Heparin Prevents the Binding of Phospholipase A_2 to Phospholipid Micelles: Importance of the Amino-Terminus†

Mitchell B. Diccianni,‡ Martha Lilly-Stauderman,‡ Larry R. McLean,§ Ambikaipakan Balasubramaniam,∥ and Judith A. K. Harmony*,‡

Department of Pharmacology and Cell Biophysics, Division of Lipoprotein Research, and Department of Surgery, College of Medicine, University of Cincinnati, Cincinnati, Ohio 45267-0575, and Marion Merrell Dow Research Institute, 2110 East Galbraith Road, Cincinnati, Ohio 45215

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ABSTRACT: The activity of the major isoform of porcine pancreatic phospholipase A_2 (PLA_2), designated B-PLA_2, against micellar substrates is inhibited by heparin. Inhibition is a consequence of binding of the enzyme to heparin, documented by a heparin-induced alteration in the intrinsic fluorescence of B-PLA_2 and in the 8-anilino-1-naphthalene sulfonate fluorescence and by the enhanced rate of chemical modification of the active site residue His-48. As a consequence of heparin binding, the conformation of B-PLA_2 at the active site and at the amino-terminus is altered, and the enzyme does not bind to phospholipid micelles. In spite of the heparin-induced conformational changes, B-PLA_2 retains its ability to catalyze the hydrolysis of monomeric phospholipid. Other glycosaminoglycans can bind to and inhibit the activity of B-PLA_2 toward organized phospholipids, but none tested is as effective as heparin. An isoform of the pancreatic enzyme, designated UB-PLA_2 and which corresponds to iso-pig PLA_2, does not bind to nor is its catalytic activity influenced by heparin. A peptide corresponding to the amino-terminal 26 residues of B-PLA_2 can rescue PLA_2 from heparin inhibition. A similar peptide corresponding to the amino-terminus of UB-PLA_2 has no effect on heparin inhibition. A model for the inhibition of B-PLA_2 by heparin is proposed in which the catalytically significant effect of heparin is to interact directly with the amino-terminus of B-PLA_2, the interfacial recognition site, to prevent the enzyme from binding to micellar substrates.

Phospholipases A_2 (PLA_2;[1] EC 3.1.1.4) comprise a class of calcium ion dependent lipolytic enzymes that catalyze lipid hydrolysis at an organized lipid–water interface. PLA_2 catalyzes the hydrolysis of the *sn*-2 fatty acyl ester linkage in diacylphospholipids, liberating free fatty acids and monoacylphospholipids (de Haas & van Deenen, 1964; Waite, 1987). Arachidonic acid, a major product of PLA_2 action, is the precursor of bioactive metabolites collectively known as the eicosanoids. Increases in arachidonic acid and monoacylphosphatidylcholine have been implicated in a number of disease states (Vadas & Pruzanski, 1986), including inflammation (Das et al., 1986), myocardial ischemia (Quinn et al., 1988), and atherosclerosis (Larrue, 1988; Smith, 1989). Cellular PLA_2s are also involved in coordinating the response of a cell to its environment (Burgoyne et al., 1987). They play a critical role in normal cellular function by participating in the metabolism and turnover of membrane phospholipids (Waite, 1985; Lapetina & Crouch, 1989) and in the regulation of the biosynthesis of specifically tailored phospholipids. PLA_2s can also help to protect cellular membranes from oxidative damage (Tan et al., 1984).

Due to the importance of PLA_2, efforts to identify regulatory mechanisms have been intense. In vivo, regulators of PLA_2 may include fatty acids or their oxidized products (Ballou & Cheung, 1983). In vitro, many agents influence PLA_2 activity, primarily at the substrate level rather than by interacting directly with the enzyme. For example, cholesterol inhibits PLA_2 by rigidfying the membrane (Vigo et al., 1980); local anesthetics, by fluidizing the membrane (Vigo et al., 1980). The glucocorticoid-induced protein lipocortin inhibits PLA_2 by sequestering substrate (Davidson et al., 1987).

