FEBS Letters 367 (1995) 228–232 FEBS 15628

Activation of a novel form of phospholipase A_2 during liver regeneration

Paul A. Wojtaszek, Vicki Van Putten, Raphael A. Nemenoff*

Department of Medicine, University of Colorado School of Medicine, Health Sciences Center, Box C281, 4200 East 9th Avenue, Denver, CO 80262, USA

Received 11 May 1995

Abstract **Activation of phospholipase A_2 (PLA_2) occurs following mitogenic stimulation of cells. This study examined PLA_2 activation during liver regeneration. Increased activity was detected within 1 h after partial hepatectomy, was maximal by 6 h, and returned to control levels by 24 h. Fractionation of cell-free extracts revealed multiple peaks of PLA_2 activity. One peak appeared identical to the previously described $cPLA_2$, and was modestly stimulated during regeneration. A higher molecular weight form ($hPLA_2$) was stimulated ~5-fold during regeneration. This enzyme was Ca^{2+}-dependent and selective for arachidonoyl-phosphatidylethanolamine. The activation of this novel form of PLA_2 represents an early event in liver regeneration, and is likely to contribute to the proliferative response.**

Key words: Phospholipase A_2; Regenerating liver; Arachidonic acid

1. Introduction

Liver regeneration has been used as a model for regulated proliferation for many years. In rats, following surgical removal of 70% of the liver mass, the remaining hepatocytes behave as a synchronized cell population, and are activated to undergo mitosis. While the factors signaling both the initiation and termination of proliferation have not been conclusively established, a number of growth factors including hepatocyte growth factor, insulin, EGF, glucagon, and vasopressin appear to be necessary for this process (see [1] for review). In addition, a number of intracellular signaling events have been shown to occur following partial hepatectomy. Within 10–60 min there is an induction of c-*fos* protooncogene mRNA [2]. Protein kinase C has been shown to translocate from the cytosol to particulate fractions within 30–60 min [3]. We have previously shown that a mitogen-stimulated serine kinase which phosphorylates ribosomal protein S6 is activated within 2 h following surgery [4]. All of these events temporally precede the entry into S phase, and the replication of DNA which occur approximately 18 h after surgery, and it is therefore likely that these events represent part of the programmed response resulting in proliferation.

The activation of phospholipase A_2 (PLA_2) has been observed to follow mitogenic stimulation of a variety of cell types in culture. In several cases it has been convincingly demonstrated that stimulation of arachidonic acid release is an obligatory event for cell growth. In 3T3 cells, stimulated arachidonic acid release through PLA_2 has been shown to be required for mitogenesis [5]. Enhanced arachidonic acid release has also been observed in transformed cells. In v-ras transformed cells Bar-Sagi and co-workers have shown that PLA_2 was activated following H-ras transformation of quiescent rat embryo fibroblasts [6], and appeared to specifically co-localize with the ras protein in these cells [7], suggesting an interaction between PLA_2 and ras protein. Arachidonic acid has also been shown to modulate the interaction between ras and rasGTPase activating protein (rasGAP) [8].

PLA_2 activation has also been associated with cell injury. Membrane breakdown and release of free fatty acids has been observed following ischemic injury in kidney, heart, and liver [9,10]. In rabbit renal proximal tubules anoxia has been shown to result in activation of PLA_2 [11], and PLA_2 activity is modulated during renal ischemia and reperfusion [12]. A novel Ca^{2+}-independent form of the enzyme specific for plasmalogens is activated during myocardial ischemia [13].

In hepatocytes, arginine vasopressin has been shown to acutely stimulate release of arachidonic acid [14]. PLA_2 stimulation has also been shown to occur following exposure of hepatocytes to carbon tetrachloride [15]. Neither the mechanism of regulation, nor the specific forms of PLA_2 involved have been identified.

We report here the identification of three isoforms of PLA_2 in liver extracts. One of these isoforms appears to be similar to the hormonally regulated $cPLA_2$ previously described [16,17,18]. However, a second, high-molecular weight form appears to represent a novel isoform of PLA_2 whose activity is markedly stimulated during the early stages of liver regeneration.

2. Experimental

2.1. Animal surgery

Partial hepatectomies were performed on male Sprague–Dawley rats (120–250 g) following ether anesthesia as previously described [4]. Sham operations involved performance of a laparotomy followed by manipulation, but not removal of the liver. All surgery was performed before 10.30 h. At various times following surgery, animals were sacrificed. The liver remnants were removed, rinsed in ice-cold phosphate buffered saline, cut into several pieces and frozen in liquid nitrogen.

