Vol. 198, No. 1, 1994
January 14, 1994
BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 220-227

REDUCED PHOSPHOLIPASE A_2 ACTIVITY IS NOT ACCOMPANIED BY REDUCED ARACHIDONIC ACID RELEASE

Howard Goldberg, Peter Maxwell, Nashrudeen Hack and Karl Skorecki*

Membrane Biology Group and Division of Nephrology, Departments of Medicine and Pediatrics, University of Toronto, Toronto, Ontario, Canada

Received November 16, 1993

Arachidonic acid release in cells highly over expressing cytosolic phospholipase A_2 has been attributed to mitogen-activated protein kinase phosphorylation of cytosolic phospholipase A_2 on serine-505. To investigate the role of cytosolic phospholipase A_2 in cellular physiology, we attempted to inhibit cytosolic phospholipase A_2 in the intact cell employing an antisense RNA strategy. Swiss 3T3 cells were stably transfected with an antisense cytosolic phospholipase A_2 expression vector. A clone of cells with reduced immunodetectable cytosolic phospholipase A_2, compared to a vector transfected cell line, was identified by Western blotting and a corresponding decrease in phospholipase A_2 activity was confirmed by enzymatic assay in cell free extracts. However, arachidonic acid release from intact cells in response to agonists was not different between antisense and control cell lines. Thus, arachidonic acid release in intact cells with decreased cytosolic phospholipase A_2 activity is likely to be modulated by rate limiting factors that are extrinsic to cytosolic phospholipase A_2.

*To whom correspondence should be addressed.

Abbreviations: PLA_2 phospholipase A_2; $cPLA_2$ cytosolic phospholipase A_2; $sPLA_2$ secretory phospholipase A2; PDGF platelet derived growth factor; PMA phorbol myristate acetate; EGF epidermal growth factor; DTT dithiothreitol; EGTA ethylene glycol bis(b-aminoethylether)-N,N,N',N'-tetraacetic acid; EDTA ethylenediaminetetraacetic acid; MAP kinase mitogen-activated protein kinase; G protein guanine nucleotide-binding regulatory protein; DMEM Dulbecco's modified Eagle's medium; CMV cytomegalovirus.

0006-291X/94 $5.00

Prostaglandins and leukotrienes, metabolites of arachidonic acid, have diverse biological roles in inflammatory processes, mitogenesis, ion channel activity and the regulation of vascular tone (1). Phospholipase A_2 (PLA_2) mediated release of arachidonic acid from the sn-2 position of membrane bound phospholipids is thought to be the rate limiting step in the generation of eicosanoids (2). Two major types of PLA_2 are recognized in mammalian cells: a class of low molecular weight secretory PLA_2's ($sPLA_2$, 14-16 kDa) and a 110 kDa cytosolic PLA_2 ($cPLA_2$). The gene encoding the latter enzyme, $cPLA_2$, was recently cloned and is thought to be responsible for agonist-stimulated cellular arachidonic acid release. In contrast to $sPLA_2$'s, it is selective for arachidonic acid, activated by nanomolar concentrations of Ca^{2+} and is insensitive to DTT (3,4). Overexpression of $cPLA_2$, but not secretory PLA_2, lead to agonist induced increases in arachidonic acid release in CHO cells (5). Several agonists, PMA, EGF, tumor necrosis factor and interleukin-1 increase both arachidonic acid release in intact cells and PLA_2 activity in cell free extracts through augmented $cPLA_2$ RNA levels and/or phosphorylation of $cPLA_2$ (6-12). Recently, regulation of $cPLA_2$ activity by agonists and agonist induced arachidonic acid release have been found to depend on phosphorylation of serine-505 of $cPLA_2$ by MAP kinase (13,14). These data suggest that the major mechanism controlling arachidonic acid release in intact cells is intrinsic to $cPLA_2$ and mediated by phosphorylation.

