Kinetic Analysis of a High Molecular Weight Phospholipase A_2 from Rat Kidney: Divalent Metal-Dependent Trapping of Enzyme on Product-Containing Vesicles†

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ABSTRACT: The kinetics of hydrolysis of 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine vesicles catalyzed by the high molecular weight phospholipase A_2 from rat kidney show an anomalous behavior. The reaction progress lasts for several minutes and then stops after only 5–10% of the available substrate has been hydrolyzed. Addition of more enzyme but not more substrate leads to a new round of hydrolysis. Although this initially suggested that the enzyme becomes inactivated during the turnover, such a conclusion could not be substantiated. Addition of buffer containing 0.15 M NaCl and bovine serum albumin to the reaction after the progress ceased leads to the re-initiation of the lipolysis. The enzyme is not strongly inhibited by the reaction products. Although the enzyme does not bind irreversibly to vesicles composed of pure 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine, it does become irreversibly trapped on vesicles that contain a critical mole percentage of reaction products. This trapping is the most likely explanation for the cessation of the reaction progress. Both the binding of enzyme to 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine vesicles and the hydrolysis of 1-stearoyl-2-[^{3}H]arachidonyl-*sn*-glycerophosphocholine contained in these vesicles require the presence of products. Furthermore, the trapping of enzyme is independent of catalytic turnover. The trapping is sensitive to the structure of the fatty acid present in the vesicles and requires the presence of divalent metals (either Ca^{2+}, Sr^{2+}, Ba^{2+}, or Mg^{2+}). Since the concentrations of the metals needed for the enzymatic activity correlate with the amounts needed to promote the trapping, it is suggested that the role of the metal is only to promote the interfacial binding of the enzyme.

Phospholipases A_2 (PLA2)[1] (EC 3.1.1.4) catalyze the hydrolysis of the ester linkage at the *sn*-2 position of phospholipids to produce a free fatty acid and a lysophospholipid (van Deenen & de Haas, 1963; Waite, 1987). The secretory enzymes from pancreas (Verheij et al., 1981), venoms (Dennis, 1983), and platelets/human synovial fluid (Kramer et al., 1989; Seilhamer et al., 1989) are the best characterized enzyme forms. These enzymes are small (molecular weights near 14K), require millimolar amounts of Ca^{2+} as a cofactor, and are related both in terms of the primary (van den Bergh et al., 1989) and in terms of the three-dimensional structure (Scott et al., 1990, 1991; Thunnissen et al., 1990; Wery et al., 1991).

More recently, an 85-kDa phospholipase A_2 has been found in human, bovine, and rabbit platelets (Kim et al., 1991a,b; Kramer et al., 1986; Takayama et al., 1991), rat macrophages (Leslie et al., 1988; Wijkander & Sundler, 1989), the human monocytic cell line U937 (Clark et al., 1990; Diez & Mong, 1990; Kramer et al., 1991; Rehfeldt et al., 1991), and rat kidney (Gronich et al., 1990). In contrast to the secretory phospholipases A_2, this high molecular weight enzyme is activated by submicromolar amounts of Ca^{2+} and preferentially hydrolyzes phospholipids that have arachidonic acid at the *sn*-2 position. In the absence of Ca^{2+}, the 85-kDa phospholipase A_2 is found in the cytosolic fraction of cells, and it relocates to the particulate fraction in the presence of submicromolar amounts of Ca^{2+} (Clark et al., 1991; Diez & Mong, 1990; Krause et al., 1991; Leslie & Channon, 1990; Rehfeldt et al., 1991; Yoshihara & Watanabe, 1990). The primary structure of the 85-kDa phospholipase A_2 from U937 cells as inferred from the cDNA clone contains no regions of homology with secreted forms of phospholipase A_2 (Clark et al., 1991; Sharp et al., 1991). Futhermore, the amino acid sequence reveals a stretch of 45 residues in the amino-terminal region that shows homology to Ca^{2+}-dependent forms of protein kinase C and GTPase activating protein. A 140 amino acid fragment of this enzyme that contains the amino terminus was shown to bind to membranes in the presence of submicromolar amounts of Ca^{2+} (Clark et al., 1991). On the basis of all these data, it is reasonable to hypothesize that this enzyme functions in a signal-transduction pathway leading to the release of arachidonic acid from membranes for the biosynthesis of eicosanoids.

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[1] Abbreviations: BSA, fatty acid-free bovine serum albumin; DOG, 1,2-dioleoyl-*sn*-glycerol; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; ^{14}C-DPPC, 1-palmitoyl-2-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; hmw-PLA2, high molecular weight phospholipase A_2 (MW 85K) from rat kidney; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)diacylphosphatidylethanolamine; OPPC, 1-oleoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine; PAPC, 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine; ^{3}H-PAPC, 1-palmitoyl-2-[5,6,8,9,11,12,14,15-^{3}H(N)]-arachidonyl-*sn*-glycero-3-phosphocholine; ^{14}C-PAPC, 1-palmitoyl-2-[1-^{14}C]arachidonyl-*sn*-glycero-3-phosphocholine; PLA2, phospholipase A_2; POPM, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphomethanol; Rh-PE, (lissamine rhodamine)phosphatidylethanolamine; ^{3}H-SAPC, 1-stearoyl-2-[5,6,8,9,11,12,14,15-^{3}H(N)]arachidonyl-*sn*-glycerol-3-phosphocholine.

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A kinetic analysis of the hmw-PLA2 from rat kidney acting on phospholipid vesicles is described in this report. A number of interesting features of the kinetics including the nonlinear reaction progress curves and the apparent enzyme inactivation during turnover (Leslie, 1991) were analyzed in detail. The results reported herein establish that the hmw-PLA2 becomes trapped on product-containined vesicles.

Experimental Procedures

Materials. The lipids 1,2-dioleoyl-*sn*-glycero-3-phosphate, DOG, DPPC, NBD-PE, OPPC, 1-palmitoyl-*sn*-glycero-3-phosphocholine, PAPC, and Rh-PE were obtained from Avanti Polar Lipids, and trioleoylglycerol was from Sigma. POPM was made from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate disodium salt (Avanti) by formation of the phosphate dimethyl ester and demethylation with lithium bromide (Jain & Gelb, 1991). The radiolabeled lipids ^{14}C-PAPC (50–55 mCi/mmol) and ^{3}H-SAPC (91 Ci/mmol) were from New England Nuclear, and ^{14}C-DPPC (100 mCi/mmol) was from Amersham. All of the unsaturated fatty acids listed in Figure 6 were obtained from Nu Chek Prep (Elysian, MN) except for arachidonic acid which was from Sigma. BSA refers to fatty acid-free bovine serum albumin (Sigma catalog no. A7511). Highly pure metal salts $SrCl_2$ and $MgCl_2$ (99.995%) and $BaCl_2$ (99.999%) were obtained from Aldrich. The PLA2 from *Naja naja naja* venom was purified as described (Hazlett & Dennis, 1985).

The preparation of the kidney homogenate was as described previously (Gronich et al., 1988, 1990) with the following additional information. Rats were sacrificed two at a time, and the four kidneys were immediately homogenized in 25 mL of ice-cold buffer containing protease inhibitors (Gronich et al., 1990). The hmw-PLA2 from rat kidney was extensively purified as described (Gronich et al., 1990) using all chromatographic steps up to and including the mono-Q column. The fractions from the peak of activity from the mono-Q column were pooled. The salt concentration of the pooled fractions was reduced by approximately 20-fold by two rounds of concentration of the sample in a Centricon-10 ultrafilter (Amicon) to a small volume and addition of buffer (10 mM HEPES/0.1 mM EDTA, pH 8.0) to give the original sample volume. The enzyme in this buffer was stored in small aliquots at −80 °C with negligible loss of enzymatic activity over several months. The enzyme was stable to at least 3 cycles of freezing and thawing. Typical yields of enzyme starting from 60 kidneys were 6000–12 000 microunits with a specific activity of 8500–17 000 microunits/mg as measured with the previously reported assay (Gronich et al., 1990) (a microunit is the amount of enzyme that produces 1 pmol of arachidonate per minute at 37 °C). These values are similar to those previously documented (Gronich et al., 1990). Numerous properties of this partially purified enzyme were essentially identical to those reported for either homogeneous or nearly homogeneous preparations (Clark et al., 1990; Diez & Mong, 1990; Gronich et al., 1990; Kramer et al., 1991; Leslie, 1991). These include the pH dependence of the activity, the effect of submicromolar and millimolar concentrations of calcium on the reaction progress curve, and the marked specificity for arachidonyl-containing phospholipids.