2.2. Preparation and fractionation of extracts

Pieces of liver were homogenized in 50 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose, 20 μM pepstatin, 20 μM leupeptin, 100 μM phenylmethylsulfonyl fluoride, and 1000 U/ml aprotinin, using a Potter-Elvesjem motor-driven pestle. Soluble extracts were prepared by differential centrifugation as previously described [16], and matched for protein concentration. Equal amounts of extract protein from regenerating and sham-operated liver were fractionated by Pharmacia Superose 12 gel filtration chromatography. Extracts containing 2–5 mg of total protein were loaded on the column, which had previously been calibrated and equilibrated in 50 mM HEPES, pH 7.5, 1 mM EGTA, 1 mM EDTA, 150 mM NaCl, as previously described [12]. 0.5 ml of extract was loaded on the column (24 ml) and 0.5 ml fractions were collected. Fractions were assayed for PLA_2 activity as

*Corresponding author. Fax: (1) (303) 270-4852.

Abbreviations: PLA_2, phospholipase A_2; PC, phosphatidylcholine; PE, phosphatidylethanolamine; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetra-acetic acid.

SSDI 0014-5793(95)00556-0

previously described [16]. A portion of each fraction was used for immunoblotting (see below). Peaks recovered from the gel filtration column were further fractionated by Mono-Q anion exchange. Peak fractions were loaded on the column which had been equilibrated in 50 mM HEPES, pH 7.5, 1 mM EGTA, 1 mM EDTA. The column was washed until the absorbance at 280 nm reached baseline (approximately 10 ml). Proteins were then eluted with a 35 ml linear NaCl gradient from 0 to 0.6 M; 1 ml fractions were collected and assayed for PLA_2 activity.

2.3. PLA_2 activity assays

In a reaction volume of 40 μl, column fractions were incubated with 15 μM 1-palmitoyl-2-[^{14}C]-arachidonoyl-phosphatidylethanoloamine (PE) or -phosphatidylcholine (PC) (specific activity ≈ 57 mCi/mmol) in the presence of 4 mM $CaCl_2$ (in excess of EDTA and EGTA) and 5% dimethyl sulfoxide for 30 min at 37°C. The reaction was quenched with addition of 40 μl unlabeled arachidonic acid (500 μg/ml) in ethanol:acetic acid (98:2), and 50 μl of each sample was spotted onto Whatman LK5D Silica Gel TLC plates. The plates were developed in ethyl acetate/isooctane/acetic acid/water (55:75:8:100) from which the lower, aqueous phase had been removed. Plates were stained with iodine vapor, and arachidonic acid peaks and PE or PC peaks were scraped with a razor blade into vials and counted by liquid scintillation counting in the presence of 250 μl water and 5 ml Opti-Fluor. Results are expressed as pmol/min. For assays using other substrates, the appropriate substrate was used at the same concentration. For studies examining divalent metal dependency and activation by NaCl, 4 mM $MgCl_2$, 4 mM $MnCl_2$ or 1 M NaCl was substituted for the 4 mM $CaCl_2$.

2.4. Determination of calcium dependency

Fractions from the gel filtration column (peaks I and II) were diluted in EGTA/Ca^{2+} buffers containing various concentrations of Ca^{2+}. PLA_2 activity was assayed using PE as a substrate, as described above. Actual Ca^{2+} concentrations were determined using fura-2 [19].

2.5. Immunoblotting

Superose 12 column fractions from 5 mg of regenerating livers extracts were run on SDS-polyacrylamide gels and transferred to nitrocellulose. Blots were probed with a polyclonal antibody raised against a recombinant fragment of the $cPLA_2$ molecule [20], followed by ^{125}I-protein A. As a positive control 10 ng of recombinant purified $cPLA_2$ was used.

2.6. Materials

Radiolabeled phospholipids were from Amersham Corp. or Dupont-New England Nuclear. Unlabeled phospholipids, arachidonic acid, and standards for thin layer chromatography, as well as standard chemicals for buffers were from Sigma. Prepacked Mono-Q (HR 5/5) and Superose 12 columns were from Pharmacia LKB Biotechnology Inc. Protein was measured using a Bio-Rad protein kit. Molecular weight markers for polyacrylamide gels were from Bio-Rad. Nitrocellulose paper (TM-NC4) and all reagents for SDS-PAGE and immunoblotting were purchased from Hoefer Scientific Instruments and Bethesda Research Laboratories.

