ELSEVIER

FEBS Letters 337 (1994) 251–254

FEBS 13533

FEBS LETTERS

Phospholipase D activity of isolated rat brain plasma membranes

Julian N. Kanfer*, Douglas McCartney

Department of Biochemistry and Molecular Biology, University of Manitoba, Winnipeg, Manitoba, R3E 0W3, Canada

Received 22 November 1993; revised version received 1 December 1993

Abstract

With [^{14}C]oleate-labeled phosphatidylcholine as a substrate for phospholipase D the hydrolytic activity was measured by phosphatidic acid formation and the transphosphatidylation activity was measured by the phosphatidylethanol formed in the presence of ethanol. The pH optimum was 6.5 with dimethylglutarate as the buffer. EGTA inhibited the transphosphatidylation activity to a greater extent than the hydrolytic activity. In contrast $CaCl_2$, $BaCl_2$, $MgCl_2$ and $SrCl_2$ stimulated the hydrolytic activity without effecting the transphosphatidylation activity. $BeCl_2$ another member of the group IIa transition metals was a very potent inhibitor of both the hydrolytic and transphosphatidylation activity. GTPγS, an activator of G protein-mediated events, was an inhibitor of both activities.

Key words: Phospholipase D; Plasma membrane; Group IIa transition metal; GTPγS; $BeCl_2$

1. Introduction

The enzymatic properties [1], and cell biological responses of phospholipase D [2–5], have been reviewed. A major justification for this intense interest is the potential of phospholipase D for the production of diacylglycerol, believed requisite for protein kinase C activations [6]. As a consequence, the bulk of these observations have been derived with a common paradigm using intact cells containing labeled endogenous substrates. There are a few reports employing subfractions obtained from broken cells or tissue homogenates. A diversity of the effects of several divalent cations have been reported. In a few instances G protein couplings for phospholipase D activity have been observed. Therefore, we decided to characterize some of the properties of phospholipase D residing in isolated rat brain plasma membranes since these membranes would be the expected site of phospholipase D activation in response to perturbants.

2. Materials and methods

Rat brain plasma membranes were prepared utilizing an aqueous 2 phase partitioning procedure [7], 5′-Nucleotidase activity was used as a plasma membrane marker [8], and glucose-6-phosphatase activity was used as an endoplasmic reticulum marker [9]. Phospholipase D activity was measured with an incubation mixture containing 50 mM dimethylglutaric acid pH 6.5, 10 mM EDTA, 25 mM NaF, where indicated 0.3 M ETOH, 2.5 mM [^{14}C]phosphatidylcholine, 4 mM oleate and approximately 80 μg membrane protein as previously described [10], protein measurement was according to the classical procedure of Lowry et al. [11], and inorganic phosphate by a published method [12]. Phosphatidic acid, the hydrolytic product, and phosphatidylethanol, the transphosphatidylation product formed in the presence of ethanol, were separated by thin layer chromatography (TLC) [13]. 1-Palmitoyl-2-[^{14}C]oleoyl glycerol-3-phosphocholine was purchased from New England Nuclear (Boston, MA), and diluted with carrier phosphatidyl choline to yield a specific activity of approximately 500 dpm/nmol, Silica gel 60 TLC plates were the product of Merck, Darmstadt. The phosphatidylethanol standard for TLC was prepared as described [10]. The other routine reagents were obtained from several suppliers. All experiments were carried out on at least 2 separate occasions and all incubations were in duplicates.

3. Results

3.1. General properties

There was a four- to sixfold enrichment of 5 nucleotidase activity and an absence of glucose-6-phosphatase activity in the isolated plasma membranes (data not shown). Maximal phospholipase D activity, measured both as hydrolysis and transphosphatidylation, was obtained with dimethylglutarate buffer at pH 6.5. The measured activity at pH 7 was reduced 80% to 90% with all buffers tested including dimethylglutarate, MES, HEPES and Tris and product formation was linear up to 100 μg of membrane protein (data not shown). The maximum phosphatidylethanol formation was obtained in the presence of 200 mM ethanol (data not shown).

3.2. Effect of divalent cations

It was of interest to determine if the plasma membrane

*Corresponding author.

Abbreviations: PtdA, phosphatidic acid; PtdETOH, phosphatidylethanol; DG, diacylglycerols.