Preparation of Substrate Vesicles. Stock solutions of substrate vesicles were prepared at a lipid concentration that was 10-fold higher than that used in the enzymatic reaction mixture. Solutions of unlabeled lipids (phospholipids, lysophospholipids, fatty acids, and DOG) in $CHCl_3$ and/or radiolabeled lipids as supplied by the manufacturer in toluene/ethanol (1:1) were mixed together in a small glass tube.

Table I

expt	description of expt[a]	cpm
1	^{3}H-SAPC/PAPC (1)	A = 1732
2	^{3}H-SAPC/PAPC (1), ^{3}H-SAPC/PAPC (29)	B = 3079
3	^{3}H-SAPC/PAPC (1), PAPC (29)	2344
4	PAPC (1), ^{3}H-SAPC/PAPC (29)	554
5	^{14}C-PAPC + ^{14}C-PAPC (10)	3220
6	^{14}C-PAPC/products + ^{14}C-PAPC (10)	2500
7	^{14}C-PAPC/products + ^{14}C-PAPC/products (10)	2120
8	^{14}C-PAPC + PAPC/products (10)	1280
9	^{14}C-PAPC/products + PAPC (10)	1960
10	^{14}C-PAPC/products + PAPC/products (10)	1260

[a] All experiments were carried out in 50 μL of buffer A. Experiments 1–4 and 5–10 were conducted with 4.5 and 4.0 microunits of hmw-PLA2, respectively. In these experiments, PAPC, ^{14}C-PAPC, and ^{3}H-SAPC/PAPC designate 6 μM phospholipid per experiment. The specific activity of ^{3}H-SAPC/PAPC was approximately 100 mCi/mmol. For experiments 5–10, "products" designates the presence of 10 mol % of the reaction products in vesicles. The numbers in parentheses designate the time in minutes either between the addition of the substrates or before the quenching of the reaction with organic solvent.

The solvent was removed with a stream of N_2 and then in vacuo for 30 min. To the residue was added 100 μL of H_2O, and the samples were lyophilized to ensure the complete removal of organic solvent. In most of the experiments, the desired amount of buffer (10 mM HEPES/0.1 mM EDTA, pH 8.0) was added to the lipid film. The tube contents were mixed briefly in a vortex mixer and then sonicated in a bath-type device (Lab Supplies, Model G11 2SPIT) for about 10 s. The lipid suspension was frozen in a dry-ice bath, and then the frozen solution was sonicated for 1 min (Jain & Gelb, 1991). In some experiments where vesicles of PAPC/DOG was used, this lipid mixture was sonicated as above in 50 mM HEPES, pH 7.4. Stock solutions of sonicated vesicles were used the same day or stored at −20 °C and resonicated as above before use. Stock solutions of arachidonic acid in $CHCl_3$ were stored in an air-tight vial under argon and were used within 1 week of preparation.

Enzymatic Reactions. All enzymatic reactions were carried out in 1.5-mL polypropylene Eppendorf tubes. Unless otherwise stated, all reactions were carried out at room temperature. The reaction buffers used were either buffer A (10 mM HEPES, 0.1 mM EDTA, and 0.6 mM $CaCl_2$, pH 8.0) or buffer B (50 mM HEPES, 150 mM NaCl, 1 mM $CaCl_2$, 2 mM 2-mercaptoethanol, and 1 mg/mL BSA, pH 7.4). For reaction progress curves, the time points represent the amount of product formed in reactions carried out in separate tubes minus the control amount produced in the absence of enzyme. The detailed conditions for the experiments with hmw-PLA2 are given in the figure and table legends. Unless stated otherwise, the reactions were initiated by the addition of substrate to each reaction tube containing buffer and enzyme. The amount of enzyme used in the experiments is reported in microunits, as defined and measured previously (Gronich et al., 1990). In experiments utilizing ^{14}C-PAPC, the specific activity of this phospholipid was always 50–55 mCi/mmol.

The general procedure for measuring the amount of fatty acid product is as follows: The enzymatic reactions were stopped by the addition of 370 μL of $CHCl_3/CH_3OH$/concentrated HCl (200:100:1 by volume). In the case of experiments 1–4 in Table I, the above solvent was precooled in a dry ice/acetone bath prior to the addition of the reaction mixture. To each of the quenched samples were added sequentially 460 μL of $CHCl_3/CH_3OH/H_2O$ (1:2:0.8 by volume), 240 μL of $CHCl_3$, 240 μL of H_2O, and finally 10 μL of a dimethyl sulfoxide solution of 400 μg/mL unlabeled ar-

achidonic acid and 50 μg/mL 2,6-di-*tert*-butyl-4-methylphenol as a carrier. The contents of the tube was mixed with a vortex mixer and then centrifuged to separate the layers. The lower phase was transferred to a glass tube, and the solvent was removed with a stream of N_2 at 37 °C. To the tube was added approximately 300 μL of low-boiling petroleum ether/diethyl ether/glacial acetic acid (70:30:1 by volume). The solution was transferred to a small column of silica gel (4–5-cm bed height of Merck silica gel 60, 230–400 mesh in a glass wool-plugged Pasteur pipet). Prior to the addition of the radiolabeled sample, the silica column was washed with about 1 column volume of the same solvent. After addition of the sample, the column was washed with 5–6 mL of the same solvent, and the eluant was collected directly into a scintillation vial. Control experiments demonstrated that this washing step was more than sufficient to elute all of the arachidonic acid with no detectable elution of either phospholipid or lysophospholipid. The solvent in the vial was removed with a stream of N_2 at 37 °C, and 4 mL of scintillation fluid was added. Samples were analyzed by counting for 2 min in a Beckman Model LS1801 scintillation counter. The counting efficiencies of ^3H and ^{14}C were 0.61 and 0.95, respectively. Control reactions in which the enzyme was omitted were always conducted in parallel with the experiments. All reported cpm represent the control-corrected values, and the cpm observed in control reactions were generally less than 10% of the cpm observed in experiments with enzyme present. All experiments were checked for reproducibility by conducting them at least in duplicate and more often in triplicate except for those in Table I which were conducted 5–6 times. In all experiments, the relative error in the cpm values was less than 10%.

Vesicle Fusion Studies. Fusion of vesicles containing DOG was investigated with a protocol adapted from a previous study (Hoekstra et al., 1984). A solution of 10 μM vesicles composed of PAPC/DOG/octadecylrhodamine (Molecular Probes, Eugene, OR) (4:1:0.16 mole ratio) was mixed with an equal volume of 100 μM PAPC/DOG vesicles (4:1 mole ratio) that lacked the dye. The fluorescence was monitored by excitation at 470 nm and emission at 535 nm. Fusion of vesicles would result in an increase in the emission intensity as the dye molecules disperse among the ensemble of vesicles and the fluorescence quenching is relieved. In a second protocol based on a previous study (Struck et al., 1981), vesicle fusion was monitored by changes in the resonance energy transfer after a solution containing 100 μM vesicles of PAPC/DOG (4:1 mole ratio) was mixed with an equal volume of a solution containing 10 μM vesicles of PAPC/DOG/NBD-PE/Rh-PE (4:1:0.04:0.04 mole ratio). The fluorescence was monitored by the excitation at 560 nm and the emission at 590 nm. Fusion of vesicles would result in a decrease in the resonance energy transfer as the two different dye probes become dispersed among the ensemble of vesicles.

Preparation and Analysis of Buffers Containing Less Than 1 μM Amounts of Free Ca^{2+}. Buffers with low concentrations of free Ca^{2+} were prepared as described elsewhere (Tsien & Pozzan, 1989). Different amounts of the $K_2CaEGTA$ solution were added to a standard buffer containing 10 mM HEPES, pH 8.0, 100 mM NaCl, and 100 μM EGTA. The concentrations of free Ca^{2+} were calculated using the published dissociation constants. The free Ca^{2+} concentrations obtained were verified by measuring the fluorescence with the Ca^{2+}-specific dye fluo-3 (Molecular Probes, Eugene, OR).

Analysis of Triacylglycerol. Twelve microunits of hmw-PLA2 was incubated with 500 μL of 6 μM ^{14}C-PAPC/3 μM DOG in buffer B for 2 h at 37 °C. The lipids were extracted as described above, and the extract was analyzed by TLC (Merck, silica 60 F-254) with the solvent (low boiling petroleum ether/diethyl ether/88% formic acid 40:10:1). Under these conditions, the reference compounds (trioleoylglycerol, DOG, and arachidonic acid) were easily separated. The silica gel was scratched off the TLC plate, and the radioactivity was detected in a scintillation counter.