3. Results

3.1. Activation of PLA_2 activity during liver regeneration

Soluble extracts were prepared from livers at various times following partial hepatectomy. Extracts were matched for protein concentration and assayed for PLA_2 activity using 1-palmitoyl-2-[^{14}C]-arachidonoyl-PC or 1-palmitoyl-2-[^{14}C]-arachidonoyl-PE as substrate in the presence of 4 mM Ca^{2+}. As shown in Fig. 1, an increase in PLA_2 activity was observed within an hour after surgery compared to sham-operated animals. Enhanced specific activity was detected using either [^{14}C]arachidonoyl-PC or [^{14}C]arachidonyl-PE as substrate, although a much larger increase was detected with [^{14}C]arachidonoyl-PE. Maximal increase occurred at 4–6 h, and PLA_2 activity returned to sham levels by 24 h. Activity from sham-operated animals was identical to control animals not undergoing any surgical procedures, and did not change as a function of time. Extracts assayed in the absence of exogenously added Ca^{2+} showed little activity (data not shown).

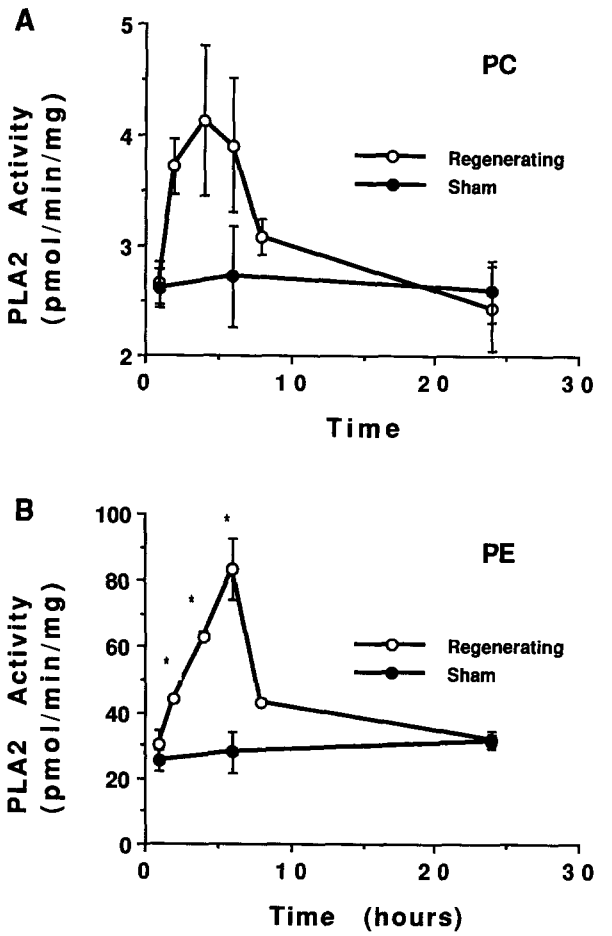

Fig. 1. Time course of stimulation of PLA_2 activity in regenerating versus sham-operated livers. Cell-free extracts were prepared from rats at the indicated times following surgery. Sham animals received a laparotomy followed by manipulation, but not removal of the liver. Extracts were matched for protein and PLA_2 activity determined by measuring the rate of hydrolysis of arachidonic acid from exogenous [^{14}C]arachidonoyl-PC (Panel A) or [^{14}C]arachidonoyl-PE (Panel B). Results represent the means of triplicate determinations from 3 separate animals at each time point. *$P < 0.05$ vs. Sham.

3.2. Fractionation of liver extracts

To characterize the specific isoforms of PLA_2 which are activated during regeneration, extracts prepared from the regenerating livers of rats sacrificed 4 and 6 h after surgery, and from sham-operated animals were fractionated by FPLC gel filtration on a Superose 12 column. Fractions were assayed for PLA_2 activity using either [^{14}C]arachidonoyl-PE or -PC, in the presence of 4 mM Ca^{2+}. When [^{14}C]arachidonoyl-PC was used as a substrate, a single major peak of activity (hereafter referred to as peak II) was observed (Fig. 2A), and a small increase in activity was apparent in regenerating compared to sham livers. However, with [^{14}C]arachidonoyl-PE as the substrate, three peaks of activity were observed, all of which were stimulated in regenerating livers compared to sham. The major activity with [^{14}C]arachidonoyl-PE migrated as a high-molecular weight