SSDI 0014-5793(93)E1450-Z

phospholipase D had a divalent cation requirement. Since divalent cations are not contained in the routine incubation mixture EGTA a known chelator was tested. Peculiarly, for any given EGTA concentration there was a greater reduction of phosphatidylethanol formation than of phosphatidic acid formation (Table 1). Diglyceride formation, presumably a measure of phosphatidic acid phosphatase activity, is similarly reduced. In contrast, EDTA did not diminish product formation, indeed there were slight increases. The inhibition by EGTA suggested a role for a divalent cation in phospholipase D activity, therefore, the effect of the addition of several divalent cations to the incubation was examined. As shown in Table 2, $CaCl_2$, $BaCl_2$, $MgCl_2$ and $SrCl_2$ increased phospholipase D hydrolysis of phosphatidylcholine. Except for 6 mM $SrCl_2$, there was increased phosphatidic acid formation in the presence of these cations with little if any stimulation of phosphatidylethanol formation. These cations are members of group IIa of the periodic chart of elements. The smallest molecular weight member of this group is beryllium. In contrast to the other members of this group, $BeCl_2$ caused a profound inhibition of phospholipase D activity (Fig. 1). Significant reductions ($P > 0.025$) of phosphatidic acid and phosphatidylethanol production were evident at 10 μM $BeCl_2$.

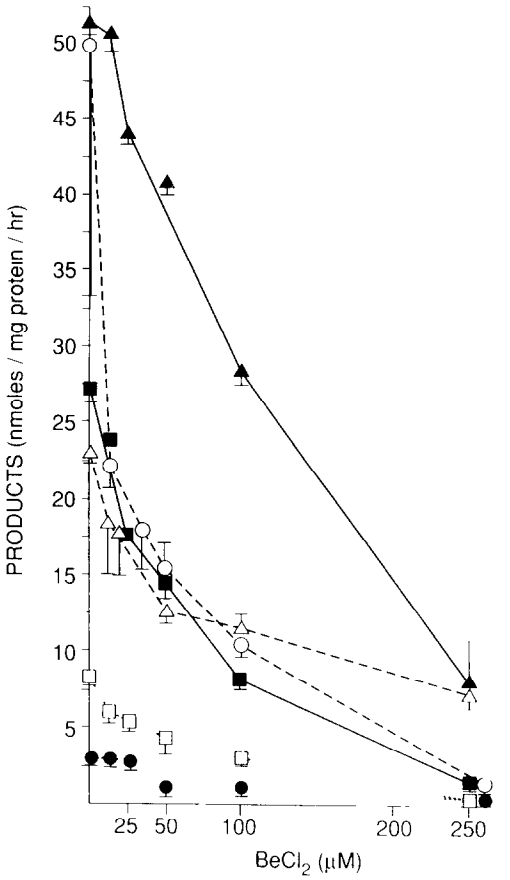

Fig. 1. The inhibition of rat brain plasma membrane phospholipase D activity by $BeCl_2$. The experimental details are provided in the text. Open symbols represent incubations in the presence of 0.2 M ETOH and filled symbols in the absence of ETOH. Squares indicate PtdA formation, circles indicate PtdETOH formation and triangles represent DG formation.

3.3. G protein involvement

In several instances receptor binding resulting in phospholipase D activation is mediated by G proteins [14]. Therefore, it seemed reasonable to determine if G protein involvement could be demonstrated with these rat brain plasma membrane preparations. As seen in Fig. 2, 50 μM GTPγS, an established activator of G proteins, results in significant reductions ($P > 0.05$) of both phosphatidic acid and phosphatidylethanol formation. The presence of 200 μg of brain cytosol plus $MgCl_2$ did not influence the GTPγS inhibition. There were similar decreases in phospholipase D activity in the presence of 1 mM GTP, ATP, UTP and GDPβS. The inclusion of 100 μM $AlCl_3$ in the incubation mixture caused a 50% reduction of phospholipase D activity (data not shown).

