant was readjusted to 7.4 with 1 M Tris-HCl. Solid ammonium sulfate was added slowly to the supernatant to 90% saturation and the mixture was stirred overnight. The precipitate was sedimented by centrifugation for 15 min at $14000 \times g$, dissolved in 5 mM sodium phosphate/10 mM β-mercaptoethanol (pH 7.2) and dialyzed for 24 h against the same buffer. Protein precipitated during dialysis was removed by centrifugation. The supernatant was used as LTP source.

Treatment of liver plasma membranes with exogenous phospholipases C [20]

Incubations were carried out with the exogenous phospholipase C for 20 min at 37°C as follows: 5 units of the enzyme were incubated with plasma membranes (1 μM phospholipids) in 5 mM $CaCl_2$, 10 mM Tris-HCl buffer (pH 7.4). The reaction was terminated by chilling and centrifugation of plasma membranes in refrigerated centrifuge at $8000 \times g$ for 30 min at 4°C. The pellet was washed twice in 10 mM Tris-HCl buffer (pH 7.4) and the partially delipidated plasma membranes were used for enrichment with different phospholipids, which was performed for 2 h at 37°C.

Assay of protein kinase activity

The assay of cAMP-dependent protein kinase A was essentially as described by Sharoni et al. [21]. The reaction mixture at final volume of 0.1 ml contained 50 mM potassium phosphate buffer (pH 6.5), 10 mM magnesium acetate, 1 mM theophylline, 1 μM cAMP, 0.2 mg Histone II A and 20 μM [γ-^{32}P]ATP (500 cpm/pmol).

Ca^{2+}-phospholipid-dependent protein kinase C was assayed as described by Pelfrey and Wassem [22]. The reaction mixture at final volume of 0.1 ml contained 50 mM Tris-HCl, 5 mM $MgSO_4$, 1 mM Ca-EGTA buffer (pH 7.4), free C^{2+} at a concentration of 5 mM, 25 μg/ml phosphatidylserine, 20 μg/ml 1,2-dioleoyl-*sn*-glycerol, 0.2 mg/ml Histone III S, 40 μg/ml leupeptin and 20 μM [γ-^{32}P]ATP (500 cpm/pmol).

Aliquots of membrane preparations (50 μg of protein) were used as sources of enzyme activity. The reaction continued for 10 min at 30°C in a shaking water-bath and was stopped by adding 2 ml of 10% trichloroacetic acid. Samples were poured onto glass-fibre filters and washed five times with 5% trichloroacetic acid, dried and counted on a Rackbeta II (LKB) scintillation counter.

The protein kinase activities were calculated as pmoles [γ-^{32}P]ATP incorporated per min per mg of membrane protein.

TABLE I

Protein kinase activities and structural order parameter (S_{DPH}) in liver plasma membranes enriched with different phospholipids by the aid of lipid transfer proteins

Values are means ± S.D. ($n = 8$). Enrichment was presented as % of incorporated phospholipid in comparison to the initial level of the same phospholipid in the membrane. (For more details see Materials and Methods). PS, phosphatidylserine; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; DOPC, dioleoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; SM, sphingomyelin.

Enrichment		S_{DPH}	Activity (pmol min^{-1} mg^{-1})	
PLs	%		protein kinase A	protein kinase C
None	-	0.680	91.8 ± 4.9	95.4 ± 13.0
PS	83	0.652 [a]	178.2 ± 11.9 [c]	279.1 ± 16.3 [c]
PG	80	0.623 [a]	518.4 ± 27.9 [c]	388.3 ± 49.0 [c]
PE	70	0.609 [b]	95.4 ± 6.7	122.4 ± 13.7 [b]
DOPC	70	0.589 [c]	203.4 ± 18.5 [c]	178.2 ± 17.1 [c]
DPPC	74	0.827 [c]	70.2 ± 12.4 [c]	88.2 ± 8.6
SM	84	0.768 [c]	70.2 ± 8.1 [c]	86.4 ± 9.9

[a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$.

Analytical methods

Lipids were extracted from plasma membranes by the method of Folch et al. [23]. The phospholipid content was determined in the total lipid extract [24]. The individual phospholipids were chromatographed on Silica gel 60 thin-layer plates (Merck AG, Darmstadt, Germany). Chloroform/methanol/isopropanol/0.25% KCl/triethylamine (30:9:25:6:19, by vol.) were used as developing solvents [25]. The protein content of plasma membranes was determined by the method of Lowry et al. [26].