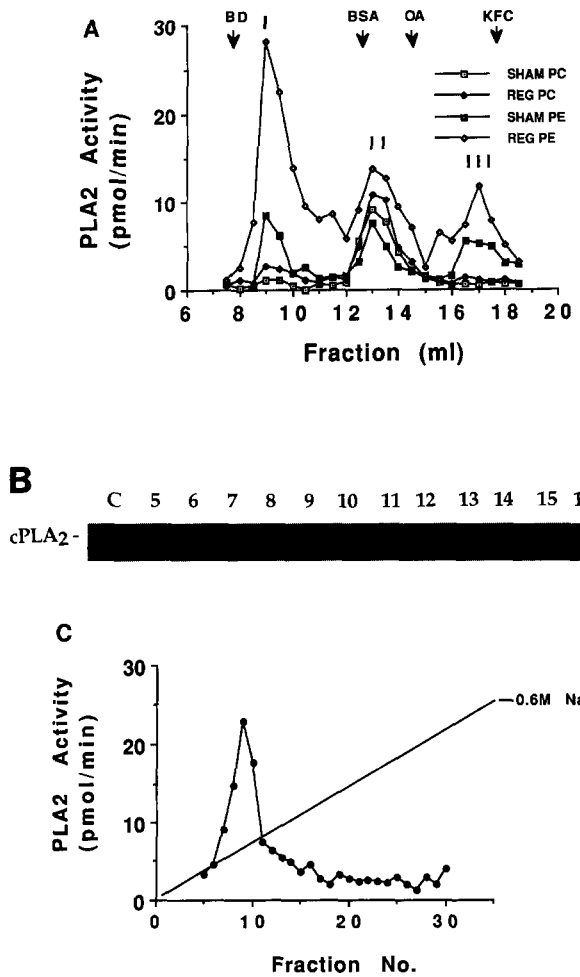

Fig. 2. Fractionation of extracts from regenerating and sham-operated liver. (A) Superose 12 gel filtration fractionation of liver extracts and determination of PLA_2 activity in fractions. One half ml of extract containing approximately 5 mg protein was loaded on the column which had been calibrated with the following standards: BD-blue dextran ($M_r = 6 \times 10^6$), BSA-bovine serum albumin ($M_r = 66{,}000$), OA-ovalbumin ($M_r = 43{,}000$), and KFC-potassium ferricyanide. Fractions (0.5 ml) were collected and assayed for PLA_2 activity as described in section 2. (B) Immunoblot of Superose 12 fractions using antibody directed against the human 85 kDa $cPLA_2$. A portion of the indicated fractions obtained in Panel A was separated on SDS-PAGE, and blotted with anti-$cPLA_2$ antibody, followed by ^{125}I-protein A. Ten ng of human recombinant $cPLA_2$ was loaded as a positive control. (C) Mono-Q anion exchange chromatography. Peak I recovered from the Superose 12 column (Fractions 8.5–10 ml) was diluted and loaded on a Mono-Q column. Proteins were eluted with a 35 ml gradient from 0 to 0.6 M NaCl. Fractions (1 ml) were assayed for PLA_2 activity using [^{14}C]arachidonoyl-PE as described above.

protein just past the excluded volume of the column (peak I); this activity was stimulated approximately 5-fold in regenerating liver. A peak corresponding to the major [^{14}C]arachidonoyl-PC peak was modestly stimulated (peak II), and a third peak (peak III) appeared at low apparent molecular weight. This peak was not further characterized.

The migration position of peak II is very similar to that of purified $cPLA_2$ isolated from rat kidney or mesangial cells [16]. To further examine the relationship between isoforms of PLA_2 detected in regenerating liver and $cPLA_2$, the Superose 12 column fractions were analyzed by immunoblotting, using a polyclonal antibody raised against recombinant $cPLA_2$ [20]. This antibody recognizes the rat form of $cPLA_2$, a 110 kDa polypeptide on SDS polyacrylamide gels, in both kidney and glomerular mesangial cell extracts (data not shown). Fig. 2B shows that this antibody recognized a 110 kDa protein in fractions corresponding to peak II (12–14 ml fractions), but not peak I (8–10 ml fractions).

To confirm that peak I represented a single enzymatic species, fractions from the gel filtration column (8.5–10 ml) were pooled and fractionated by FPLC Mono-Q anion exchange, utilizing a linear NaCl gradient. A single peak of PLA_2 activity eluted at about 0.15 M NaCl (Fig. 2C). Previous studies have shown that purified $cPLA_2$ elutes from this column at approximately 0.37 M NaCl using the same buffer conditions [16].