We recently discovered that heparin can inhibit PLA_2 activity (Diccianni et al., 1990). Heparin, a glycosaminoglycan (GAG), is a highly *N*- and *O*-sulfated polysaccharide comprised of repeating subunits of L-iduronic acid and D-glucosamine joined by α1–4 linkages (Chakrabarti & Park, 1980). Heparin is heterogeneous, varying in extent and location of the sulfate groups. The high degree of sulfation gives heparin a net negative charge, which is believed to be important in its interaction with proteins (Cardin & Weintraub, 1989). Heparin has been shown to regulate smooth muscle cell proliferation in vitro (Hoover et al., 1980) and in vivo (Guyton et al., 1980) and to regulate Schwann cell (Ratner et al., 1985) and endothelial cell proliferation (Thornton et al., 1983). Heparin also can alter angiogenesis in vitro (Azizkhan et al., 1980). In addition, the activities of numerous enzymes have been shown to be regulated in vitro by heparin (Hara et al., 1981; Hathaway et al., 1980; Chrisman et al., 1981; Ishii et al., 1982).

Since agents that can regulate PLA_2 directly may have a significant physiological function, as well as important pharmacological applications, we have elucidated the mechanism

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* Address correspondence to this author.
‡ Department of Pharmacology and Cell Biophysics, University of Cincinnati.
§ Marion Merrell Dow Research Institute.
∥ Department of Surgery, University of Cincinnati.

[1] Abbreviations: ANS, 8-anilino-1-naphthalene sulfonate; BPB, *p*-bromophenylacyl bromide; B-PLA_2, heparin-binding isoform of porcine pancreatic phospholipase A_2; BSA, bovine serum albumin; cmc, critical micelle concentration; de-*N*-SO_4 heparin, de-*N*-sulfated heparin; dH_2O, distilled and deionized H_2O; di-C7-PC, diheptanoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DMSO, dimethyl sulfoxide; GAG, glycosaminoglycan; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HF, hydrofluoric acid; IRS, interfacial recognition site; LMW, low molecular weight; monoacyl-PC, monoacylphosphatidylcholine, myristoyl; NP-40, nonidet P-40; PLA_2, porcine pancreatic phospholipase A_2; TFA, trifluoroacetate; UB-PLA_2, non-heparin-binding isoform of porcine pancreatic phospholipase A_2.

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by which heparin inhibits PLA_2. Heparin inhibition of PLA_2 is dependent on ionic strength and pH but independent of substrate concentration (Diccianni et al., 1990). Furthermore, two isoforms of porcine pancreatic PLA_2 exist that differ in their affinity for and susceptibility to inhibition by heparin. The predominant isoform is a heparin-binding isoform (B-PLA_2); the minor isoform (UB-PLA_2) does not bind heparin (Diccianni et al., 1991). The data presented in this report indicate that heparin inhibits B-PLA_2 activity through a direct interaction with the enzyme. Using UB-PLA_2 as a control, we have established that the interaction of heparin with B-PLA_2 induces conformational changes at the enzyme active site and amino-terminus. The heparin–PLA_2 complex does not bind to organized phospholipid interfaces, although the active site remains functional and can catalyze the hydrolysis of water-soluble phospholipids.

Materials and Methods

Materials

Porcine pancreatic PLA_2 was purchased from Sigma Chemical Co. or Calbiochem. PLA_2 was separated into isoforms by chromatography on heparin-Affi-Gel as previously described (Diccianni et al., 1991). Dimyristoylphosphatidylcholine (DMPC), bovine serum albumin (BSA), histone, and the glycosaminoglycans [heparin (from porcine mucosa; H-3125, lot 34F-0742; M_r ~15000), low molecular weight heparin (H-5640, lot 47F-0677; M_r ~5000), de-*N*-sulfated heparin (D-4776, lot 66F-0417), and chondroitin-6-sulfate (from shark cartilage; C-4384, lot 102F-0418)] were obtained from Sigma Chemical Co. The hexasaccharide (lot 02-165; M_r 2630), produced as a degradation product of heparin, was obtained from Hepar Industries. Diheptanoylphosphatidylcholine (di-C7-PC; 850–306, lot C70-20) was purchased from Avanti Polar Lipids and checked for purity by TLC, using the solvent system of $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (25:15:4:2, v/v). Electrophoresis supplies and equipment were purchased from Bio-Rad Laboratories. Radiolabeled phosphatidylcholine (L-α-1-stearoyl-2-[5,6,8,9,11,12,14,15-^{3}H]arachidonyl-phosphatidylcholine, ^{3}H-PC, 91 Ci/mmol) was obtained from New England Nuclear. All *N*-α-Boc-amino acids with benzyl-based side-chain protecting groups and the synthesis reagents were obtained commercially and used without further purification.