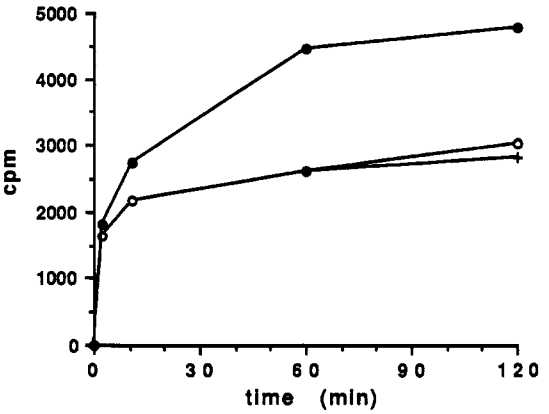

FIGURE 1: Reaction progress curve for the action of hmw-PLA2 (4 microunits) on 6 μM ^{14}C-PAPC in 50 μL of buffer A (open circles). Same as above but with a second addition of 6 μM ^{14}C-PAPC at 60 min (plus sign). Same as the curve given by open circles but with 2 mg/mL fatty acid-free BSA in the buffer (closed circles).

RESULTS

Reaction Progress Curves under Various Conditions. The reaction progress curve for the action of hmw-PLA2 on sonicated vesicles of pure ^{14}C-PAPC shows an anomalous behavior (Figure 1, open circles). A burst of activity was seen at early times, and the reaction ceased after about 10–20 min when only 5–10% of the substrate was hydrolyzed. This type of behavior was also observed with vesicles of unlabeled PAPC containing ^3H-SAPC (not shown) which demonstrates that the nonlinear curve is not unique to the ^{14}C-PAPC substrate. The reaction progress curve shown in Figure 1 is similar to those reported previously for the action of an analogous PLA2 from rat macrophages acting on vesicles of 1-*O*-hexadecyl-2-[^3H]arachidonyl-*sn*-glycero-3-phosphocholine (Leslie, 1991; Leslie & Channon, 1990; Leslie et al., 1988). The cessation of the reaction progress is not due to the instability of the enzyme over the time period of the experiments (data not shown). The possibility of nonspecific absorption of the enzyme to the walls of the reaction vessel was ruled out on the basis of the following observations. The same results were obtained when the reactions were conducted in glass or polypropylene tubes. Furthermore, identical kinetic results were obtained when the reaction was initiated by the dilution of enzyme into the mixture of substrate and buffer or when the enzyme was first diluted in buffer without substrate and allowed to sit for a variable amount of time (5–30 min) followed by transferring aliquots to separate tubes and then adding substrate to initiate the reaction.

A partial hydrolysis of the substrate may be due to a limited availability of substrate, for example, in multilamellar aggregates where only a fraction of the total substrate is accessible to the enzyme. This was ruled out by demonstrating that a maximum of about 60% of the ^{14}C-PAPC substrate was hydrolyzed after the addition of an excess amount of cobra venom PLA2 (not shown). This is the expected yield of product if the ^{14}C-PAPC is present in the form of small unilamelar vesicles (Jain et al., 1986). In addition, no additional product formation occurred following the addition of more

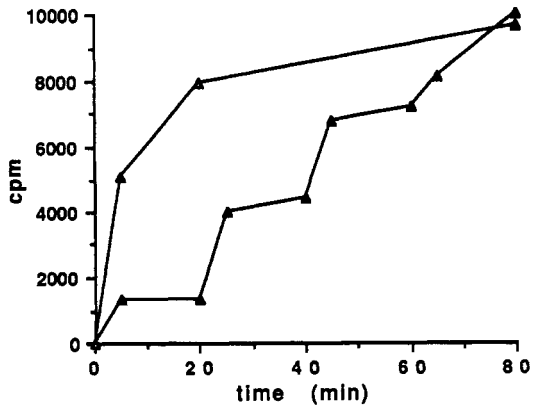

FIGURE 2: Each time point corresponds to a reaction containing 10.8 microunits of enzyme added to 6 μM ^{14}C-PAPC in 50 μL of buffer A (open triangles). Same as above except that the enzyme was added in four portions, 2.7 microunits each, at t = 0, 20, 40, and 60 min (closed triangles).

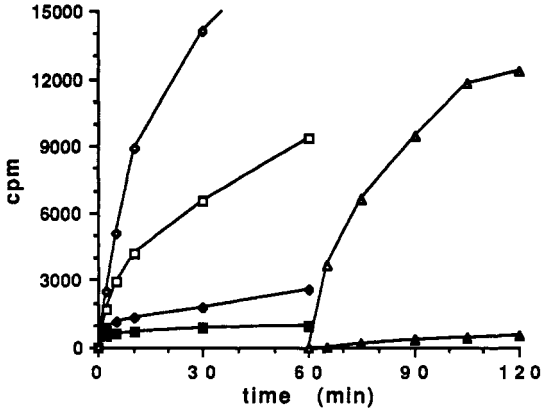

FIGURE 3: Reaction progress curve for the action of hmw-PLA2 (0.9 microunits) on 50 μL of 6 μM ^{14}C-PAPC in buffer A at 37 °C (closed squares). Same as above except that the vesicles contained either 3 μM DOG (closed diamonds), or buffer B was used (open squares), or both (open diamonds). The enzyme was first incubated in 50 μL of 6 μM unlabeled PAPC in buffer A at room temperature for 1 h. Then 50 μL of 6 μM ^{14}C-PAPC containing 3 μM DOG in buffer A was added, and the incubation was continued for another 1 h at 37 °C (closed triangles). Same as for closed triangles except the second substrate was in buffer B (open triangles).

^{14}C-PAPC to the reaction mixture with hmw-PLA2 after the initial reaction ceased (Figure 1, plus signs).

The results in Figure 2 (closed triangles) show that after an initial round of hydrolysis of ^{14}C-PAPC vesicles, addition of more hmw-PLA2 resulted in a second round of hydrolysis. The sequential addition of four equal portions of enzyme produced a similar amount of product to that formed by the addition of the same total amount of enzyme added in a single portion at the beginning [Figure 2, open triangels; see also Leslie (1991)].

The results so far can be explained by postulating that the enzyme becomes inactivated during the catalytic turnover either by irreversible modification of the enzyme or by precipitation of the enzyme together with an insoluble lipid aggregate. However, there are additional possibilities that need to be considered. The cessation of the hydrolysis can also be explained by classical competitive product inhibition by one or both of the reaction products. Another possibility is that the enzyme is operating on PAPC vesicles in the scooting mode (Jain et al., 1986). In this type of hydrolysis, the enzyme remains tightly bound to the vesicle, and therefore only a limited amount of substrate is hydrolyzed if the number of vesicles exceeds the number of enzymes. In a variation on the scooting model, the enzyme is initially able to hop from one vesicle to another. If the enzyme has a higher affinity for vesicles that contain products, it eventually becomes trapped on these vesicles, leading to a cessation in the reaction progress. These two types of processive behavior are considered under Kinetic Tests for Scooting Behavior and under Kinetic Tests for the Trapping of Enzyme on Product-Containing Vesicles.

The 85-kDa PLA2s from human platelets and U937 cells have been assayed with radiolabeled PAPC vesicles containing 33 mol % DOG in a buffer containing $CaCl_2$, BSA, NaCl, and 2-mercaptoethanol (buffer B) (Kramer et al., 1986, 1991). The addition of DOG to the vesicles is based on the observation that this lipid enhances the enzymatic activity by approximately 2–5-fold (Kramer et al., 1987). The reaction progress curve for the action of hmw-PLA2 under these conditions is markedly different than that observed in buffer A and in the absence of DOG (Figure 3, compare open diamonds to closed squares). The reaction progress curve remains linear for several minutes and starts to show curvature only after a significant fraction of the ^{14}C-PAPC has been hydrolyzed. To further examine the possibility that the limited hydrolysis of PAPC in buffer A is due to irreversible enzyme inactivation, the hmw-PLA2 was first allowed to incubate for 1 h with unlabeled PAPC in buffer A followed by the addition of an equal volume of buffer B containing ^{14}C-PAPC/DOG. As shown in Figure 3 (open triangles), the enzymatic activity was recovered following this treatment. The slope of this reinitiated reaction progress curve was only about 2-fold smaller than the slope of the reaction progress curve obtained by adding buffer B containing ^{14}C-PAPC/DOG immediately after the enzyme was added to PAPC (not shown). It is clear from this study that the complete cessation of the reaction progress seen with PAPC in buffer A is not due to substantial irreversible enzyme inactivation.