Fluorescence assays

1,6-Diphenyl-1,3,5-hexatriene (DPH) was used as a fluorescent probe for estimation of plasma membrane fluidity. The steady-state anisotropy of fluorescence (r_s) of DPH was estimated from the equation [27]:

$$r_s = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp})$$

The lipid structural order parameter (S_{DPH}) was calculated by an empirical method described by Van Blitterswijk et al. [28].

Fluorescent measurements have been performed at 355 nm (excitation beam) and 425 nm (emission beam) on a Perkin-Elmer 3000 fluorescence spectrometer.

Statistical analysis was performed using Student's *t*-test.

Results

Table I presents the changes in the structural order parameter (S_{DPH}) and protein kinase A and C activities induced by incorporation of different phospholipids into rat liver plasma membranes in the presence of lipid transfer proteins. Incubation of plasma membranes with different phospholipids caused marked elevation of the corresponding phospholipid fraction (Table I). The percent of this enrichment was approximately similar for all phospholipid fractions – about 70 to 80%. In addition the incorporation of the exogenous phospholipids into the membrane induced alteration in the lipid structural order parameter (S_{DPH}) of the bilayer (Table I).

Our data showed a great similarity in the effect of a given phospholipid fraction on protein kinase A and C activities. Phosphatidylglycerol (PG) was found to be the most effective activator, followed by phosphatidylserine (PS) and dioleoylphosphatidylcholine (DOPC). The effect of sphingomyelin (SM) and dipalmitoylphosphatidylcholine (DPPC) was particularly interesting.

TABLE II

Phosphatidylglycerol content, structural order parameter (S_{DPH}) and protein kinase activities in pretreated with exogenous phospholipases A_2, C and D liver plasma membranes

Values are means ± S.D. ($n = 8$). For more technical details see Materials and Methods. PLC, phospholipase C; PLA_2, phospholipase A_2; PLD, phospholipase D; PKA, protein kinase A; PKC, protein kinase C; PG, phosphatidylglycerol.

Treatment	Enrichment with PG	PG (μg/mg protein)	S_{DPH}	Activity (pmol min^{-1} mg^{-1})	
				PKA	PKC
Control	–	15.67 ± 1.07	0.712 ± 0.002	94.6 ± 4.2	102.1 ± 5.2
	+	23.50 ± 2.04 [c]	0.697 ± 0.002 [c]	518.4 ± 27.9 [c]	388.3 ± 49.0 [c]
PLC	–	7.14 ± 1.03	0.802 ± 0.009	35.6 ± 1.4	153.1 ± 4.9
	+	14.90 ± 1.34 [c]	0.722 ± 0.007 [c]	242.0 ± 29.1 [c]	463.3 ± 65.5 [c]
PLA_2	–	7.31 ± 2.16	0.754 ± 0.007	52.1 ± 7.7	61.5 ± 4.8
	+	13.15 ± 1.68 [c]	0.703 ± 0.006 [c]	251.5 ± 20.7 [c]	354.5 ± 13.2 [c]
PLD	–	10.66 ± 0.80	0.732 ± 0.003	73.2 ± 4.2	89.6 ± 5.2
	+	16.44 ± 1.45 [c]	0.697 ± 0.009 [c]	284.1 ± 17.4 [c]	333.6 ± 14.5 [c]

[c] $P < 0.001$.

These two phospholipids reduced the activity of both protein kinases, their effect being more pronounced for protein kinase A, which was inhibited by about 25%.

Since PG turned out to be the most efficient phospholipid effector we studied in detail its role in the activation of the two protein kinases.

The changes in the level of PG in membranes treated with exogenous phospholipases A_2, C and D are shown in Table II. Phospholipase treatment of plasma membranes reduced the PG level by about 50%. The subsequent enrichment of partially delipidated plasma membranes with PG led to a complete restoration to the control level and it always induced membrane fluidization (i.e. diminution of S_{DPH}).