The role of PLA_2 products in cellular processes has previously been examined by testing the effect of inhibitors of cyclooxygenase, lipoxygenase or cytochrome P450 (1). However, these inhibitors may have non-specific effects and the results obtained with them are complicated by increased metabolism of arachidonic acid through alternate pathways, when one pathway is blocked. In order to clarify the role of eicosanoids in cellular physiology, we sought to specifcally inhibit $cPLA_2$ activity by expressing antisense $cPLA_2$ RNA (15).

Of interest, we found that reduced expression of $cPLA_2$ at the protein level and reduced PLA_2 activity in cell free extracts was not accompanied by reduced arachidonic acid release from intact cells. This unexpected observation has important implications for understanding the control of arachidonic acid release.

METHODS

Selection of antisense clones. Murine $cPLA_2$ cDNA (3) (generously provided by Dr Lih-Ling Lin and Dr J. L. Knopf, Genetics Institute, Cambridge) was excised from its pMT2 vector with Sal 1 and EcoR1 (nucleotides 1 to 2384), filled in with Klenow and ligated in the antisense orientation into the Xba 1 site of pRcCMV (Invitrogen), which was also

filled in with Klenow to form the plasmid pASP (Fig 1). The pRcCMV vector uses a CMV promoter to express cDNA and also carries a neomycin resistance gene driven by an SV40 promoter. Swiss 3T3 cells (ATCC) were transfected with the antisense RNA expression vector, pASP, or the vector, pRcCMV, using a modified calcium phosphate technique (16). The cells were selected in 600 μg/ml G418 and individual clones isolated with a cloning cylinder.

Western blotting The corresponding vector or antisense cells were lysed in a buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 10 % glycerol, 1% Triton X100, 10 μg/ml leupeptin and 2 mM PMSF. Five μg of protein, (17) from each lysate was separated by SDS-PAGE on a 7.5% gel and transferred to Immobilon (Millipore). Blots were probed with anti-cPLA$_2$ antibody (#7905 generously provided by Dr Lih-Ling Lin and Dr J. L. Knopf, Genetics Institute) diluted 1/5000, incubated with goat anti-rabbit antibody linked to horseradish peroxidase and developed by ECL (Amersham). The results were quantitated by densitometry.

PLA$_2$ activity in cell extracts These assays were similar to those previously outlined (6-9). Confluent cells in 100 mm dishes were preincubated for 40 minutes in 40 mM Hepes, pH 7.3, 1 mg/ml BSA and DMEM. The cells were washed and homogenized with 50 mM Hepes, pH 7.5, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose, 2 mM PMSF, 40 μg/ml leupeptin, 40 μg/ml pepstatin, spun at 1000 g to eliminate nuclei and then at 100,000 g to obtain cytosol. PLA$_2$ was assayed in homogenization buffer containing 20 μg of protein, 5 mM calcium, 5 mM DTT, 15 μM 1-stearoyl-2-[^{14}C]arachidonyl phosphatidylcholine (Amersham) substrate (dissolved in DMSO) in a volume of 40 μl for 15 minutes at 37°C. Arachidonic acid and phospholipids were then separated by thin layer chromatography.

Arachidonic acid release As previously described (18) confluent cells in 60 mm dishes were labelled for 24 hours with 0.5 μCi [^{3}H]arachidonic acid per dish. They were then washed with 40 mM Hepes, pH 7.3, DMEM and 5 mg/ml BSA and preincubated in 40 mM Hepes, pH 7.3, DMEM and 1 mg/ml BSA for 40 minutes. The media was changed and agonists added for 30 minutes at 37°C. The media was collected, centrifuged at 16,000 g to eliminate cellular debris and then counted. Results are expressed as a percent of the basal arachidonic acid release in each cell type.