3.3. Substrate specificity and divalent cation requirement

To characterize the substrate specificity of peak I, appropriate fractions were pooled as above and activity determined

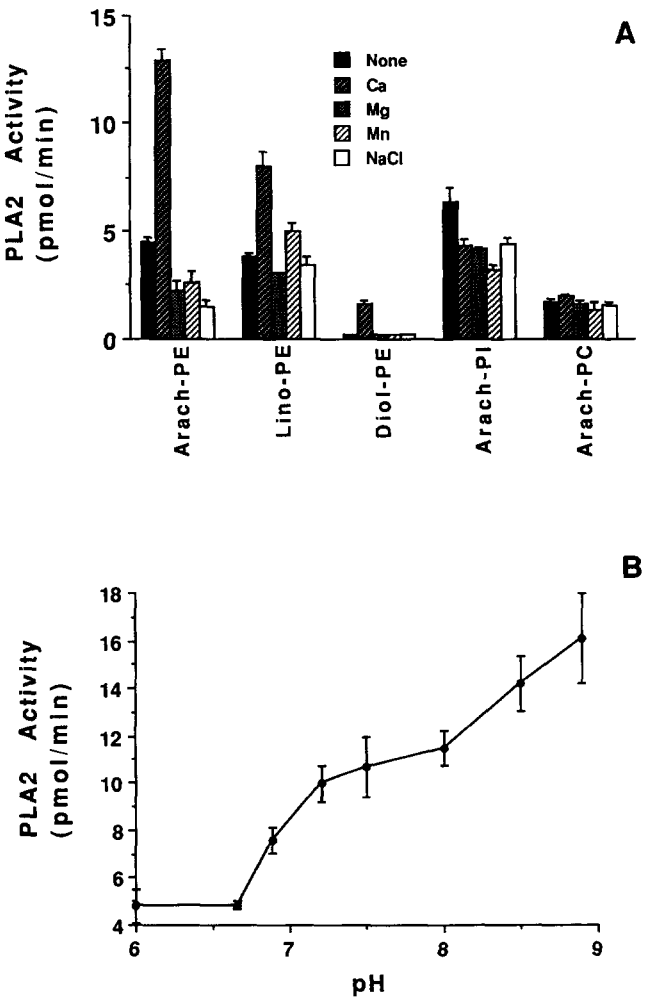

Fig. 3. (A) Substrate and ion selectivity of peak I. Peak I recovered from the Superose 12 column was diluted with 50 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM EGTA, and activity against various substrates (15 μM) was determined as described above in the presence of 4 mM of divalent cation salts, 1 M NaCl, or chelators alone (none). (B) pH dependence of peak I PLA2 activity. Activity was determined using [^{14}C]arachidonoyl-PE as a substrate and adjusting the pH with either Tris base or acetic acid. Results represent the mean of triplicate determinations with the standard error indicated.

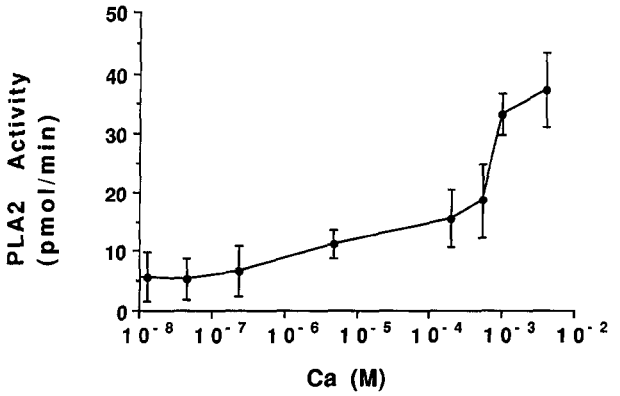

Fig. 4. Dependence of peak I PLA_2 activity on Ca^{2+} concentration. Peak I recovered from the Superose 12 column was diluted with 50 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM EGTA. Various amounts of buffer or 20 mM $CaCl_2$ were added to obtain 200 μl aliquots at each Ca^{2+} concentration. PLA_2 activity was determined using [^{14}C]arachidonoyl-PE as a substrate as described above. A portion of each aliquot was used to determine the actual free Ca^{2+} concentration using fura-2.

against phospholipids with different headgroups (ethanolamine, choline, or inositol), and with different unsaturated fatty acids at the *sn-2* position (arachidonate, linoleate, or oleate) (Fig. 3A). The data show a selectivity for ethanolamine as the headgroup, and for arachidonate as the *sn-2* fatty acid.

When activity was assayed in the presence of divalent cations other than Ca^{2+}, a clear selectivity for Ca^{2+} was seen when the phosphatidylethanolamine lipids were used as substrates. Such selectivity was not clearly seen with phosphatidylcholine lipid, which, however, was a poor substrate for the enzyme under all conditions assayed. Activity against the 1-stearoyl-2-[^{14}C]-arachidonoyl-phosphatidylinositol was slightly higher, but was not dependent upon divalent cations and was, in fact, somewhat inhibited by them (Fig. 3A). This activity may therefore be distinct from the activity against phosphatidylethanolamine lipids, perhaps representing coelution with a distinct, Ca^{2+}-independent phosphoinositidase.