Next, a dissection of the ^{14}C-PAPC/DOG/buffer B assay was carried out in order to determine the component(s) necessary for the stimulated and prolonged reaction progress. In the absence of DOG, the hydrolysis of ^{14}C-PAPC in buffer B was markedly stimulated compared to the hydrolysis of the same vesicles in buffer A (Figure 3, compare open and closed squares). The hydrolysis of ^{14}C-PAPC vesicles containing 33 mol % DOG in buffer A showed a less significant stimulation (Figure 3, closed diamonds); however, in the presence of DOG, the enzymatic reaction did not show the cessation seen with ^{14}C-PAPC alone. The largest stimulation was seen when both DOG and buffer B were used (Figure 3, open diamonds). In this case, omission of 2-mercaptoethanol from buffer B did not change the reaction progress curve, but omission of either BSA or NaCl led to a significant decrease in the initial slope of the reaction (not shown), indicating that both of these components stimulate the enzymatic reaction. After the hmw-PLA2 acted on unlabeled PAPC vesicles for 1 h, the addition of ^{14}C-PAPC/DOG vesicles without buffer B led to only a small fraction of the re-initiation in the enzymatic reaction that was seen when both ^{14}C-PAPC/DOG and buffer B were added (Figure 3, compare closed and open triangles).

Another possible explanation for the cessation of the reaction progress on ^{14}C-PAPC in buffer A is the irreversible inactivation of the hmw-PLA2 by covalent modification by either substrate or fatty acid product. Addition of DOG to the substrate may offer an alternative acceptor group for acylation and therefore may prevent inactivation by a putative fatty acyl linkage to the enzyme. This possibility was examined by allowing the enzyme to act on ^{14}C-PAPC/DOG in buffer B and to analyze an extract of the reaction mixture by TLC for radiolabeled triacylglycerol. No radioactivity was detected

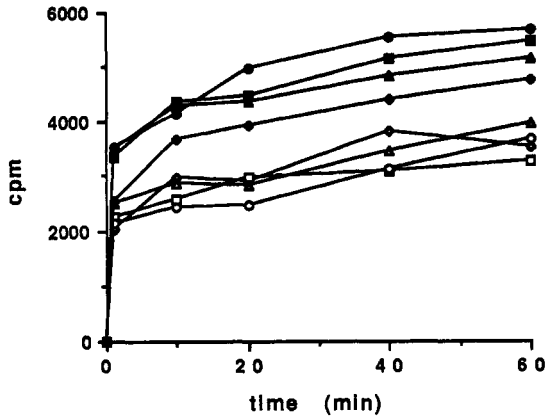

FIGURE 4: Each time point corresponds to the action of 4 microunits of hmw-PLA2 on different concentrations of substrate vesicles with or without added reaction products (1:1 mixture of 1-palmitoyl-*sn*-glycero-3-phosphocholine + arachidonic acid) in 50 μL of buffer A: 3 μM ^{14}C-PAPC (closed diamonds); 6 μM ^{14}C-PAPC (closed triangles); 12 μM ^{14}C-PAPC (closed squares); 24 μM ^{14}C-PAPC (closed circles); 3 μM ^{14}C-PAPC + 10 mol % reaction products (open diamonds); 6 μM ^{14}C-PAPC + 10 mol % reaction products (open triangles); 12 μM ^{14}C-PAPC + 10 mol % reaction products (open squares); 24 μM ^{14}C-PAPC + 10 mol % reaction products (open circles).

in the region of the TLC where the reference trioleoylglycerol migrated. This result strongly suggests that the role of the DOG is not to remove an acyl-linked fatty acid from the enzyme.

Further evidence against a turnover-dependent inactivation of the hmw-PLA2 stems from the observation that the extent of the reaction was increased by inclusion of BSA (2 mg/mL) in buffer A (Figure 1, closed circles). In addition, the results in Figure 4 (upper four curves) show that the extent of reaction became larger as the concentration of the ^{14}C-PAPC vesicles was increased from 3 to 24 μM in buffer A. Such results are not expected if the enzyme undergoes an inactivation event for every fixed number of turnover cycles.

Kinetic Tests for Scooting Behavior. Next, the possibility of the enzyme operating on PAPC vesicles in the highly processive scooting mode was examined. If the enzyme is able to absorb irreversibly to the substrate interface, it will remain bound to the vesicles that it first encounters and will be unable to operate on a population of vesicles that are added at some time later. This model is consistent with the observations that addition of fresh enzyme but not fresh substrate led to the onset of further vesicle hydrolysis (Figures 1 and 2). Such behavior has been previously documented for the action of a number of 14-kDa PLA2s acting on vesicles composed of anionic phospholipids (Jain et al., 1991a). To investigate this, vesicles of unlabeled PAPC and ^{3}H-SAPC/PAPC were used. If the enzyme is operating in a perfectly processive fashion, addition of enzyme to PAPC followed by the addition of ^{3}H-SAPC/PAPC will not yield any radiolabeled product. Likewise, addition of enzyme to ^{3}H-SAPC/PAPC followed by the addition of PAPC will result in the production of the same amount of radioactive product that is formed when the enzyme is added to ^{3}H-SAPC/PAPC alone. In this experimental approach, it is reasonably assumed that the phospholipid molecules do not undergo intervesicle exchange (Cevc & Marsh, 1987).

In order to interpret these experiments, it is necessary to determine the amounts of radioactive product formed both before and after the addition of the second substrate. The amount of radioactive product in these two time periods was obtained by using ^{3}H-SAPC/PAPC alone and stopping the enzymatic reaction after 1- and 30-min incubations. The amounts of cpm formed after 1- and 30-min periods are designated by *A* and *B*, respectively. In the experiment in which enzyme is incubated with ^{3}H-SAPC/PAPC for 1 min followed by incubation with PAPC for an additional 29 min, the expected cpm of product formed if the enzyme operates in a perfectly processive mode will be *B*. Likewise, if the enzyme is first added to PAPC and ^{3}H-SAPC/PAPC is added last, zero cpm will be produced. It can be seen in Table I (experiments 1–4) that this is not the case.

If the enzyme operates in a completely hopping mode, equal amounts of product will come from the PAPC and ^{3}H-SAPC/PAPC vesicles during the second time period in which both are present in equal amounts. Under such conditions, the amount of cpm produced in experiments 3 and 4 of Table I will be $(B + A)/2$ and $(B - A)/2$, respectively. Using the experimental values of *A* and *B* (experiments 1 and 2, Table I), the calculated cpm values for experiments 3 and 4 of 2405 and 673 compare well to the experimental values of 2344 and 554. These results establish that after incubation of the hmw-PLA2 with PAPC vesicles for 1 min, the enzyme has little tendency to remain bound to these vesicles.

The dependency of the reaction extent on the concentration of pure ^{14}C-PAPC vesicles (upper four curves in Figure 4) also supports the notion that the enzyme is not scooting on these vesicles. If the enzyme binds irreversibly to ^{14}C-PAPC vesicles and if the number of vesicles is at least 3–5-fold larger than the number of enzymes so that there are very few vesicles with more than one enzyme bound, the extent of the reaction will be independent of the concentration of vesicles. Because of the limited amount of purified hmw-PLA2 available, it is difficult to get an accurate estimate of the amount of enzyme per experiment. However, if the number of enzymes is comparable or exceeds the number of vesicles, the extent of reaction would be more sensitive to the concentration of vesicles than that shown in Figure 4 (top four curves). Furthermore, the fraction of available substrate hydrolyzed would be much larger than the 5–10% seen in Figures 1 and 4.

Kinetic Tests for the Trapping of Enzyme on Product-Containing Vesicles. The results described so far establish that the cessation of the reaction progress on vesicles of PAPC is not due to the limited availability of substrate, enzyme inactivation, or scooting on PAPC vesicles. A number of observations suggested that the enzymatic reaction products (arachidonic acid or lysophospholipid, or both) played a role in inducing a limited extent of vesicle hydrolysis. The increase in the extent of the reaction by the addition of BSA to buffer A (Figure 1, closed circles) may be due to the removal of the products from the vesicles since this protein is known to bind fatty acids and lysophospholipids (Peters, 1986). The possibility that one or both of the reaction products are functioning as tight-binding competitive inhibitors of the hmw-PLA2 was examined by preparing vesicles of ^{14}C-PAPC that were cosonicated with 10 mol % each of arachidonic acid and 1-palmitoyl-2-*sn*-glycero-3-phosphocholine. The reaction progress curves for the hydrolysis of these product-containing vesicles were studied, and the results are presented in Figure 4 (bottom four curves). It can be seen that the initial velocities for the hydrolysis of product-containing vesicles are comparable to the initial velocities measured with pure ^{14}C-PAPC vesicles. These results strongly suggest that the enzymatic reaction products are not functioning as classical tight-binding competitive inhibitors.

