Volume 284, number 1, 27–30 FEBS 09795 June 1991

ADONIS 0014579391005078

Calcium is sufficient but not necessary for activation of sheep platelet cytosolic phospholipase A_2

Lori A. Zupan, Kelly K. Kruszka and Richard W. Gross

Molecular and Cellular Cardiovascular Biochemistry Section, Washington University School of Medicine, St. Louis, MO 63110, USA

Received 14 March 1991; revised version received 4 April 1991

In this study we demonstrate that: (1) although the major phospholipase A_2 present in sheep platelets is activated by calcium ions, it can effectively catalyze hydrolysis of the *sn*-2 ester linkage in phospholipids in the absence of calcium; (2) expression of calcium-independent phospholipase A_2 activity can be induced by NaCl utilizing purified (but not crude) cytosolic enzyme; and (3) calcium-independent phospholipase A_2 activity is regulated by a reconstitutable cytosolic protein. Collectively, these results underscore the fundamental catalytic differences between extracellular and intracellular calcium-dependent phospholipases A_2 and demonstrate that calcium is sufficient, but not necessary, for the activation of this class of intracellular phospholipases A_2

Phospholipase A_2; Calcium; Platelet; Arachidonic acid; Plasmalogen

1. INTRODUCTION

Phospholipase A_2 (PLA_2) catalyzed hydrolysis of choline and ethanolamine glycerophospholipids represents the predominant mechanism responsible for the release of arachidonic acid mass during signal transduction in most mammalian cells (e.g. [1–5]). Since the release of arachidonic acid from endogenous phospholipid storage depots is the rate-limiting step for the production of biologically active eicosanoids [6–8], the biochemical mechanisms responsible for the regulation of intracellular PLA_2 activity have been the focus of intense investigation. Sheep platelets contain an intracellular PLA_2 which is activated by physiologic increments in calcium ions and possesses a remarkable substrate specificity [9]. Since extracellular phospholipases A_2 possess an obligatory requirement for calcium ions for catalysis, it was initially assumed that this prototypic calcium-dependent intracellular PLA_2 utilized an analogous catalytic mechanism to facilitate polarization of the *sn*-2 carbonyl during hydrolysis. We now demonstrate that calcium-independent PLA_2 activity can be induced by high concentrations of several monovalent and divalent salts utilizing purified (but not crude) cytosolic enzyme and that calcium-independent hydrolysis is modulated by a cytosolic regulatory protein.

Correspondence address: R.W. Gross, Molecular and Cellular Cardiovascular Biochemistry, Washington University School of Medicine, 660 South Euclid Avenue, Box 8020, St. Louis, MO 63110, USA.

2. EXPERIMENTAL

2.1. *Preparation and purification of sheep platelet cytosolic PLA_2*

Sheep platelets PLA_2 were prepared as described previously and cytosolic PLA_2 was purified utilizing sequential DEAE-cellulose, Superose 12 and Mono Q chromatographies [9].

2.2. *Synthesis of phospholipids and assay of PLA_2 and lysophospholipase activities*

Synthesis of 1-*O*-(*Z*)-hexadec-1′-enyl-2-[9,10-^{3}H]-octadec-9′-enoyl-*sn*-glycero-3-phosphocholine ([^{3}H]plasmenylcholine) was performed by acylation of reverse-phase purified 1-*O*-(*Z*)-hexadec-1′-enyl-*sn*-glycero-3-phosphocholine as described previously [10]. Assays of phospholipase and lysophospholipase activities were performed by injection of 10 μl of radiolabeled phospholipid (dissolved in ethanol) into 100 mM Tris-HCl buffer (pH 7.6) as described previously [11]. Released radiolabeled fatty acids were extracted with butanol, separated by TLC and quantified by scintillation spectrometry [11].