Further, in contrast to $cPLA_2$, which is activated under high-salt conditions [21], activity of peak I in the presence of 1 M NaCl was strongly inhibited. In contrast to the secreted PLA_2's, peak I was relatively insensitive to disruption of disulfide bonds, demonstrating only a 15% decrease in activity after 5 min of treatment with 50 mM DTT at 37°C (not shown). The pH sensitivity of peak I favored the alkaline range (Fig. 3B).

3.4. Ca^{2+} dependence

To determine the Ca^{2+} dependence of peak I, appropriate fractions (8.5–10 ml) from the Superose 12 column were pooled, and PLA_2 activity against 1-palmitoyl-2-[^{14}C]-arachidonoyl-PE was assayed over a wide range of Ca^{2+} concentrations (Fig. 4). Peak I required relatively high concentrations of Ca^{2+} for enzymatic activity, with the half-maximal concentration for activation occurring at approximately 500 μM. This is significantly higher than the half-maximal Ca^{2+}concentration for $cPLA_2$ which is approximately 500 nM [16].

3.5. Sensitivity to known PLA_2 inhibitor

A trimethyl ketone analogue of arachidonic acid ($AACOCF_3$) has recently been shown to strongly inhibit the human platelet $cPLA_2$ but not the low molecular weight, secretable $sPLA_2$ [22]. $AACOCF_3$ was a potent inhibitor of both peak I and peak II activity (Fig. 4A). Peak II was somewhat more strongly inhibited by $AACOCF_3$, with 50% inhibition occurring at approximately 7 μM, and 80% inhibition occurring at 30 μM. For peak I, half-maximal inhibition was observed at approximately 15 μM $AACOCF_3$, and 60% inhibition at 30 μM. The methyl ketone derivative of arachidonic acid ($AACOCH_3$) partially stimulated peak II activity at low concentrations and had a minor inhibitory effect at 30 μM (Fig. 4B). In contrast this compound was a potent inhibitor of peak I, with half-maximal inhibition occurring at approximately 15 μM, and 70% inhibition at 30 μM (Fig. 5).

4. Discussion

The data presented suggest that multiple isoforms of PLA_2 are present in rat liver extracts. One of these, designated as peak

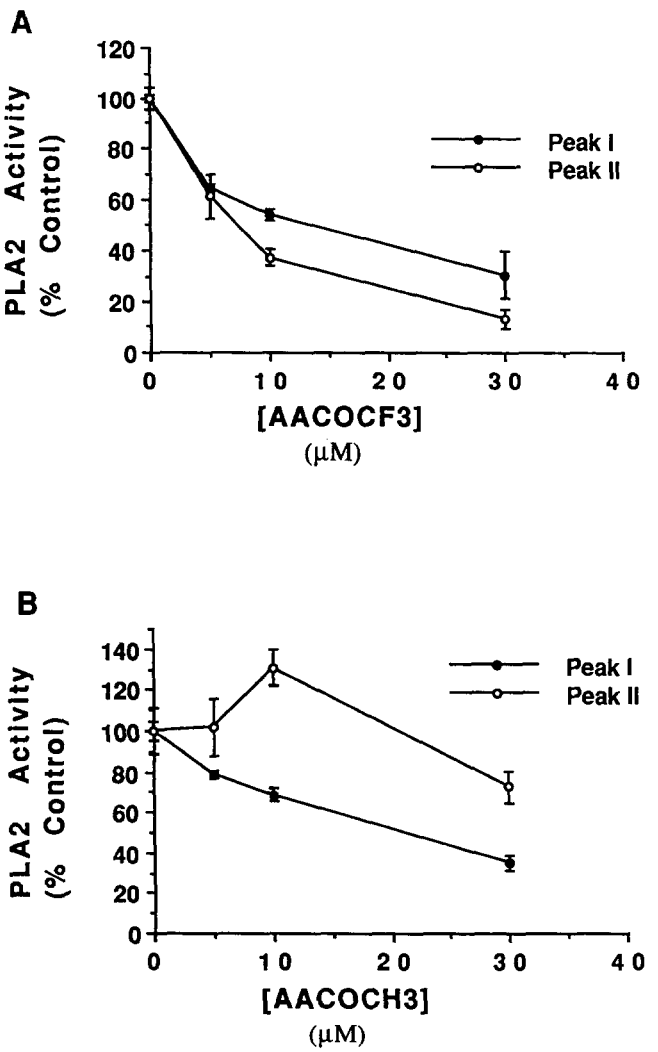

Fig. 5. Effect of trifluoroketone ($AACOCF_3$) and methyl ketone ($AACOCH_3$) arachidonic acid analogues on PLA_2 activities of peaks I and II. Pooled fractions from the Superose 12 column (peak I = fractions 8.5–10 ml, peak II = fractions 12.5–14.5 ml) were incubated with various concentrations of $AACOCF_3$ (Panel A) or $AACOCH_3$ (Panel B), the 300 μM stocks of which had been sonicated immediately before adding. PLA_2 activity using [^{14}C]arachidonoyl-PE was determined.