The accumulation of products may lead to a cessation in the reaction progress if the hmw-PLA2 has a higher affinity

for product-containing vesicles than that for pure PAPC vesicles. Under such conditions, the enzyme may be hopping from one PAPC vesicle to another at early times in the reaction progress, but as time goes on, an increasing fraction of the enzyme will get trapped on product-containing vesicles, resulting in the termination of the reaction. In experiments 5–10 (Table I), various types of vesicles, either PAPC or ^{14}C-PAPC, both with or without 10 mol % reaction products, were premixed. The enzyme was added last, and the reaction was stopped after a 10-min incubation. The results of experiments 5–7 show that the cpm produced after 10 min decreased as the fraction of product-containing vesicles was increased even though the total lipid concentration was the same in these experiments. This effect can also be seen when the reaction progress curves on product-containing vesicles are compared to those for pure ^{14}C-PAPC vesicles (Figure 4, compare upper four curves to the lower four curves) and will be discussed further below. The cpm produced in experiment 8 in which the enzyme was added to a mixture of ^{14}C-PAPC and PAPC vesicles containing 10 mol % products were significantly less than those observed in experiment 9 in which the products were present in the ^{14}C-PAPC vesicles but not in the PAPC vesicles. This clearly shows that a larger fraction of the enzyme is bound to product-containing vesicles than to pure PAPC vesicles.

Although the data in Table I (experiments 1–4) establish that the enzyme is hopping initially in the reaction progress, this hopping cannot proceed at longer times since the enzymatic hydrolysis eventually stops when only a small fraction of the vesicles have been hydrolyzed (Figures 1 and 4). The results in Table I (experiments 5–10) suggest that the enzymatic reaction may stop due to the trapping of the enzyme on product-containing vesicles. In this model, the dependency of the extent of reaction on the concentration of ^{14}C-PAPC vesicles (Figure 4, upper four curves) may be due to the following reason. It is expected that the average mole percent of products in the vesicles will be lower if the number of vesicles is higher in the reaction mixture. In this case, the higher number of enzymatic turnovers required to produce enough product to trap the enzyme leads to an increase in the observed reaction extent as the concentration of substrate is increased. The presence of reaction products in the vesicles at time zero lowers the number of turnovers required for trapping. Furthermore, the extent of reaction will be less sensitive to the concentration of the product-containing vesicles. All of these features were observed as shown by the lower four curves in Figure 4.

Trapping of Enzyme on Product-Containing Vesicles in the Absence of Catalytic Turnover. Although the above experiments are consistent with the product-trapping model, the effects of product addition to the vesicles or the generation of product due to enzymatic turnover are hard to separate. Therefore, the possible trapping of enzyme by product was investigated by using vesicles composed of phospholipids that are not hydrolyzed by the hmw-PLA2. Since the enzyme has been reported to prefer phospholipids that have arachidonyl at the 2-position (Clark et al., 1990, 1991; Diez & Mong, 1990; Kim et al., 1991b; Leslie, 1991; Wijkander & Sundler, 1989), the hydrolysis of vesicles composed of pure ^{14}C-DPPC and ^{14}C-DPPC containing 10 mol % each arachidonic acid and 1-palmitoyl-*sn*-glycero-3-phosphocholine was examined. With both types of vesicles, no radioactive palmitate (less than 100 cpm more than background) was detected after a reaction time of 20 min using 4.5 microunits of hmw-PLA2. Only very small amounts of radioactive arachidonic acid were produced from unlabeled DPPC vesicles containing 0.05 mol % ^{3}H-SAPC

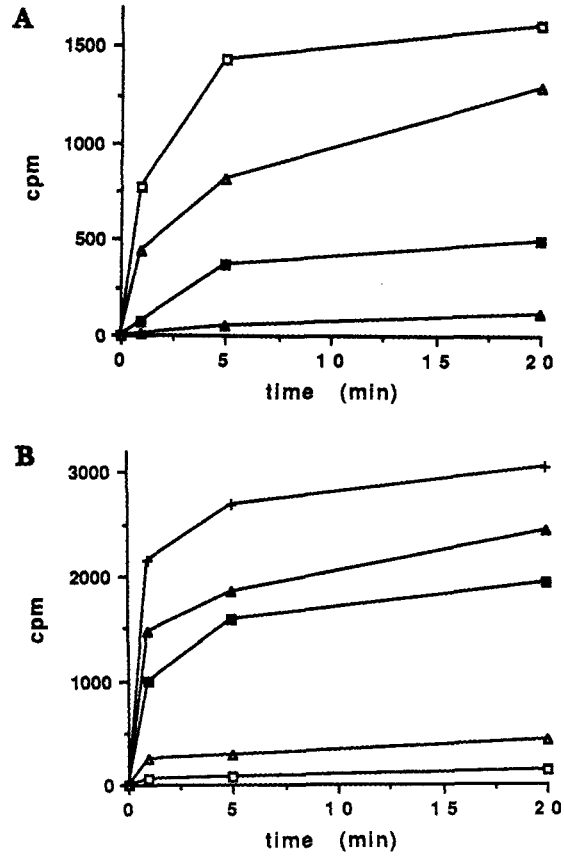

FIGURE 5: (A) Each time point corresponds to the action of 3 microunits of hmw-PLA2 on 6 μM phospholipid substrate vesicles containing ^{3}H-SAPC (specific activity of 50 mCi/mmol based on total phospholipid present) with or without reaction products (1:1 mixture of 1-palmitoyl-*sn*-glycero-3-phosphocholine + arachidonic acid) in 50 μL of buffer A: DPPC/^{3}H-SAPC (closed triangles); DPPC/^{3}H-SAPC + 10 mol % products (open triangles); OPPC/^{3}H-SAPC (closed squares); OPPC/^{3}H-SAPC + 10 mol % products (open squares). (B) Three microunits of hmw-PLA2 was first added to 50 μL of buffer A containing 6 μM either OPPC or DPPC vesicles with or without 10 mol % reaction products. After 1 min, 5 μL of a stock solution of 60 μM ^{14}C-PAPC was added, and the reactions were quenched after 20 min. Enzyme preincubated with DPPC (closed triangles); enzyme preincubated with DPPC + 10 mol % products (open triangles); enzyme preincubated with OPPC (closed squares); enzyme preincubated with OPPC + 10 mol % (open squares). The reaction progress curve for the action of the same amount of enzyme on 6 μM ^{14}C-PAPC in the absence of unlabeled vesicles is shown by the plus signs.

(specific activity of 50 mCi/mmol based on total lipid). The amount of ^{3}H-product increased dramatically (12-fold) if the vesicles contained 10 mol % of the products mentioned above (Figure 5A, closed and open triangles). Similar results were observed with vesicles of OPPC containing ^{3}H-SAPC except that even in the absence of added products there was a considerable amount of ^{3}H-arachidonate produced (Figure 5A, closed and open squares). These results reinforce the notion that the PAPC reaction products are not functioning as tight-binding competitive inhibitors of hwm-PLA2. This apparent activation of ^{3}H-SAPC hydrolysis by products may be due to an increase in the fraction of hmw-PLA2 bound to the vesicles.

In order to investigate this further, the ability of product-containing DPPC or OPPC vesicles to trap the enzyme was studied in the following way. The enzyme was first added to DPPC or OPPC vesicles either with or without 10 mol % products. Then, vesicles of ^{14}C-PAPC were added, and the amount of ^{14}C-product was determined after various incubation times. The results in Figure 5B show that the product-con-

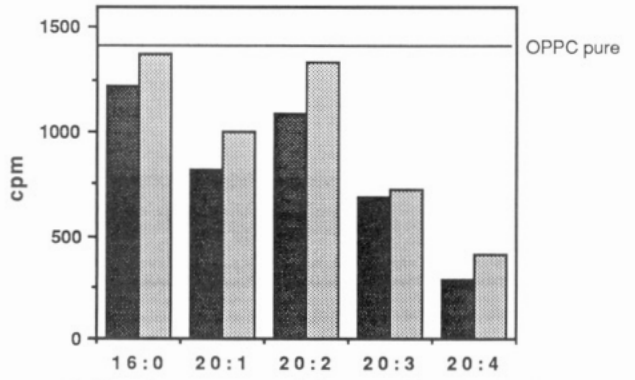

FIGURE 6: Experiments were carried out exactly as described in Figure 5B except that arachidonic acid was replaced by the various fatty acids shown. Shown is the trapping on OPPC vesicles with 10 mol % fatty acid + 1-palmitoyl-*sn*-glycero-3-phosphocholine (dark bars) and with 10 mol % fatty acid and 1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine (light bars). The fatty acids used were as follows: 16:0, palmitic; 20:1, *cis*-11-eicosenoic; 20:2, *all-cis*-11,14-eicosadienoic; 20:3, *all-cis*-8,11,14-eicosatrienoic; 20:4, *all-cis*-5,8,11,14-eicosatetraenoic (arachidonic). The horizontal line shows the cpm produced after preincubation of the enzyme as above on OPPC vesicles without added products.

taining vesicles (open triangles and squares) are able to efficiently trap the enzyme so that the amount of product produced from the ^{14}C-PAPC vesicles is barely detectable. The enzyme has not become inactivated under these conditions since addition of buffer B along with ^{14}C-PAPC/DOG vesicles after the enzyme was incubated with OPPC/product vesicles for 20 min led to the immediate reinitiation of the reaction progress (not shown). In marked contrast, enzyme is not trapped well on vesicles of DPPC or OPPC without products (Figure 5B, closed triangles and squares). These results strongly suggest that the role of the reaction products in promoting the hydrolysis of ^{3}H-SAPC in OPPC or DPPC vesicles (Figure 5A) is to enhance the binding of the enzyme to the vesicles. Since it has already been shown that there was no detectable hydrolysis of DPPC by the hmw-PLA2, this fact and the results in Figure 5B establish that product trapping of enzyme on vesicles can occur in the absence of catalytic turnover.