Fluorescence Assays

ANS Fluorescence. Fluorescence experiments were performed at 37 °C in 50 mM HEPES (pH 7.0) with a Perkin-Elmer 450-10S or a SLM-4800 spectrofluorimeter, with stirred and thermostated 1-cm path length quartz cuvettes. The fluorescence probe 8-anilino-1-naphthalene sulfonate (ANS, 60 μM) was excited at 390 nm; excitation and emission slit widths were 8 nm. Changes in fluorescence intensity, corrected for dilution, are expressed in arbitary fluorescence units.

Intrinsic Fluorescence. The interaction of PLA_2 with heparin or phospholipid in 50 mM HEPES (pH 7) was determined by monitoring changes in fluorescence intensity or wavelength maximum. PLA_2, in a stirred thermostated (37 °C) quartz cuvette, was excited at 295 nm; excitation and emission slit widths were 8 nm. Changes in fluorescence, corrected for dilution, are expressed in arbitary fluorescence units.

Phospholipase A_2 Assays

Detergent Assay. The activity of PLA_2 against detergent–phospholipid micelles was determined by the release of tritiated fatty acid from radiolabeled ^{3}H-PC, essentially as previously described (Diccianni, et al., 1990). A typical reaction proceeded for 1 h at 37 °C and, unless specified otherwise, contained (final concentration) 80–86 μg/mL PLA_2, 2.5 mM DMPC, 50 mM HEPES (pH 7), 0.8% Nonidet P-40 (NP-40), 1 mM $CaCl_2$, 0.02 μCi of ^{3}H-PC, and additions such as GAG. The enzyme was assayed under conditions of low specific activity, typically 200 pmol of PC hydrolyzed/(μg·h), to facilitate determination of heparin effects. Heparin also inhibits the activity of PLA_2 in nondetergent micellar systems (Diccianni et al., 1990), against which the enzyme has a much higher specific activity. All hydrolysis values were corrected for background (uncatalyzed hydrolysis), typically 1–5% of the total radioactivity added. The percent change in activity relative to control was determined as [1 – (nmol PC test/nmol PC control)] × 100. Half-maximal inhibition values were obtained by nonlinear least-squares fit analysis.

Modified PLA_2 Assays. *p*-Bromophenacyl bromide (BPB) treated PLA_2 (10 μg) prepared as described below was incubated at 37 °C for 90 min in a reaction mixture (290 μL) containing (final concentration) 50 mM HEPES (pH 7), 0.8% NP-40, 2.5 mM DMPC, 0.02 μCi of ^{3}H-PC, 50 mM $CaCl_2$, and 400 μg/mL protamine sulfate. This modification prevented both continued inactivation of PLA_2 by BPB and heparin inhibition during the assay. Calcium ion (50 mM) prevented BPB inactivation of PLA_2 in the assay time of 90 min (not shown), as reported by Pieterson et al. (1974). Protamine sulfate binds heparin and prevents its interaction with PLA_2 (Diccianni et al., 1990). Assay conditions were also modified to measure the activity of PLA_2 in the presence of synthetic peptides. The assay mixture consisted of buffer (50 mM glycylglycine and 50 mM Tris-HCl, pH 7) with 1 mM $CaCl_2$, 0.8% NP-40, 2.5 mM PC, and 30% dimethyl sulfoxide (DMSO) in a final volume of 0.25 mL. The synthetic peptides were dissolved in DMSO before addition to the assay. DMSO, at the final concentration of 30% (v/v), did not affect enzyme activity.