Table 1
Inhibition of phospholipase D activity of rat brain plasma membranes by EGTA*

Additions	PtdA	PtdETOH	DG
None	24.68 ± 0.59	0.98 ± 0.12	39.6 ± 3.5
+ 0.2 M ETOH	6.1 ± 0.39	23.66 ± 2.11	12.47 ± 2.3
+ 10 mM EGTA	22.07 ± 0.83 (11)[a]	1.3 ± 0.49	35.56 ± 11.35 (11)
+ 10 mM EGTA, + 0.2 mM ETOH	5.36 ± 0.33 (12)	20.26 ± 2.0 (15)[c]	12.0 ± 6.71 (4)
+ 25 mM EGTA	20.96 ± 1.6 (16)[a]	2.04 ± 0.66	27.85 ± 3.4 (30)[a]
+ 25 mM EGTA, + 0.2 mM ETOH	3.61 ± 0.39 (41)	13.14 ± 1.22 (45)[b]	6.95 ± 1.24 (45)
+ 50 mM EGTA	16.5 ± 2.2 (33)[b]	0.48 ± 0.34	20.77 ± 0.94 (48)[b]
+ 50 mM EGTA, 0.2 mM ETOH	3.58 ± 0.92 (42)	9.26 ± 2.86 (61)[b]	5.97 ± 1.5 (52)[d]
50 mM EDTA	23.54 ± 0.08	2.58 ± 0.5	50.05 ± 6.1 (26%)[d]
50 mM EDTA, + 0.2 mM ETOH	6.13 ± 0.57	27.5 ± 1.3 (16)[c]	13.8 ± 0.15

*Values expressed as nmol formed/mg protein/h, values in parenthesis are the % inhibitions compared to corresponding incubations not containing EGTA or EDTA. [a]$P = < 0.005$; [b]$P = < 0.001$; [c]$P = < 0.025$; [d]$P = < 0.01$

3.4. Phorbol esters and amphiphilic cations

Low concentrations of phorbol myristate acetate and phorbol dibutyrate, from 10 μM to 100 μM, did not influence phospholipase D activity, however, at 1 mM there was complete inhibition (data not shown). Several amphiphilic cations had been shown to activate phospholipase D activity of cultured cells [13]. There was a doubling of phospholipase D activity in the presence of 1 mM oleylamine but not in the presence of an equivalent amount of either sphingosine or chlorpromazine (data not shown).

4. Discussion

The phospholipase D activity of post nuclear supernates of HL 60 cells was estimated by measuring phosphatidylethanol formation and found maximally active in the presence of 5 mM Ca^+ and 100 μM GTPγS [15]. The phospholipase D activity of canine cerebral cortical microsomes was stimulated at 20 μM GTPγS but was inhibited slightly by Ca^{2+} and Mg^{2+} [16]. Millimolar Ca^{2+} and Mg^{2+} inhibited the phospholipase D activity of membranes prepared from human amniotic tissues [17], rat brain frontal cortex and primary cultures of this tissue [18], as well as rat sciatic nerve [19]. Mg^{2+} but not Ca^{2+} stimulated the phospholipase D activity of rat brain synaptic membranes incubated at a pH of 7.2 [20]. A partially purified rat brain phospholipase D activity was stimulated by millimolar concentrations of Ca^{2+} and Fe^{2+} [21]. GTPγS stimulated phospholipase D activity of membranes prepared from hepatocytes [22–24], and there may be an additional enhancement of this