II appears to be the liver homolog of $cPLA_2$ [16,17,23,24]. Its migration position on both anion exchange and gel filtration are identical to the kidney form of $cPLA_2$, and it is recognized by antibodies raised against the human $cPLA_2$. The second form, designated peak I, is greatly stimulated within the first several hours of liver regeneration. This enzyme appears to represent an novel form of PLA_2, not previously well characterized. By a number of criteria it can be distinguished from $cPLA_2$, the best characterized high-molecular weight form of PLA_2. Peak I shows much greater activity towards arachidonoyl-PE than PC, as distinct from $cPLA_2$ which has approximately equal activity against either substrate. It elutes earlier from anion exchange columns, is inhibited rather than activated by 1 M NaCl, and migrates at a higher molecular weight by gel filtration. Specific antibodies against $cPLA_2$ do not appear to recognize this enzyme.

This novel form appears to require much higher concentrations of Ca^{2+} for activation in vitro than does $cPLA_2$ [25]. In fact, the Ca^{2+} dependency of this enzyme is similar to the Ca^{2+} requirements of the $sPLA_2$ family of enzymes [26]. However, our data strongly suggest that peak I is not an aggregate of $sPLA_2$. On two successive fractionation procedures the enzymatic activity migrates as a single peak, with no detectable breakdown. Secondly, the sensitivity of peak I to inhibitory arachidonic acid analogs is very different from $sPLA_2$. These compounds are poor inhibitors of $sPLA_2$ [22], but potent inhibitors of peak I. In fact the methyl ketone derivative is a more potent inhibitor of peak I than of $cPLA_2$. Finally, peak I is insensitive to reducing agents. It is therefore more likely that peak I represents a novel form of PLA_2, distinct from both $cPLA_2$ and $sPLA_2$ specific to certain tissues. We have tentatively designated this enzyme $hPLA_2$ (hepatic PLA_2).

Since the Ca^{2+} dependency of this enzyme is so high, it raises the question as to how it is activated under normal or growth factor-stimulated levels of Ca^{2+}. It is conceivable that interactions with other proteins or additional cofactors regulate the Ca^{2+} dependency, allowing activation at more physiologic Ca^{2+} concentrations. Alternatively, during the process of tissue disruption and fractionation we have damaged the enzyme and altered its true Ca^{2+} dependency.

The mechanisms causing activation of PLA_2 during liver regeneration remain to be determined. The kinetics of the increase in activity (detectable at 1 h following surgery) suggest that post-translational alteration of pre-existing enzyme is likely to be involved. It has been shown that $cPLA_2$ is phosphorylated on serine residues in response to growth factor stimulation [27]. We and others have recently demonstrated that both protein kinase C and p42 MAP kinase can phosphorylate and activate purified human $cPLA_2$ [28,29]. Both of these protein kinases have been shown to be activated within the first hour during liver regeneration [30] and therefore are potential candidates mediating the observed activation of peak I. The observation that PLA_2 activity is rapidly increased during liver regeneration suggests an important role for arachidonic acid or its metabolites in the regenerative response. Alternatively, lysophospholipids produced by PLA_2 may be converted to lysophosphatidic acid, which has mitogenic properties [31].

To our knowledge, this is the first demonstration of activation of PLA_2 associated with mitogenesis in vivo. Studies using selective inhibitors of either the cyclooxygenase pathway or the lipoxygenase pathway to block liver regeneration will indicate whether these metabolites, as opposed to free arachidonic acid itself, are critical for the observed stimulation of DNA synthesis.

Acknowledgments: We are grateful to Eric Weider for carrying out fura-2 measurements of intracellular Ca^{2+}. This work was supported by grants from the NIH (DK 39902, DK 19928).