3. RESULTS

3.1. *Expression of latent calcium-independent PLA_2 activity by NaCl*

Incubation of sheep platelet cytosol with [^{3}H]plasmenylcholine resulted in the calcium-dependent release of radiolabeled fatty acid from the *sn*-2 position (Fig. 1, left). Incubation of cytosol in the combined presence of NaCl and EGTA did not result in a significant increase in fatty acid release in comparison to incubations with EGTA alone. Anion-exchange column chromatographic separation of sheep platelet cytosolic proteins resulted in a 90-fold purification of sheep platelet phospholipase A_2 activity in a quantitative yield. Similar to results obtained with cytosol, incubation of the active fractions with [^{3}H]plasmenylcholine substrate in the presence of EGTA resulted in only

diminutive amounts of fatty acid released in comparison to that manifest in the presence of calcium ions (Fig. 1, center). Remarkably, incubation of the active fractions from anion-exchange chromatography in the combined presence of 10 mM EGTA and 2 M NaCl resulted in similar amounts of fatty acid released in comparison to incubations conducted in the presence of calcium ions alone (Fig. 1, center). Addition of 2 M NaCl to incubations containing calcium ions modestly diminished PLA_2 activity in comparison to incubations containing calcium alone (Fig. 1, center). The possibility that ambient calcium was responsible for salt-inducible calcium-independent PLA_2 activity was excluded, since addition of increasing amounts of EGTA (up to 100 mM) did not attenuate salt-inducible calcium-independent PLA_2 activity. The induction of calcium-independent PLA_2 activity by NaCl was both concentration-dependent (dose-dependent activation was manifested from 200 mM to 2 M NaCl) and reversible (dialysis of NaCl-treated cytosol restored its calcium-dependent activation). Furthermore, salt-inducible calcium-independent PLA_2 activity was not specific for NaCl since a variety of other salts (e.g. KCl, K_2PO_4, Na_2SO_4, $LiSO_4$ and $MgSO_4$) also effectively induced calcium-independent PLA_2 activity after anion-exchange chromatography in multiple independent preparations. Since salt-inducible PLA_2 activity was not observed in crude cytosol but was present following anion-exchange chromatography, we incubated [^{3}H]plasmenylcholine with mixtures containing equal volumes of dialyzed cytosol and anion-exchange column eluents. Although additive amounts of PLA_2 activity were observed in the presence of calcium, incubation of these mixtures in the presence of either EGTA alone or in the combined presence of EGTA and 2 M NaCl did not result in the release of substantive amounts of radiolabeled fatty acid (Fig. 1, right). Pretreatment of platelet cytosol with *Staphylococcus aureus* endoprotease (strain V8) completely ablated the ability of cytosol to inhibit the expression of salt-inducible calcium-independent PLA_2 activity in DEAE-cellulose column eluents. In stark contrast, neither snake venom (*Naja naja naja*) nor bee venom PLA_2 catalyzed the hydrolysis of [^{3}H]plasmenylcholine in the combined presence of NaCl (2 M) and EGTA (10 mM). Collectively, these results demonstrate that purified, but not cytosolic, sheep platelet phospholipase A_2 can effectively catalyze hydrolysis of phospholipids in the absence of calcium ions and that salt-induced expression of calcium-independent phospholipase A_2 activity is inhibited by a cytosolic protein.

Application of dialyzed sheep platelet cytosol to tandem columns comprised of Superose 12 resin resolved the modulating factor from PLA_2 catalytic activity

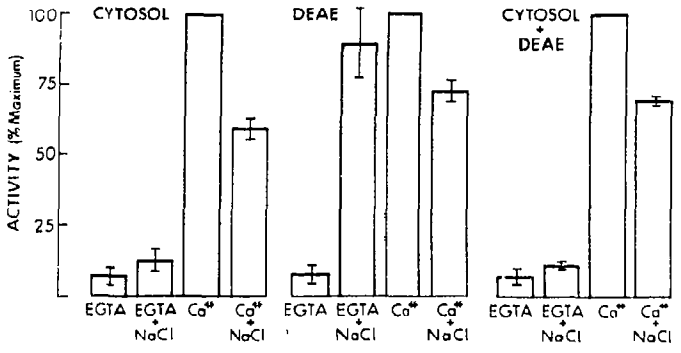

Fig. 1. Calcium-independent PLA_2 activity is expressable with NaCl utilizing purified (but not crude) cytosolic phospholipase A_2. Dialyzed sheep platelet cytosol (Left), dialyzed DEAE-cellulose-purified phospholipase A_2 (Center) or equal volumes of dialyzed cytosol and DEAE-purified phospholipase A_2 (Right) were incubated with [^{3}H]plasmenylcholine substrate in the presence of either 10 mM EGTA, 10 mM EGTA and 2 M NaCl, 10 mM $CaCl_2$ alone, or 10 mM $CaCl_2$ and 2 M NaCl. Phospholipase A_2 activity was assessed by quantifying fatty acid release after butanol extraction, TLC and scintillation spectrometry. The vertical bars indicate fatty acid release (expressed as a percentage of maximum calcium-dependent release) in the presence of NaCl, calcium, or EGTA alone, or in combination as indicated. Typical specific activities of phospholipase A_2 in cytosol and DEAE peak fractions were 0.02–0.04 and 2–4 nmol/mg·min, respectively. Data represent $\bar{X} \pm$ S.E. of 3 independent preparations.