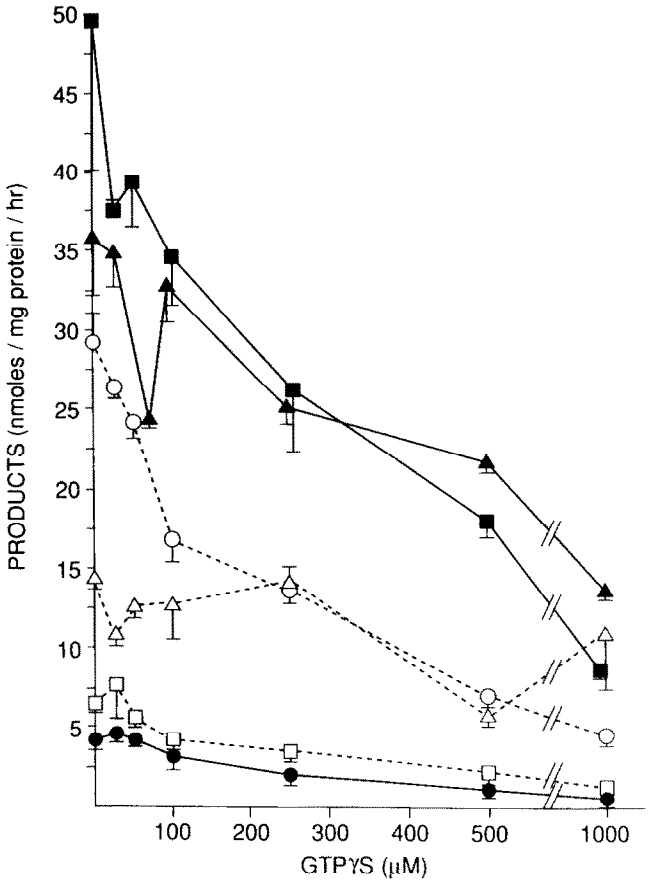

Fig. 2. The inhibition of rat brain plasma membrane phospholipase D activity by GTPγS. The experimental details are provided in the text. Open symbols represent incubations in the presence of 0.2 M ETOH and filled symbols in the absence of ETOH. Squares indicate PtdA formation, circles indicate PtdETOH formation and triangles represents DG formation.

Table 2
The effect of Ba^{2+}, Ca^{2+}, Mg^{2+} and Sr^{2+} upon the phospholipase D activity of rat brain plasma membranes*

	PtdA	PtdETOH	DG
N.A.	32.15 ± 5.1	3.75 ± 2.5	44.3 ± 15
+ 0.2 M ETOH	6.2 ± 1	24 ± 2.2	15.2 ± 11.3
2 mM $CaCl_2$	38.9 ± 4.5	3.3 ± 1.2	50.5 ± 9.8
2 mM $CaCl_2$ + 0.2 M ETOH	6.7 ± 1.8	28.6 ± 2.5	14.3 ± 7.5
6 mM $CaCl_2$	48.3 ± 4.7	2.2 ± 0.61	35.2 ± 7.5
6 mM $Ca2Cl_2$ + 0.2 M ETOH	7.8 ± 1.3	24.3 ± 1.6	14.9 ± 3
2 mM $MgCl_2$	42.8 ± 1.5	2.4 ± 0.6	48.3 ± 2.9
2 mM $MgCl_2$ + 0.2 M ETOH	9.63 ± 2.6	32.5 ± 1.8	14.1 ± 3.4
6 mM $MgCl_2$	58.9 ± 5.5	3.9 ± 0.6	38.9 ± 8.3
6 mM $MgCl_2$ + 0.2 M ETOH	7.3 ± 0.14	25.9 ± 3.2	8.3 ± 1.5
2 mM $BaCl_2$	37.2 ± 2.2	1.9 ± 0.5	44.9 ± 16.4
2 mM $BaCl_2$ + 0.2 M ETOH	5.5 ± 1	27.2 ± 1.3	9.9 ± 17
6 mM $BaCl_2$	50.9 ± 8.5	4.4 ± 0.8	42.6 ± 4.3
6 mM $BaCl_2$ + 0.2 M ETOH	9.1 ± 2.1	31.9 ± 12	14.2 ± 2.3
2 mM $SrCl_2$	45.9 ± 3.7	4.1 ± 2	23.6 ± 3.4
2 mM $SrCl_2$ + 0.2 M ETOH	7.8 ± 0.6	26.9 ± 0.4	14.1 ± 0.8
6 mM $SrCl_2$	63.8 ± 13	4 ± 2	7.9 ± 4
6 mM $SrCl_2$ + 0.2 M ETOH	17.2 ± 1.9	42.1 ± 1	7.8 ± 1

*Values expressed as nmol product/mg protein/h
N.A. = No additions

GTPγS stimulation by soluble proteins [25–27]. The properties of the rat brain plasma membrane phospholipase D is different from the above. There is a lack of GTPγS stimulation, indeed there is a profound inhibition, which is unaltered by the presence of rat brain cytosol (Fig. 2). There are no inhibitions by millimolar Ca^{2+} or Mg^{2+} but rather a stimulation which is also found with Ba^{2+} and Sr^{2+} (Table 2). These observations indicates that the properties of phospholipase D resident in different membranes prepared from different cells or tissue may not be identical. Therefore, comparisons of observations made with different perturbants in different cell systems occasionally may be more complicated than originally believed.