RESULTS AND DISCUSSION

Murine Swiss 3T3 cells were selected for study since intracellular signalling has been extensively characterized in these cells. Murine cPLA$_2$ cDNA, subcloned in the antisense orientation into the expression vector pRcCMV (Fig 1), was transfected into Swiss 3T3 cells and 42 G418 resistant clones were selected. Immunodetectable cPLA$_2$ was determined by Western blotting The clone with the greatest reduction in cPLA$_2$ expression was selected for further characterization and is designated "antisense", while a clone of cells transfected with the pRcCMV vector alone was used as a control. Western blotting (Fig 2) demonstrated a 65 % reduction in cPLA$_2$ expression at the protein level in the antisense cell line compared to the vector clone. Clones with greater degrees of reduction in PLA$_2$ activity may have grown too slowly to be isolated by this protocol.

PLA$_2$ activity was assayed in cell free extracts from these cells in the presence of DTT. Previous work by other investigators has shown cPLA$_2$ to

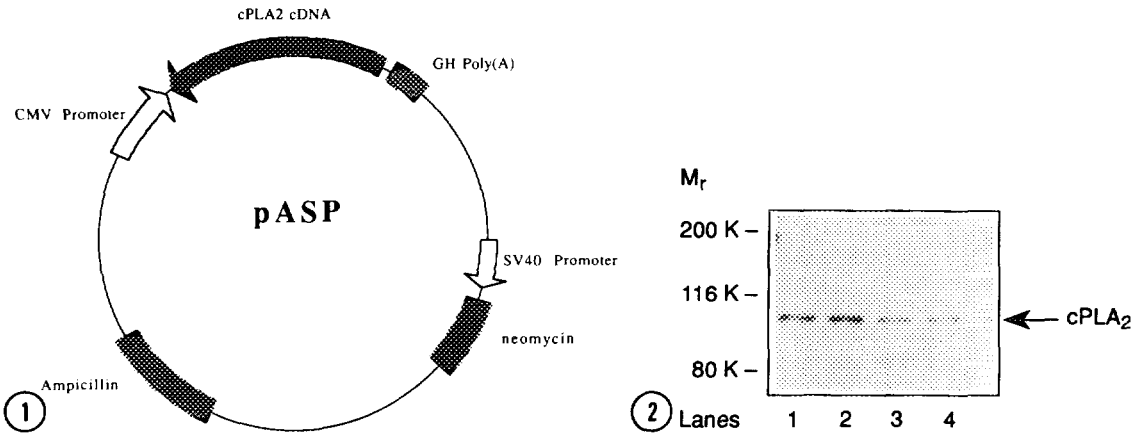

Fig. 1. *Schematic representation of the antisense cPLA$_2$ RNA expression vector, pASP.*

Fig. 2. *Western blot of cPLA$_2$.* 5 μg of protein from the cell lines stably transfected with the pRcCMV vector (lanes 1 and 2) or the antisense cPLA$_2$ RNA expression vector, pASP, (lanes 3 and 4) was transferred to Immobilon and probed with anti-cPLA$_2$ as described in Methods.

be the major component of DTT insensitive PLA$_2$ activity in cell free extracts derived from cells in culture (6,7,19). After 40 minutes of serum starvation (conditions similar to those used for arachidonic acid release) PLA$_2$ activity in cell free extracts was 102 ± 14 pmol/mg/min in the vector cell line and 56 ± 7 pmol/mg/min (3 experiments, mean ± S.E., $p < .01$) in the antisense cell line, corresponding to a 46 % reduction in PLA$_2$ activity. There was minimal stimulation of phospholipase A$_2$ activity in cell free extracts in either antisense or vector cell lines. Serum starvation did not lead to the differences in PLA$_2$ activity, since cell free PLA$_2$ activity was reduced by 63 % from 46 ± 3 pmol/mg/min in the vector cell line to 12 ± 1 pmol/mg/min in the antisense cell line (4 experiments, mean ± .S.E., $p < .01$, different batch of substrate). The parallel reduction in cPLA$_2$ protein expression and PLA$_2$ activity in cell free extracts further supports the hypothesis that cPLA$_2$ may be regulated by changes in transcription as well as by direct phosphorylation (10).