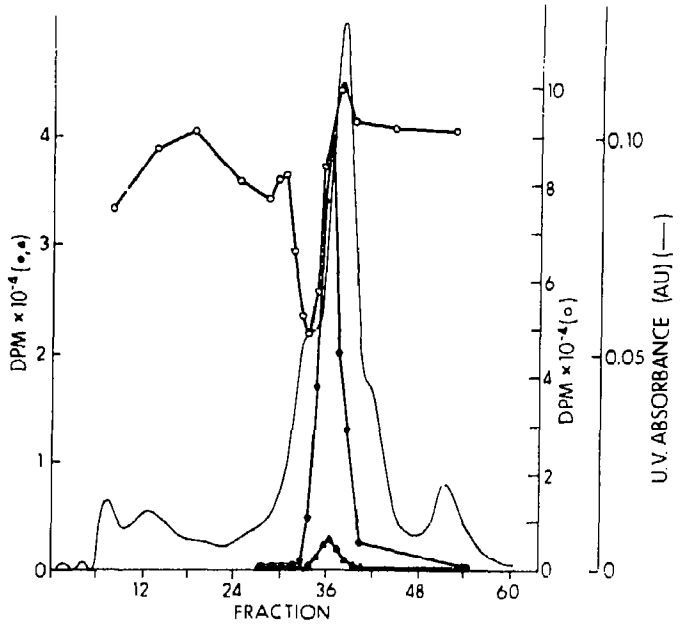

Fig. 2. Gel filtration chromatographic separation of PLA_2 catalytic and regulatory polypeptides. Dialyzed sheep platelet cytosol was loaded onto tandem columns comprised of Superose 12 resin and eluted at 12 ml/h. Column eluates were assayed directly for enzymic activity in the presence of either 10 mM $CaCl_2$ (●), or 10 mM EGTA (▲). Reconstitution experiments were performed by co-incubating equal volumes of DEAE-purified phospholipase A_2 with the indicated column chromatographic fractions in the combined presence of 10 mM EGTA and 2 M NaCl (○). Fatty acid released from [^{3}H]plasmenylcholine substrate was extracted with butanol, separated by TLC and quantified by scintillation spectrometry. Results are representative of 3 independent preparations. (——), ultraviolet absorbance at 280 nm.