Treatment of plasma membranes with exogenous phospholipases was accompanied by marked inhibition of protein kinase A which corresponded well to the decrease of PG in these membranes (Table II). The restoration of the PG level augmented about 3-fold the enzyme activity. Partial delipidation with exogenous phospholipases A_2 and D also induced reduction of protein kinase C activity. In phospholipase-C-treated membranes, however, a significant activation of prtoein kinase C was observed. Membrane enrichment with PG led to reactivation of this enzyme of about 3–4-times. This effect was more pronounced in membranes pretreated with phospholipase C.

Discussion

The role of protein kinases in the control of cell growth and development [1,2] makes the investigation of their regulation particularly important. Since these enzymes are known to associate with the membranes under definite conditions the influence of the lipid environment is of special interest. There is a great deal of evidence illustrating the essential role of PS for protein kinase C activation [2,8–11]. However, the role of different phospholipids in the regulation of protein kinases has been investigated so far mainly by addition of phospholipid liposomes to the incubation mixture. In contrast, the approach we used was to enrich rat liver plasma membranes with definite phospholipids, thus modulating the enzyme lipid environment.

Our results indicated that PG was the most effective activator of membrane-bound protein kinases A and C. This was confirmed in the experiments using PG-enriched plasma membranes, as well as membranes in which PG and most of the other phospholipids have been significantly reduced by exogenous phospholipases A_2, C and D. Partial delipidation of membranes was accompanied by a significant inactivation of both protein kinases A and C. The only exception was protein kinase C in membranes treated with phospholipase C. However, this result was not unexpected. Protein kinase C exhibited quite a high activity in these membranes, in comparison with the native membranes. This was apparently due to the enhanced content of diacylglycerols caused by phospholipase C treatment. There are many papers devoted to the activating effect of diacylglycerols on protein kinase C [2–4,29]. Our data further confirmed this effect. The accumulation of diacylglycerols (due to phospholipase C treatment of plasma membranes) followed by enrichment with exogenous PG induced a strong activation of protein kinase C. In addition, we observed that some other phospholipids, which were incorporated into the membranes, such as PS, DOPC and PE, also activated protein kinase C, whereas SM and DPPC exhibited a slight inhibitory effect. It should be pointed out that PS and DOPC displayed a similar effect on protein kinase A activity. Membrane enrichment with SM and DPPC, which are known to reduce membrane fluidity was accompanied by marked inhibition of this protein kinase. The augmentation of the structural order parameter S_{DPH}, due to membrane treatment with exogenous phospholipases was also followed by a significant inhibition of protein kinase A activity. Therefore it seems likely that the activity of this enzyme was influenced by membrane fluidity. Similar effect was also observed for protein kinase C. In this respect Szamel et al. [30] reported that the incorporation of polyunsaturated fatty acids into plasma membrane phospholipids resulted in long-term activation of protein kinase C. The more fluid or destabilized bilayer might allow protein kinase C to penetrate deeper into the membrane, altering the protein conformation and changing its activity. In addition diacylglycerols can act as fluidizers increasing the lipid spacing, thus promoting penetration of protein kinase C into the lipid bilayer [31,32]. Protein kinase C has been reported to associate and subsequently penetrate lipid monolayers [33] and bilayers [34] which might provoke alteration of the protein conformation and results in an active lipid-protein complex. Protein kinase C was established to have numerous cystine residues in its regulatory region which are important for lipid and phorbol ester binding [35,36]. The lipid composition of membranes appears to influence the selection of different interacting cystine rich regions [33].

Our results imply that in addition to membrane fluidization PG most probably acts as a specific activator of membrane-associated protein kinase C. Thus, if PS is necessary for the initial activation of this enzyme as well as for its attachment to the membrane, it seems quite likely that PG, which is also negatively charged like PS, performs an additional activation for achievement of optimal enzyme activity. In this respect, we agree with Brumfeld and Lester [32] who suggested that a reconsideration of the mechanism of protein kinase C activation is required.

Unfortunately there are no data available in literature showing that protein kinase A is phospholipid-dependent. Our experiments indicated that this enzyme was greatly activated by PG and significantly inhibited by SM and DPPC.

In conclusion, we suggest that besides some specific phospholipid activators, protein kinases C and A prefer a more fluid lipid environment in order to display an optimal activity.