It was anticipated that arachidonic acid release would be reduced in the antisense cell line concomitant with the reduction in cPLA$_2$ activity. Analysis of total neutral lipids showed equal incorporation of [^{3}H]arachidonic acid into phospholipids in both the vector and antisense cell lines (data not shown). Measurements of arachidonic acid release, using the same passage of cells as the cell free PLA$_2$ assay, revealed no difference in the response to the Ca^{2+} ionophore, A23187, PDGF, PDGF + A23187 or PMA + A23187 (Fig 3).

This unexpected result indicates that arachidonic acid release is not directly proportional to cPLA$_2$ activity, as predicted by recent results when mutant and wild type cPLA$_2$ were highly overexpressed in CHO cells (13).

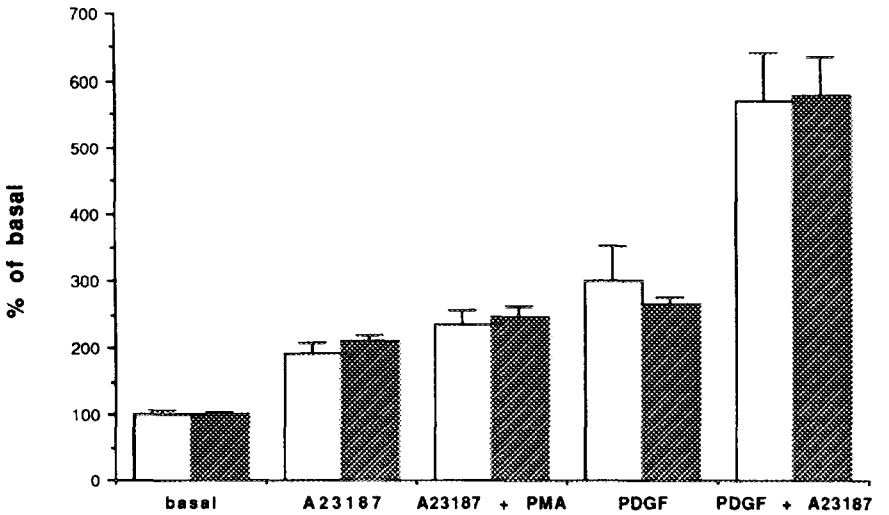

Fig. 3. *Arachidonic acid release.* The vector (open bars) or antisense cell lines (hatched bars) were labelled with [^{3}H]arachidonic acid, serum starved for 40 minutes, stimulated with A23187(2 μM), A23187(2 μM) + PMA(300 nM), PDGF(50 ng/ml) or PDGF(50 ng/ml) + A23187(2 μM) for 30 minutes and the media radioactivity was counted. Results are expressed as a percent of basal [^{3}H] counts in each cell type. All agonists caused a significant increase in [^{3}H] arachidonic acid release compared to the basal level.

A large body of evidence suggests that cellular arachidonic acid release is primarily mediated by the action of PLA_2. Mepacrine, an inhibitor of PLA_2, decreases arachidonic acid release (20). Our own previous studies, which utilized double labelling of glomerular mesangial cell lipids and the measurement of lysophospholipids, also indicate that agonist-stimulated arachidonic acid release derives principally from activation of PLA_2 (21). In fact, the biosynthesis of lysophospholipids, the hallmark of PLA_2 activity, has been detected by many investigators in response to agonists (22).