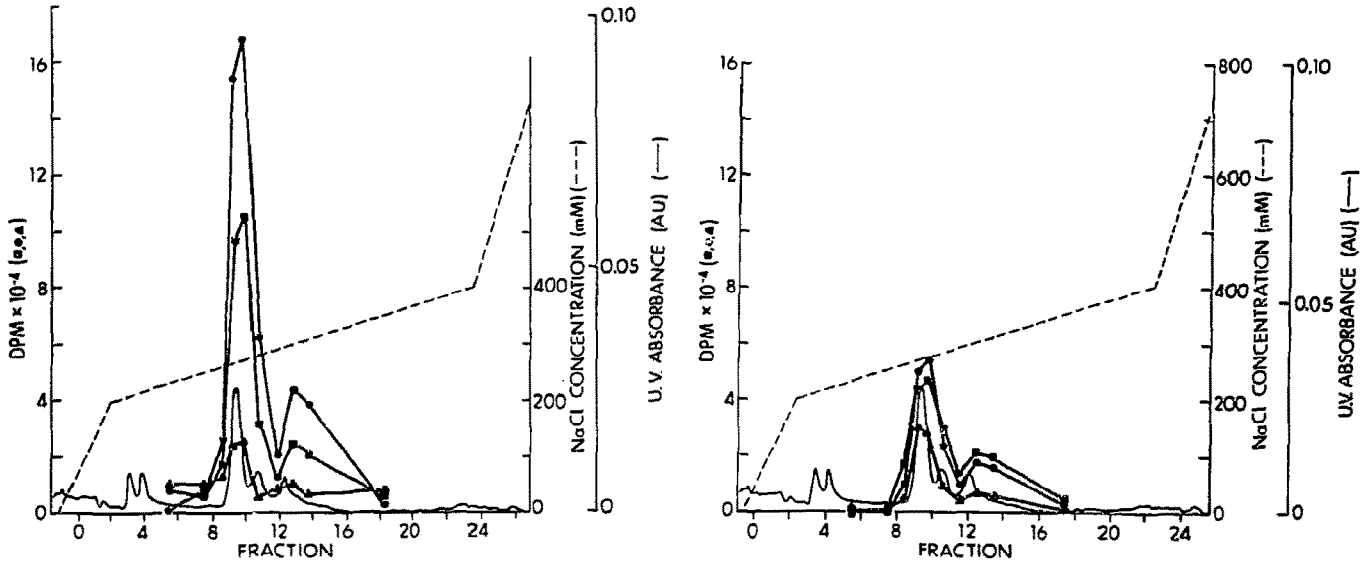

Fig. 3. Co-chromatography of calcium-dependent PLA_2 activity, NaCl-inducible calcium-independent PLA_2 activity and lysophospholipase activity. Active fractions from gel filtration chromatography were purified to near-homogeneity utilizing a Mono Q column. Phospholipase A_2 activity (Left) or lysophospholipase activity (Right) were assessed by incubating column chromatographic fractions with 5 μM [^{3}H]plasmenylcholine (left) or [^{14}C]lysophosphatidylcholine (right) in the presence of 10 mM $CaCl_2$ (●), 10 mM EGTA (▲), or 10 mM EGTA plus 2 M NaCl (■). Similar results were present utilizing other concentrations of calcium ions (e.g. 1 μM) or EGTA (e.g. 25 mM). Released fatty acid was extracted with butanol, separated by TLC and quantified by scintillation spectrometry. (——), ultraviolet absorbance at 280 nm; (– – –) NaCl gradient.

(Fig. 2). Co-incubation of aliquots of column eluents from gel-filtration chromatography of platelet cytosol with the pooled active fractions from anion-exchange chromatography (in the combined presence of 10 mM EGTA and 2 M NaCl) demonstrated that the cytosolic factor responsible for inhibition of salt-inducible calcium-independent PLA_2 activity eluted with an apparent $M_r = 90K$ ($n = 3$), just prior to PLA_2 activity.

To determine whether calcium-dependent and calcium-independent PLA_2 activities were catalyzed by the same polypeptide, the enzyme was purified to near-homogeneity utilizing a Mono Q stationary phase. Calcium-dependent and salt-inducible calcium-independent phospholipase A_2 activity co-eluted at 270–280 mM NaCl (Fig. 3) in two chromatographically resolved peaks of activity corresponding to the previously described 30 kDa isoforms [9]. Although the purified enzyme possessed an obligatory requirement for calcium ions to hydrolyze 1-palmitoyl-2-[1-^{14}C]arachidonyl-*sn*-glycero-3-phosphocholine or 1-palmitoyl-2-[1-^{14}C]arachidonyl-*sn*-glycero-3-phosphoethanolamine in the absence of NaCl, it could effectively catalyze the hydrolysis of their *sn*-2 ester linkages in the presence of 10 mM EGTA and 2 M NaCl (Fig. 3). Furthermore, the purified isoforms also displayed some lysophospholipase activity (utilizing monomeric substrate) which was activated by calcium ions but which also efficiently catalyzed the hydrolysis of the *sn*-1 acyl linkage in the combined presence of 2 M NaCl and 10 mM EGTA (Fig. 3). Calcium-dependent and salt-inducible calcium-independent PLA_2, as well as lysophospholipase activities, co-chromatographed in every fraction of every column chromatographic step employed (e.g. Fig. 3).

4. DISCUSSION

Although calcium ions are sufficient for activation of sheep platelet PLA_2, these results demonstrate that calcium is not a necessary co-factor for catalysis mediated by this purified intracellular PLA_2. These findings stand in stark contrast to the obligatory requirement of extracellular phospholipases A_2 for calcium ions [12]. Prior studies have demonstrated the binding of calcium to the active site of extracellular PLA_2 (e.g. [13,14]), have identified its chelation sphere [15], and have demonstrated the participation of calcium ions in the polarization of the *sn*-2 carbonyl [16]. Although a variety of divalent cations bind to extracellular PLA_2, expression of its catalytic activity is absolutely dependent upon the presence of calcium ions [17]. Thus, the catalytic domains responsible for hydrolysis of the *sn*-2 ester in phospholipids mediated by intracellular and extracellular calcium-dependent phospholipases A_2 are comprised of functionally distinct catalytic elements.