References

1 Hunter, T. (1987) Cell 50, 823–829.
2 Nishizuka, Y. (1986) Science 233, 305–311.
3 Kishimoto, A., Takai, Y., Mori, T., Kikkawa, Y. and Nishizuka, Y. (1980) J. Biol. Chem. 255, 2273–2276.
4 Hannun, Y.A. and Bell, R.M. (1989) Clin. Chim. Acta 185, 333–346.
5 Epand, R.M. and Lester, D.S. (1990) Trends Pharmacol. Sci. 11, 317–320.
6 Wolf, M., Levine, M., III, May, S., Jr., Cuatrecasas, P. and Sayhoun, N. (1985) Nature 317, 546–549.
7 Lee, M.H. and Bell, R.M. (1989) J. Biol. Chem. 264, 14799–14805.
8 Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T. and Nishizuka, Y. (1979) J. biol. Chem. 254, 3692–3695.
9 Hannun, Y.A., Loomis, C.R. and Bell, R.M. (1986) J. Biol. chem. 261, 7184–7190.
10 Huang, K.-P. and Huang, F.L. (1990) J. Biol. Chem. 265, 730–744.
11 Kaibuchi, K., Takai, J. and Nishizuka, Y. (1981) J. Biol. Chem. 256, 7146–7149.
12 Krebs, E.G. and Beavo, A.G. (1979) Annu. Rev. Biochem. 48, 923–959.
13 Klemm, D.J. and Elias, L. (1988) Arch. Biochem. Biophys. 265, 506–513.
14 McOsker, C.C., Weiland, G.A. and Zilversmit, D.B. (1983) J. Biol. Chem. 258, 13017–13026.
15 Kimelberg, H.K. (1975) Biochim. Biophys. Acta 413, 143–152.
16 Momchilova, A.B., Petkova, D.H. and Koumanov, K.S. (1986) Int. J. Biochem. 18, 945–952.
17 Petkova, D.H., Momchilova, A.B. and Koumanov, K.S. (1986) Biochimie 68, 1195–1200.
18 Vankatesan, S.O., Galagher, G. and Mitropoulos, K.A. (1983) Biochim. Biophys. Acta 756, 72–82.
19 Wisher, M.H. and Evans, W.H. (1975) Biochem. J. 146, 375–388.
20 Momchilova-Pankova, A.B., Markovska, T.T. and Koumanov, K.S. (1990) Biochimie 72, 863–866.
21 Sharoni, J., Feldman, B., Teuerstein, I. and Levy, J. (1984) Endocrinology 115, 1918–1924.
22 Palfrey, H.C. and Wassem, A. (1985) J. Biol. Chem. 260, 16021–16029.
23 Folch, J., Lees, M. and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497–507.
24 Kahovkova, J. and Odavic, R. (1969) J. Chromatogr. 40, 90–95.
25 Touchstone, J.C., Chen, J.C. and Beaver, K.M. (1980) Lipids 15, 61–62.
28 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
27 Shinitzky, M. and Barenholz, Y. (1974) Biochemistry 249, 2652–2657.
28 Van Blitterswijk, W.J., Van Hoeven, R.P. and Van der Meer, B.M. (1981) Biochim. Biophys. Acta 644, 323–332.
29 Molleyres, L.P. and Rando, R.R. (1988) J. Biol. Chem. 263, 14832–14838.
30 Szamel, M., Rehermann, B., Krebs, B., Kurrle, R. and Resch, K. (1989) J. Immunol. 143, 2806–2813.
31 Lester, D.S., Doll, L., Brumfeld, V. and Miller, I.R. (1990) Biochim. Biophys. Acta 1039, 33–41.
32 Brumfeld, V. and Lester, D.S. (1990) Arch. Biochem. Biophys. 277, 318–323.
33 Tran, P.L., Ter-Manassian-Saraya, L., Madelmont, G. and Castagna, M. (1983) Biochim. Biophys. Acta 727, 31–38.
34 Das, S. and Rand, R.P. (1986) Biochemistry 25, 2882–2889.
35 Nishizuka, Y. (1988) Nature 334, 661–665.
36 Ono, Y., Fujii, T., Igarashi, K., Kuno, T., Tanaka, C., Kikkawa, U. and Nishizuka, Y. (1989) Proc. Natl. Acad. Sci. USA 86, 4868–4871.