The four stimulatory cations are transition metals members of the group II a, Be^{2+} the other member of this group is a robust inhibitor (Fig. 1). Industrial exposure of workers to beryllium dust results in a pathological condition [28]. Be^{2+} inhibits nuclear protein phosphorylation [29], and microsomal cytochrome *p-450* hydroxylations [30]. The fluoride salt of beryllium, in a manner

similar to aluminium fluoride, will bind to G proteins and ATPases [31]. Perhaps an inhibition of phospholipase D thus reducing a cells capacity to produce diacylglycerol or phosphatidic acid in response to receptor occupancy is a contributory factor to beryllium toxicity.

Acknowledgements: Supported by a grant from the Medical Research Council of Canada.

References

[1] Kanfer, J.N. (1989) Phosphatidylcholine Metabolism: (Vance, D.E. Ed.) pp. 65–86, CRC Press.
[2] Loffelholz, K. (1989) Curr. Aspects Neurosci. 2, 1–52.
[3] Exton, J. (1990) J. Biol. Chem. 265, 1–4.
[4] Dennis, E., Rhee, S.O., Billah, M. and Hannun, Y.A. (1991) FASEB J. 5, 2068–2077.
[5] Liscovitch, M. (1992) Trends Biochem. Sci. 17, 393–398.
[6] Nishizuka, G. (1992) Science 258, 607–614.
[7] Perrson, A. and Jergil, B. (1992) Ann. Biochem. 204, 131–136.
[8] Avruch, J. and Wallace, D.F.H. (1971) Biochim. Biophys. Acta 233, 334–347.
[9] Hubscher, G. and West, G.R. (1965) Nature 205, 799–800.
[10] Kobayashi, M. and Kanfer, J.N. (1987) J. Neurochem. 48, 1597–1603.
[11] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[12] Ames, B.N. and Dubin, D.T. (1960) J. Biol. Chem. 235, 769.
[13] Singh, I.N., Massarelli, R. and Kanfer, J.N. (1993) J. Lipid Mediators 7, 85–96.
[14] Cockroft, S. (1992) Biochim. Biophys. Acta 1113, 135–160.
[15] Anthes, J.C., Eckerl, S., Siegel, M.I., Egan, A.W. and Billah, M.M. (1989) Biochem. Biophys. Res. Commun. 163, 657–669.
[16] Qian, Z., Reddy, P.V. and Drewes, L.R. (1990) J. Neurochem. 54, 1632–1638.
[17] Inamori, K., Sagawa, N., Hasegawa, M., Itoh, H., Ueda, H., Kobayashi, F., Ihara, Y. and Mori, T. (1993) Biochem. Biophys. Res. Commun. 191, 1270–1277.
[18] Nishida, A., Shimizu, M., Kanaho, Y., Nozawa, Y. and Yamowaki, S. (1992) Brain Res. 595, 12–16.
[19] Chattopadhyay, J., Natarajan, V. and Schmid, H.O. (1991) J. Neurochem. 57, 1429 1436.
[20] Chalifa, V., Mohn, H. and Liscovitch, M. (1990) J. Biol. Chem. 265, 17512–17519.
[21] Taki, T. and Kanfer, J.M. (1979) J. Biol Chem. 254, 9761–9765
[22] Irving, H.R. and Exton, J.H. (1987) J. Biol. Chem. 262, 3440–3443.
[23] Hurst, K.M., Hughes, B.P. and Barrett, G.J. (1990) Biochem J 272, 749–753.
[24] Siddiqui, R.A. and Exton, J.H. (1992) Eur. J. Biochem. 210, 601–607.
[25] Anthes, J.C., Wang, P., Siegel, M.I., Egan, R.W. and Billah, M.M. (1991) Biochem. Biophys. Res. Commun. 175, 236–242.
[26] Olson, S.C., Bowman, E.P. and Lambeth, J.D. (1991) J. Biol Chem. 266, 17236–17242.
[27] Geny, B., Fensome, A. and Cockroft, S. (1993) Eur. J. Biochem. 215, 389–396.
[28] Hardy, H.L. (1980) Environ. Res. 21, 1–9.
[29] Williams, B.E. and Shilleter, D.N. (1983) Biosci. Reports 3, 955–962.
[30] Teixeira, C.F.P., Yasaka, W.J., Silva, L.F., Oshiro, T. and Oga, S. (1990) Toxicology 61, 293–301.
[31] Chabre, M. (1990) Trends Biochem. Sci. 15, 6–10.