Calcium-independent activation of sheep platelet PLA_2 by salt may be mediated by interactions of salt with enzyme, salt with substrate, or by combinations of these phenomena. Several lines of evidence indicate that

salt-inducible calcium-independent PLA_2 activity is the result of salt-induced alterations in PLA_2 catalytic activity modulated by a regulatory protein and is not mediated by the interaction of salt with phospholipid substrate. First, a variety of different phospholipid substrates in different physical states (i.e. monomer, bilayer and inverted hexagonal phase) have identical salt titration profiles for calcium-independent PLA_2 activity. It seems highly unlikely that the interactions of salt with different phospholipid classes and subclasses aggregated in distinct physical states are similar to the interactions of salt with monomeric lysophosphatidylcholine. Second, salt-inducible calcium-independent PLA_2 activity is present utilizing purified but not crude cytosolic enzyme employing identical substrates. Third, salt-inducible calcium-independent PLA_2 activity is ablated by reconstitution of the purified enzyme with an endogenous cytosolic protein, thus strongly mitigating against direct effects of NaCl on substrate.

Collectively, these experiments underscore the fundamental catalytic differences between extracellular and intracellular calcium-responsive phospholipase A_2, and demonstrate a previously unsuspected level of complexity in the regulation of intracellular calcium-responsive phospholipases A_2 which are mediated by associated protein regulatory elements. In this regard, we note that Pollock et al. have demonstrated activation of a platelet PLA_2 in the absence of increases in cytosolic calcium ion content [17]. Accordingly, these results underscore the possibility that elevations in intracellular calcium ion concentration are not an obligatory precondition for the release of arachidonic acid by calcium-responsive intracellular phospholipases A_2.

Acknowledgements: This research was supported by NIH Grant HL35864 and Monsanto. R.W.G. is the recipient of an Established Investigator award from the American Heart Association.

REFERENCES

[1] Samuelsson, B., Granstrom, E., Green, K., Hamberg, M. and Hammarstrom, S. (1975) Annu. Rev. Biochem. 44, 669-695.
[2] Jesse, R.L. and Cohen, P. (1976) Biochem. J. 158, 283-287.
[3] Bills, T.K., Smith, J.B. and Silver, M.J. (1977) J. Clin. Invest. 60, 1-6.
[4] Dennis, E.A. (1983) Enzymes 16, 307-353.
[5] Chilton, F.H. and Murphy, R.C. (1986) J. Biol. Chem. 261, 7771-7777.
[6] Lands, W.E.M. and Samuelsson, B. (1968) Biochim. Biophys. Acta 164, 426-429.
[7] Marcus, A.J., Ullman, H.L. and Safier, L.B. (1969) J. Lipid Res. 10, 108-114.
[8] Samuelsson, B., Dahlen, S.E., Lindgren, J.A., Rouzer, C.A. and Serhan, C.N. (1987) Science 237, 1171-1176.
[9] Loeb, L.A. and Gross, R.W. (1986) J. Biol. Chem. 261, 10467-10470.
[10] Wolf, R.A. and Gross, R.W. (1985) J. Lipid Res. 26, 629-633.
[11] Hazen, S.L., Loeb, L.A., and Gross, R.W. in: Methods of Enzymology, (Dennis, E.A., ed.) vol. 197, pp. 400-411, Academic Press, San Diego, California.
[12] Hershberg, R.D., Reed, G.H., Slotboom, A.J. and De Haas, G.H. (1976) Biochemistry 15, 2268-2274.
[13] Pieterson, W.A., Volwerk, J.J. and De Haas, G.H. (1974) Biochemistry 13, 1439-1445.
[14] Volwerk, J.J., Pieterson, W.A. and De Haas, G.H. (1974) Biochemistry 13, 1446-1454.
[15] Dijkstra, B.W., Kalk, K.H., Hol, W.G.J. and Drenth, J. (1981) J. Mol. Biol. 147, 97-123.
[16] Thunnissen, M.M.G.M., Ab, E., Kalk, K.H., Drenth, J., Dijkstra, B.W., Kuipers, O.P., Dijkman, R., De Haas, G.H. and Verheij, H.M. (1990) Nature 347, 689-691.
[17] Pollock, W.K., Rink, T.J. and Irvine, R.F. (1986) Biochem. J. 235, 869-877.