Recent evidence indicates that among the various species of PLA_2, it is $cPLA_2$, which accounts for agonist-responsive arachidonic acid release following its phosphorylation on serine-505 by MAP kinase (5,13,14). The present finding may be considered consistent with $cPLA_2$ being the major enzyme responsible for cellular arachidonic acid release in Swiss 3T3 cells. It is unlikely that the vector and antisense cells have different degrees of phosphorylation of serine-505 in the intact cell versus cell free extracts, since changes in phosphorylation of this site are reflected in assays of cell free extracts (13, HG unpublished). Therefore, we postulate that factors extrinsic to $cPLA_2$ are able to increase arachidonic acid release by $cPLA_2$ in the antisense cell line relative to the vector cell line. The net effect is that

the vector and antisense cell lines release equivalent amounts of arachidonic acid in vivo despite discordant PLA_2 activity in vitro. Several possibilities may account for this finding. Firstly, numerous reports show GTP analogues, pertussis toxin, dominant negative G proteins or dominant negative RAS proteins modulate arachidonic acid release suggesting that $cPLA_2$, like phospholipase C, may be directly regulated by a G protein (1, 23-26). It is possible that there may be differential effects of such a G protein in the intact cell, which are not mirrored in PLA_2 assays in cell free extracts. However, G proteins are generally not thought to be rate limiting for signal transduction. A second possibility is that there is another protein, such as PLA_2 activating protein, which regulates $cPLA_2$ in intact cells, whose effect is not apparent in cell free PLA_2 assays (27). Lipocortin inhibits PLA_2 activity in vitro by binding to lipid substrate (28), however its role in vivo is unclear.

The above results could also be due to limitation of arachidonic acid release by substrate availability or rapid reacylation of released arachidonic acid in vivo. Arachidonic acid release may vary depending on subcellular compartmentalization of arachidonic acid and its incorporation into different phospholipids in vivo (29, 30). The subcellular distribution of the [^{3}H]arachidonic acid used to label the cells in this study and the accessibility of $cPLA_2$ to it remains to be characterized.

A final possibility for our anomalous result is that a major proportion of arachidonic acid may be released by enzymes other than $cPLA_2$, such as calcium independent forms of PLA_2 (31), secretory PLA_2 or diacylglycerol lipase (32).

Although there have been no previous reports of the expression of antisense $cPLA_2$ RNA, other experiments provide indirect support for our hypothesis that arachidonic acid release in intact cells is not directly proportional to intrinsic $cPLA_2$ activity. Forskolin, which stimulates a rise in cAMP concentration, strongly inhibits arachidonic acid release in intact glomerular mesangial cells, but has no effect on PLA_2 activity in a cell free assay (6,HG unpublished). Furthermore, it has recently been demonstrated that cycloheximide and actinomycin can substantially inhibit arachidonic acid release in response to PDGF in Swiss 3T3 cells, without affecting PLA_2 activity in a cell free assay (33).

In summary, the finding of reduced PLA_2 activity with unchanged arachidonic acid release is informative. It is likely that arachidonic acid release in intact cells is limited by both direct phosphorylation of $cPLA_2$ and by less well characterized factors extrinsic to $cPLA_2$. The latter extrinsic factors, once identified, may be considered as potential targets for anti-inflammatory drugs.

Acknowledgments: We wish to thank Dr. Lih-Ling Lin and Dr. J. L. Knopf (Genetics Institute, Cambridge, Mass.) for the $cPLA_2$ antibody and the murine $cPLA_2$ cDNA. We are grateful to Paula Clayman for excellent technical assistance. This work was supported by grants from the Kidney Foundation of Canada and the Medical Research Council of Canada.