Fatty Acid Specificity of Product Trapping. Besides arachidonic acid, a variety of different fatty acids together with either 1-palmitoyl-*sn*-glycero-3-phosphocholine or 1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine in OPPC vesicles were tested as enzyme traps. Experiments involving the addition of fatty acid or lysophospholipid alone were not attempted since it is generally found that these lipids in the absence of the other can undergo intervesicle exchange (Cevc & Marsh, 1987; Jain et al., 1982a; Micol et al., 1990). The results of these experiments are summarized in Figure 6. It can be seen that all of the 20-carbon unsaturated fatty acids together with either of the 2 different lysophospholipids promoted some degree of enzyme trapping and that arachidonic acid was the most potent. The ability to function as a trapping agent is markedly influenced by minor structural changes of the fatty acid. For example, 8,11,14-eicosatrienoic acid, which differs from arachidonic acid only by the absence of a double bond in the C-5 position, was significantly less potent than arachidonic acid as a trapping agent. OPPC vesicles containing lysophospholipid and the saturated 16-carbon palmitic acid produced no significant trapping. These results also serve to illustrate that the trapping does not require the 1-acyl linkage in the lysophospholipid since a similar degree of trapping was seen when the 1-*O*-alkyllysophospholipid was present. This point is noteworthy because the 85-kDa PLA2 from rat macrophages

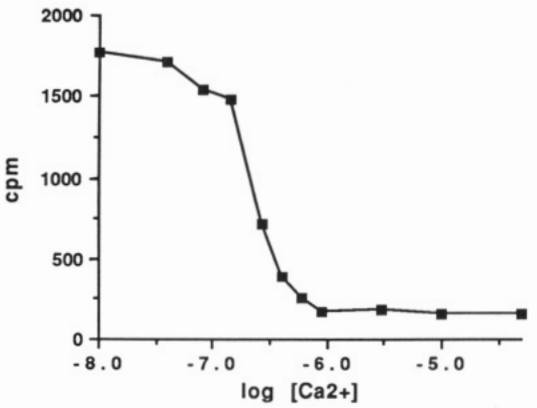

FIGURE 7: Enzyme (2.5 microunits) was incubated for 1 min with 6 μM OPPC containing 10% products (1:1 mixture of arachidonic acid + 1-palmitoyl-*sn*-glycero-3-phosphocholine) in 45 μL of buffer containing the indicated amounts of free calcium ions (see Experimental Procedures). Then, 5 μL of 60 μM ^{14}C-PAPC was added, and after an additional 1 min, 1 μL of 25 mM $CaCl_2$ was added to bring the Ca^{2+} concentration to approximately 0.5 mM. After 20 min, the reaction was quenched and analyzed for products.

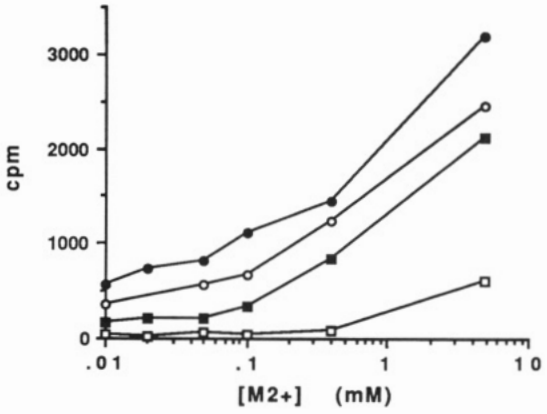

FIGURE 8: Enzyme (2.5 microunits) was added to 50 μL of calcium-free buffer A containing 6 μM ^{14}C-PAPC and the indicated concentrations of metal ions in concentrations excess to the EGTA. After 20 min at room temperature, the amount of radioactive product was analyzed. The following metals were used $CaCl_2$ (closed circles); $SrCl_2$ (open circles); $BaCl_2$ (closed squares); $MgCl_2$ (open squares).

has detectable lysophospholipase activity (Leslie, 1991). Thus, the trapping of enzyme does not involve the lysophospholipase activity of the hmw-PLA2.

Divalent Metal Requirement for Product Trapping. The requirement for calcium in the trapping of enzyme on product-containing vesicles (Figure 5B) was investigated by mixing the hmw-PLA2 with OPPC vesicles containing 10 mol % arachidonic acid and 1-palmitoyl-*sn*-glycero-3-phosphocholine in buffer with various amounts of free calcium. This was followed by addition of ^{14}C-PAPC vesicles and sufficient calcium to give a concentration of approximately 500 μM. After incubation for 20 min, the amount of ^{14}C-product was determined. In these experiments, concentrations of calcium of 5 μM or less were controlled by using an EGTA/calcium buffer as described under Experimental Procedures. Higher calcium concentrations (10–500 μM) were obtained by adding the appropriate amount of $CaCl_2$ in excess of the EGTA. The results in Figure 7 show that the trapping of hmw-PLA2 on OPPC/product vesicles requires calcium and that complete trapping of enzyme occurs with free calcium concentrations of about 400 nM or higher.

The dependency of the enzymatic activity on the Ca^{2+} ion concentration is shown in Figure 8 (closed circles). In these experiments, the metal ion concentrations in the range of 10

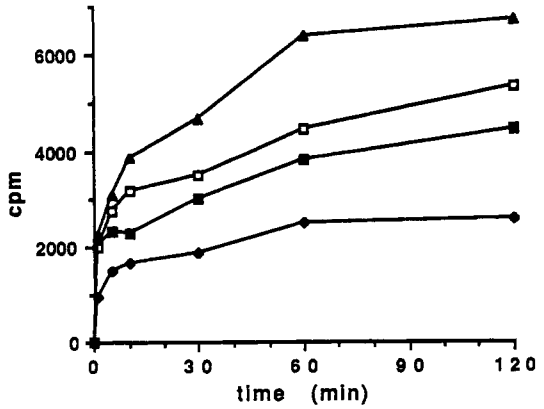

FIGURE 9: Reaction progress curves for the action of 4.5 microunits of hmw-PLA2 on 6 μM ^{14}C-PAPC in 50 μL of buffer A in the presence of 0 mM $(NH_4)_2SO_4$ (closed diamonds), 100 mM $(NH_4)_2SO_4$ (closed squares), 200 mM $(NH_4)_2SO_4$ (open squares), and 400 mM $(NH_4)_2SO_4$ (closed triangles).

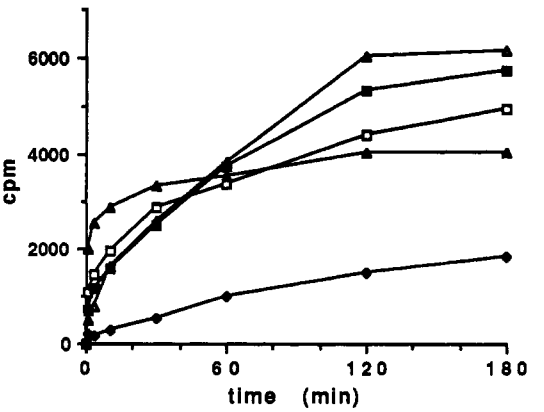

FIGURE 10: Reaction progress curves for the action of 4.5 microunits of hmw-PLA2 on vesicles of 6 μM ^{3}H-SAPC/PAPC (50 mCi/mmol based on total lipid) containing various amounts of POPM in 50 μL of buffer A: pure ^{3}H-SAPC/PAPC (closed triangles); 20 mol % POPM in ^{3}H-SAPC/PAPC (open squares); 40 mol % POPM in ^{3}H-SAPC/PAPC (open triangles); 60 mol % POPM in ^{3}H-SAPC/PAPC (closed squares); ^{3}H-SAPC/POPM (closed diamonds).