References

[1] Michalopoulos, G.K. (1990) FASEB J. 4, 176–178.
[2] Thompson, N.L., Mead, J.E., Braun, L., Goyette, M., Shank, P.R. and Fausto, N. (1986) Cancer Res. 46, 3111–3117.
[3] Buckley, A.R., Putnam, C.W., Evans, R., Laird, H.E., Shah, G.N., Montgomery, D.W. and Russell, D.H. (1987) Life Sci. 41, 2827–2834.
[4] Nemenoff, R., Mendelsohn, M., Gunsalus, J., Carter, E. and Avruch, J. (1988) J. Biol. Chem. 263, 19455–19560.
[5] Millar, J.B.A. and Rozengurt, E. (1990) J. Biol. Chem. 265, 19973–19979.
[6] Bar-Sagi, D. and Feramisco, J.R. (1986) Science 233, 1062–1068.
[7] Bar-Sagi, D., Suhan, J.P., McCormick, F. and Feramisco, J.R. (1988) J. Cell Biol. 106, 1649–1658.
[8] Tsai, M.-H., Yu, C.-L., Wei, F.-S. and Stacey, D.W. (1989) Science 243, 522–526.
[9] Matthys, E.Y., Patel, Y., Kreisberg, J., Stewart, J.H. and Venkatachalam, M. (1984) Kidney Int. 26, 153–161.
[10] Chien, K.R., Han, A., Sen, A., Buja, L.M. and Willerson, J.T. (1984) Circ. Res. 54, 313–322.
[11] Portilla, D., Mandel, L.J., Bar-Sagi, D. and Millington, D.S. (1992) Am. J. Physiol. 262, F354–F360.
[12] Nakamura, H., Nemenoff, R.A., Gronich, J.H. and Bonventre, J.V. (1991) J. Clin. Invest. 87, 1810–1818.
[13] Hazen, S.L., Stuppy, R.J. and Gross, R.W. (1990) J. Biol. Chem. 265, 10622–10630.
[14] Hughes, B.P., Polverino, A.J., Lim, F. and Barritt, G.J. (1987) Horm. Metab. Res. 19, 15–20.
[15] Glende, E.A. and Pushpendran, C.K. (1986) Biochem. Pharmacol. 19, 3301–3307.
[16] Gronich, J.H., Bonventre, J.V. and Nemenoff, R.A. (1990) Biochem. J. 271, 37–43.
[17] Leslie, C.C., Voelker, D.R., Channon, J.Y., Wall, M.M. and Zelarney, P.T. (1988) Biochim. Biophys. Acta 963, 476–492.
[18] Clark, M.A., Ozgur, L.E., Conway, T.M., Dispoto, J., S.T., C. and Bomalaski, J.S. (1991) Proc. Natl. Acad. Sci. USA 88, 5418–5422.
[19] Grynkiewicz, G., Poenie, M. and Tsien, R.Y. (1985) J. Biol. Chem. 260, 3440–3450.
[20] Winitz, S., Gupta, S.K., Qian, N.-X., Heasley, L.E., Nemenoff, R.A. and Johnson, G.L. (1994) J. Biol. Chem. 269, 1889–1895.
[21] Reynolds, L.J., Hughes, L.L., Louis, A.I., Kramer, R.M. and Dennis, E.A. (1993) Biochim. Biophys. Acta 1167, 272–280.
[22] Bartoli, F., Lin, H.-K., Ghomashchi, F., Gelb, M.H., Jain, M.K. and Apitz-Castro, R. (1994) J. Biol. Chem. 269, 15625–15630.
[23] Kramer, R.M., Roberts, E.F., Manetta, J. and Putnam, J.E. (1991) J. Biol. Chem. 266, 5268–5272.
[24] Clark, J.D., Lin, L.-L., Kriz, R.W., Ramesha, C.S., Sultzamn, L. A., Lin, A.Y., Milona, N. and Knopf, J.L. (1991) Cell 65, 1043–1051.
[25] Channon, J.Y. and Leslie, C.C. (1990) J. Biol. Chem. 265, 5409–5413.
[26] Mayer, R.J. and Marshall, L.A. (1993) FASEB J. 7, 339–348.
[27] Lin, L.-L., Lin, A.Y. and Knopf, J.L. (1991) J. Cell. Biochem. 15B, 161.
[28] Nemenoff, R.A., Winitz, S., Qian, N.-X., Van Putten, V., Johnson, G. L. and Heasley, L.E. (1993) J. Biol. Chem. 268, 1960–1964.
[29] Lin, L.-L., Wartmann, M., Lin, A.Y., Knopf, J.L., Seth, A. and Davis, R.J. (1993) Cell 72, 269–278.
[30] Sasaki, Y., Hayashi, N. and Kamada, T. (1990) Adv. Second Messenger Phosphoprotein Res. 24, 345–351.
[31] van Corven, E.J., Groenink, A., Jalink, K., Eichholtz, T. and Moolenaar, W.H. (1989) Cell 59, 45–54.