REFERENCES

1. Bonventre J.V. (1992) J. Am. Soc. Nephrol. 3, 128-150
2. Irvine R.F. (1982) Biochem. J. 204, 3-16
3. Clark JD, Lin L-L, Kriz RW, Ramesha CS, Sultzman MA, Lin AY, Millona N, Knopf JL. (1991) Cell 65:1043-11049
4. Sharp J.D. et al (1991) J. Biol. Chem. 266, 14,850-14,853
5. Lin L-L, Lin AY, Knopf J. (1991) Proc.Natl. Acad. Sci. 89:6147-6151
6. Gronich, J.H., Bonventre, J.V. and Nemenoff, R.A. (1988) J.Biol. Chem. 263, 16645-16652
7. Bonventre, J.V., Gronich, J.H. and Nemenoff, R.A. (1990) J. Biol. Chem. 265, 4934-4938
8. Goldberg H, Viegas M, Margolis B, Schlessinger J, Skorecki KL. (1990) Biochem. J. 267:461-465
9. Hack N, Margolis B, Ullrich A, Schlessinger J, Skorecki K. (1991) Biochem. J. 275 (3):563-467
10. Maxwell, A.P., Goldberg, H.J., Tay, A.H., Li, Z., Arbus, G. and Skorecki, K.L. submitted
11. Lin, L-L, Lin, A.Y. and Dewitt, D.L. (1992) J. Biol. Chem. 267, 23451-23454
12. Hoeck, W.G., Ramesha, C.S., Chang, D.J., Fan, N. and Heller, R.A. (1993) Proc. Natl. Acad. Sci. 90, 4475-4479
13. Lin, L-L, Wartmann, M., Lin, A.Y., Knopf, J.L. and Davis, R. (1993) Cell 72, 269-278
14. Nemenoff, R.A., Winitz, S., Qian, N-X., Putten, V.V., Johnson, G.L. and Heasley, L.E. (1993) J. Biol. Chem. 268, 1960-1964
15. Watkins, D.C., Johnson, G.L. and Malbon, C.C. (1992) Science 258, 1373-1375
16. Goldberg, H., Helaakoski, T., Garrett, L.A., Karsenty, G., Pellegrino, A., Lozano, G., Maity, S. and de Crombrugghe, B. (1992) J. Biol. Chem. 267, 19622-19630
17. Bradford, M. (1976) Anal. Biochem. 72, 248-254
18. Margolis B, Bonventre J, Kremer S, Kudlow J, Skorecki KL. (1988) Biochem. J. 249:587-592
19. Clark JD, Millona N, Knopf JL. (1990) Proc. Natl. Acad. Sci. 87:7708-7712
20. Takuwa, N., Kumada, M., Yamahita, K. and Takuwa, Y. (1991) J. Biol. Chem. 266, 14237-14243
21. Margolis B, Holub B, Troyer D, Skorecki KL. (1988) Biochem. J. 256:469-474
22. Asaoka, Y., Oka, M., Yoshida, K. Sasaki, Y. and Nishizuka, Y. (1992) Proc. Natl. Acad. Sci. 89, 6447-6451
23. Hack N, Clayman P, Skorecki K. (1990) Bioscience Reports 10 (4):353-362
24. Teitelbaum, I. (1990) J. Biol. Chem. 265, 4218-4222
25. Gupta, S.K., Diez, E., Heasley, L.E., Osawa, S. and Johnson, G.L. (1990) Science 249, 662-666
26. Warner, L., Hack, N., Egan, S., Goldberg, H. Weinberg, R.A. and Skorecki, K.L. (1993) Submitted
27. Clark, M.A., Ozgur, L.E., Conway, T.M., Dispoto, J. Crooke, S.T. and Bomalaski, J.S. (1991) Proc. Natl. Acad. Sci. 88, 5418-5422
28. Davidson, F.F., Dennis, E.A., Powell, M. and Glenney, J. (1987) J. Biol. Chem. 262, 1698-1705

29. Laposta M., Kaiser S.L. and Capriotti, A.M. (1988) J. Biol. Chem. 263, 3266-3273
30. Capriotti, A.M., Furth, E.E., Arrasmith, M.E. and Laposta, M. (1988) J. Biol. Chem. 263, 10029-10034
31. Hazen, S.L. and Gross, R.W. (1991) J. Biol. Chem. 266, 14526-14534
32. Balsinde J., Diez, E. and Mollinedof (1991) J. Biol. Chem. 266, 15638-15643
33. Domin, J. and Rozengurt, E. (1993) J. Biol. Chem. 268, 8927-8934