μM–5 mM were obtained by using the desired amount of metal salt in excess of the EGTA concentration. The activity versus concentration of calcium profile is essentially identical to that reported previously (Clark et al., 1990; Diez & Mong, 1990; Gronich et al., 1990; Kim et al., 1991b; Kramer et al., 1991; Krause et al., 1991; Leslie et al., 1988). An onset of activity was seen with calcium concentrations between 0 and 10 μM followed by a further rise in the activity as the metal ion concentration was increased above 50 μM.

Other divalent metal ions were tested for their abilities to promote enzymatic activity of the hmw-PLA2 and to allow the enzyme to become trapped on product-containing vesicles. The results are summarized in Figure 8. In all of these experiments, the metal ion concentrations given along the abscissa in Figure 8 represent the concentration in excess of the 100 μM EGTA present in the buffers. Barium and strontium ions both proved to serve as cofactors in activating hmw-PLA2 to hydrolyze ^{14}C-PAPC whereas magnesium ions were markedly less active. The activity obtained with 10 μM Ca^{2+} was reached by using 100 μM Sr^{2+}, 400 μM Ba^{2+}, or 5 mM Mg^{2+}. Activation of the enzyme by the presence of trace amounts of Ca^{2+} was ruled out by the fact that the results in Figure 8 were obtained by using highly purified metal salts (>99.995%) and essentially identical results were obtained by using metal salts of lower purity (99%). In addition, the stability constant of the EGTA–Ca^{2+} complex is 10^2-fold higher than that for Ba^{2+} and Sr^{2+} and 10^5-fold higher than that for Mg^{2+}.

Next, these metal ions were examined for their ability to promote the trapping of the enzyme on OPPC vesicles containing 10 mol % reaction products. $BaCl_2$ and $SrCl_2$ produced considerable trapping at a concentration of 10 μM whereas a much higher concentration of $MgCl_2$ was required. The exact concentrations of these metals required for trapping were not obtained since well-defined buffers for these metals are not readily available.

Effect of Salt and Anionic Phospholipids on the Reaction Progress Curve. As shown in Figure 9, the enzymatic activity on ^{14}C-PAPC vesicles was markedly influenced by the addition of $(NH_4)_2SO_4$ in the range of 100–400 mM. As in the absence of salt, the reaction progress stopped after only a small fraction of the available substrate became hydrolyzed; however, the reaction extent increased with increasing salt concentration. The results in Figure 10 show that the reaction progress curve was influenced by the addition of the negatively charged phospholipid POPM to the ^{14}C-PAPC vesicles. The addition of POPM produced two effects on the reaction progress curve. The initial enzymatic velocity decreased as the mole percent POPM increased. Despite this inhibitory effect, the addition of POPM (20–60 mol %) to the vesicles prolonged the reaction progress so that the amount of product formed eventually increased beyond that seen with pure ^{14}C-PAPC vesicles. Addition of 1,2-dioleoyl-*sn*-glycero-3-phosphate produced effects similar to that seen with POPM (data not shown).

Discussion

The present study was initiated in order to understand the reason(s) for the cessation in the reaction progress observed when the 85-kDa class of PLA2 operates on sonicated vesicles of PAPC in buffer A [Figure 1 and Leslie (1991) and Leslie et al. (1988)]. Although some of the data are consistent with turnover-dependent enzyme inactivation [see Reaction Progress Curves under Various Conditions and Leslie (1991)], such a conclusion could not be substantiated in additional studies (see Reaction Progress Curves under Various Conditions and Figures 3 and 4).

The possibility that the hmw-PLA2 catalyzes the hydrolysis of vesicles in the scooting mode (Jain & Berg, 1989; Berg et al., 1991; Jain et al., 1986) has not been previously considered. A detailed analysis (See Kinetic Tests for Scooting Behavior, experiments 1–4 in Table I, and the top four curves in Figure 4) revealed that the enzyme was not operating in the scooting mode soon after it was added to the reaction mixture. Therefore, scooting on PAPC vesicles cannot be the reason for the cessation of the reaction progress. In addition, the results in Figure 4 (bottom four curves) suggest that the reaction products do not function as tight-binding competitive inhibitors of hmw-PLA2.

On the basis of all of the results presented in this study, the most likely explanation for the anomalous progress curve (Figure 1) is that the hmw-PLA2 becomes trapped on product-containing vesicles. This model is consistent with the results in Table I (experiments 5–10 and see Kinetic Tests for the Trapping of Enzyme on Product-Containing Vesicles) which establish that hmw-PLA2 has a higher affinity for product-containing vesicles than for pure PAPC vesicles. Interestingly, the extent of the reaction increased slightly as the concentration of PAPC vesicles was increased, but this was not observed with the vesicles containing 10 mol % product initially (Figure 4). Such a result demonstrates that the enzyme is not scooting on pure PAPC vesicles (see Kinetic Tests for Scooting Behavior) but is consistent with the product-

trapping model (see Kinetic Tests for the Trapping of Enzyme on Product-Containing Vesicles). The experiments under Trapping of Enzyme on Product-Containing Vesicles in the Absence of Catalytic Turnover and in Figure 5 provide the strongest demonstration of product trapping. These data establish that the reaction products have a dual role; they function as apparent activators by promoting the binding of enzyme to the interface and as apparent inhibitors by not allowing the enzyme to hop among the ensemble of vesicles.

The reason for the observed arachidonic acid specificity of enzyme trapping (see Fatty Acid Specificity of Product Trapping and Figure 6) is not known, and further work will be needed to better understand this phenomenon. Interestingly, Ca^{2+} ions are needed to induce enzyme trapping (Divalent Metal Requirement for Product Trapping, Figure 7). Using EGTA buffers to control the free calcium concentration, it was shown that approximately 400 nM Ca^{2+} was sufficient to cause the enzyme to bind tightly to OPPC vesicles containing product (Figure 7). Furthermore, the results in Figure 8 confirm earlier reports that the enzymatic activity of the 85-kDa PLA2s shows a biphasic dependency on the concentration of Ca^{2+} (Clark et al., 1990; Diez & Mong, 1990; Gronich et al., 1990; Kim et al., 1991b; Kramer et al., 1991; Wijkander & Sundler, 1989). With low metal ion concentrations, the onset of enzymatic activity occurs with a K_{Ca} of about 500 nM. The observation of similar K_{Ca}'s for enzymatic activity and interfacial binding suggests that the role of the metal ion is to promote the binding of the hmw-PLA2 to the substrate interface rather than to function as a cofactor required for the lipolysis. This suggestion is also supported by the observations that Ba^{2+}, Sr^{2+}, and Mg^{2+} were able to substitute for Ca^{2+} in promoting the enzymatic activity and the trapping of enzyme on OPPC/product vesicles. Furthermore, the concentrations of divalent cations required for the enzymatic activity correlated well with the concentrations required for product trapping (data not shown). It has been recently shown that even high concentrations (2 M) of NaCl can activate the cytosolic PLA2 in sheep platelets (Zupan et al., 1991). These results are in marked contrast to the observations that other divalent metals cannot substitute for Ca^{2+} as an essential cofactor of the low molecular weight PLA2s (Dennis, 1983; Verheij et al., 1981). In these cases, it is suggested that the Ca^{2+} plays a role in binding the transition-state form of the substrate (Jain et al., 1991b; Scott et al., 1990; Volwerk et al., 1974).

The extent of hydrolysis of PAPC vesicles increased when $(NH_4)_2SO_4$ was added to buffer A (see Effect of Salt and Anionic Phospholipids on the Reaction Progress Curve and Figure 9), which is consistent with the idea that ionic interactions between the enzyme and the product-containing vesicles are responsible for the cessation of the reaction progress. Thus, the trapping of enzyme would require a higher mole percent of products to accumulate in the vesicles, and this would lead to an increase in the reaction extent. Furthermore, the high concentration of NaCl present in buffer B contributed to the prolonged enzymatic reaction seen with the PAPC/DOG assay. In addition, the divalent metals Ca^{2+}, Ba^{2+}, Sr^{2+}, and Mg^{2+} at concentrations of 0.1–5 mM all led to a similar increase in enzymatic activities (Figure 8). These results suggest that the metal ions are functioning in a similar fashion as $(NH_4)_2SO_4$ by competing with the hmw-PLA2 for the interfacial binding to product-containing vesicles.

Interestingly, the addition of the anionic phospholipid POPM to ^{3}H-SAPC/PAPC vesicles led to a decrease in the initial velocity and an increase in the extent of the reaction (see Effect of Salt and Anionic Phospholipids on the Reaction Progress Curves and Figure 10). Further studies are needed to determine whether the lower initial enzymatic velocities in POPM-containing vesicles are due to competitive inhibition by POPM or to a reduced binding affinity of the hmw-PLA2 to POPM-containing interfaces. In a previous study, it was shown that anionic phospholipids such as phosphatidic acid and phosphatidylinositol activated the 85-kDa PLA2 from rat macrophages (Leslie & Channon, 1990). The results in Figure 10 show that the addition of anionic phospholipids to PAPC vesicles would give the impression of enzyme activation in a single time point taken late in the reaction progress. However, it is premature to conclude that anionic phospholipids function as specific activators of hmw-PLA2. The observed effect may very well be the result of the poorer trapping of enzyme by product when anionic lipids are present in the interface. In addition, it is also possible that the mixed POPM/PAPC vesicles are somewhat larger than pure PAPC vesicles and this may lead to a larger reaction extent.

From the present results, it is difficult to fully understand the molecular details of the trapping of enzyme on product-containing vesicles. To accomplish such a task would require knowledge of the fraction of the substrate that becomes hydrolyzed in an enzyme-containing vesicle. This information can only be obtained when larger amounts of pure hmw-PLA2 become available so that the absolute amount of enzyme in the experiment is precisely known. Furthermore, a full analysis of the reaction progress curves with respect to product-trapping requires a knowledge of the distribution of the mole percent of product present in the ensemble of vesicles as a function of time. It is for this reason that the analysis of interfacial catalysis by PLA2s in the hopping mode is fraught with difficulties (Jain & Berg, 1989). On the basis of these arguments, it is difficult to fully understand the data in Figure 2 which show that the addition of more enzyme to the PAPC vesicles leads to a new round of substrate hydrolysis. One possibility is that the rate constant for the binding of hmw-PLA2 to the vesicles is not very sensitive to the mole percent of products in the vesicles and that the dissociation of enzyme from vesicles with high product mole percent is slower than that from vesicles of pure PAPC. Under such circumstances, the newly added enzyme would catalyze a new round of lipolysis as it randomly binds to the various types of vesicles present, and it becomes trapped only when it encounters a vesicles containing a high mole percent of products. This would be true with consecutive additions of enzyme (Figure 2) as long as the probability of finding a vesicle with a high mole percent of products is relatively small. The data in Figure 2 can also be explained if most of the product produced in the first round of hydrolysis is part of an enzyme–product complex. In this way, the portions of enzyme added consecutively are prevented from binding to the product already present in this complex. The structure of this enzyme–product complex could involve a "patch" of segregated product molecules that are in direct contact with a bound enzyme.

It is interesting to note that both protein kinase C and the hmw-PLA2 contain a Ca^{2+}-dependent lipid binding domain (Clark et al., 1991; Coussens et al., 1986; Sharp et al., 1991) and the former enzyme has recently been shown to induce the Ca^{2+}-dependent segregation of acidic phospholipids when it binds to membranes (Bazzi & Nelsestuen, 1991). In addition, it has been shown that the pig pancreatic PLA2 binds to segregated patches of fatty acids that form in vesicles of phosphatidylcholine near the phase transition temperature of the phospholipid (Jain et al., 1989). The latency period ob-

served in the pig pancreatic PLA2-catalyzed hydrolysis of phosphatidylcholine vesicles is due to the increase in binding affinity of the enzyme to the vesicles as the mole percent fatty acid builds up in the vesicles (Apitz-Castro et al., 1982). The enzyme from *Naja melanoleuca* venom binds more tightly than the pig enzyme to phosphatidylcholine vesicles in the absence of products, and no latency period is observed in the reaction progress curve with the cobra enzyme (Jain et al., 1982b). The absence of an observed latency in the hydrolysis of PAPC vesicles by hmw-PLA2 (Figure 1) suggests that this enzyme is also able to bind reasonably tightly to the zwitterionic vesicles; however, the results in Table I (experiments 1–4) demonstrate that this binding is not irreversible.

The role of DOG in prolonging the reaction progress (Figure 3) is not yet understood. It may enhance the activity of the 85-kDa PLA2 in cells that generate diacylglycerols in response to external stimuli (Kramer et al., 1987). However, the activation by DOG is probably due to a nonspecific effect of this lipid on the phase behavior of the mixed phospholipid vesicles. The possibility of DOG-promoted fusion of vesicles was considered since this would lead to an apparent enzyme activation by allowing the enzyme that is trapped on product-containing vesicles to come in contact with nonhydrolyzed PAPC vesicles. Furthermore, diacylglycerols are known to induce a bilayer-to hexagonal-phase transition when present in vesicles (Sen et al., 1982), and this may promote fusion. However, when the fusion of PAPC vesicles containing 20 mol % DOG was examined with two protocols described under Experimental Procedures, no fusion was observed over a 10–20-min period. It seems more likely that the DOG somehow alters the ability of the products to induce the trapping of enzyme. For example, if the enzyme trapping is due to the segregation of anionic fatty acids, the DOG may act to prevent these arrangements of the products from occurring.

It is clear from the present study that the PAPC/buffer A assay system for measuring the activity of the hmw-PLA2 is not suitable for the quantitative analysis of activators, inhibitors, and substrate preferences. It has already been mentioned that the activation by DOG and anionic phospholipids is probably not due to a specific interaction of these additives with the enzyme. In studies with the 14-kDa class of PLA2s, the advantages of studying the enzyme in the scooting mode have been developed in detail (Berg et al., 1991; Ghomashchi et al., 1991; Jain & Gelb, 1991). The present study suggests that the hmw-PLA2 hydrolyzes ^{3}H-SAPC present in OPPC/product vesicles without leaving the interface. This type of analysis may lead to a full appreciation of the catalytic properties of the 85-kDa class of PLA2s.

Registry No. PLA2, 9001-84-7; PAPC, 35418-58-7; SAPC, 35418-59-8; DOG, 24529-88-2; DPPC, 63-89-8; OPPC, 59491-62-2; POPM, 100295-16-7; 20:4, 506-32-1; 16:0, 57-10-3; 20:1, 5561-99-9; 20:2, 5598-38-9; 20:3, 1783-84-2; Ca, 7440-70-2; Mg, 7439-95-4; Sr, 7440-24-6; Ba, 7440-39-3; 1-palmitoyl-2-*sn*-glycero-3-phosphocholine, 98703-68-5; 1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine, 52691-62-0.

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Structural Requirements of Lyngbyatoxin A for Activation and Downregulation of Protein Kinase C†

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ABSTRACT: Structure–activity studies of novel synthetic analogues of lyngbyatoxin A reveal that the lactam ring but not the 7-linalyl moiety of lyngbyatoxin A is essential for the in vitro stimulation of protein kinase C (PKC). (−)-Indolactam V (ILV), which contains no hydrophobic substituent at C-7, or analogues containing either a linalyl or *n*-hexyl group at C-7 were equally efficacious in stimulating HeLa cell PKC in vitro and in competing with phorbol 12,13-dibutyrate for binding to PKC in intact cells. The hydrophobicity of alkyl groups at C-7, however, influenced the potency of these compounds to bind to and activate PKC. In addition, these compounds exhibited differences in their ability to translocate PKC. Lyngbyatoxin A (0.1 μM) like TPA induced a rapid translocation of PKC from the cytosol to the membrane and subsequently led to a sustained decrease in both cytosolic and membrane PKC activity. In contrast, (−)-*n*-hexylILV (0.1 μM) and (−)-ILV (1 μM) produced a transient and attenuated decrease in cytosolic PKC activity. At concentrations that produced half-maximal PKC stimulation, (−)-ILV did not cause any downregulation of PKC whereas lyngbyatoxin A and (−)-*n*-hexylILV led to 60% and 40% PKC downregulation, respectively. Western blot analyses with monoclonal antibodies to PKC isoforms indicated that reduction in PKC activity by chronic exposure to TPA or lyngbyatoxin A analogues could be explained by downregulation of PKCα. Constitutive expression of PKCβ and PKCγ isoforms was low in HeLa cells and was not affected significantly by TPA or lyngbyatoxin A analogues. These results indicate that the structural requirements for PKC activation may be separated from those required for PKC downregulation.

Protein kinase C (PKC),[1] a calcium-activated, phospholipid-dependent enzyme, is an important element in signal transduction (Nishizuka, 1986). It phosphorylates a variety of proteins and regulates many cellular processes including cell proliferation, differentiation, and gene expression (Nishizuka, 1986). PKC is now recognized as a family of closely related polypeptides with at least eight members including α, βI, βII, and γ (Nishizuka, 1988; Huang et al., 1986; Jaken & Kiley, 1987).

The activity of PKC is highly regulated. Both calcium and acidic phospholipids like phosphatidylserine are required for its activity (Kishimoto et al., 1980). Diacylglycerol (DAG),

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[1] Abbreviations: PKC, protein kinase C; DAG, diacylglycerol; ILV, indolactam V; PDBu, phorbol 12,13-dibutyrate; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; DMSO, dimethyl sulfoxide; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid.

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