Nondetergent Monomer and Micelle Hydrolysis. The activity of PLA_2 toward monomeric and micellar L-diheptanoylphosphatidylcholine (di-C7-PC; 0.25–5.0 mM) was determined at 37 °C in a 5-mL reaction mixture containing 2 mM HEPES (pH 7), 1 mM $CaCl_2$, 0.1% BSA, and 100 mM NaCl. Generally, 2.5-10 μg of the enzyme in 25–100 μL of buffer was added to the reaction mixture to initiate hydrolysis. Aliquots of heparin (10 mg/mL dialyzed into 2 mM HEPES, pH 7) were added to reaction mixtures at t = 0 to give a final concentration of 200 μg/mL. Titration with 4 mM NaOH was carried out under a nitrogen atmosphere, with a Radiometer pH stat (titrator, TTT 80; autoburette, ABU 80; pH meter, PHM 84). Enzyme activities, expressed in μmol/(min·mg), were analyzed directly by linear regression with a Hewlett-Packard 85B computer. Between experiments, the electrodes and hydrolysis chamber were washed with 10% Clorox, rinsed with dH_2O, and dried.

Critical Micelle Concentrations. The critical micelle concentration (cmc) of di-C7-PC was determined, by using ANS and the method of de Vendittis et al. (1981).

Chemical Modification of PLA_2

Inactivation Procedures. Experiments to measure the inactivation of PLA_2 (500 μg/mL) by BPB were performed at 25 °C in 50 mM HEPES (pH 7) and 100 mM EGTA. At t = 0, BPB (1 mg/mL in acetonitrile) or acetonitrile was added to a final concentration of 19.6 μg/mL. Prior to BPB/acetonitrile addition and at specific time intervals thereafter, 10 μg of PLA_2 was removed and assayed by using the modified PLA_2 assay. The rate of PLA_2 inactivation was determined

by analysis of the activity vs time of incubation with BPB curves.

Peptide Synthesis

The peptides Ala-Leu-Trp-Gln-Phe-Arg-Ser-Met-Ile-Lys-Cys-Ala-Ile-Pro-Gly-Ser-His-Pro-Leu-Met-Asp-Phe-Asn-Asn-Tyr-Gly and Ala-Leu-Trp-Gln-Phe-Arg-Ser-Met-Ile-Lys-Cys-Thr-Ile-Pro-Gly-Ser-Asp-Pro-Leu-Leu-Asp-Phe-Asn-Asn-Tyr-Gly, corresponding to the amino-terminal (N_t) 26 residues of B-PLA_2 and UB-PLA_2, respectively (Diccianni et al., 1991), were synthesized as described previously (Balasubramaniam et al., 1990), with an Applied Biosystems model 430A synthesizer. Briefly, benzhydrylamine resin (0.45 mmol of the NH_2 group) was placed in the reaction vessel of the peptide synthesizer, and the protected amino acids were coupled automatically, with the standard program provided by the manufacturer. All amino acids were coupled as preformed symmetrical anhydrides (2.2 equiv) except Arg, Asn, and Gln, which were double coupled as preformed 1-hydroxybenzotriazole esters (4.4 equiv) to avoid side reactions. At the end of the synthesis, the *N*-α-Boc group was removed, the peptide resin was dried in vacuo overnight, and the free peptide was obtained by treating the peptide resin with HF (~10 mL) containing *p*-cresol (~0.8 g), *p*-thiocresol (~0.2 g), and dimethyl sulfide (~0.8 mL) for 1 h at −2 to −4 °C. HF was evacuated, and the residue was transferred to a fritted filter funnel with diethyl ether, washed with diethyl ether (3 × 15 mL), and extracted with 30% acetic acid (2 × 15 mL). The acetic acid extract was diluted to 10% and lyophilized. Crude peptide was purified in batches of 30 mg on a Vydac semipreparative column (250 × 10 mm, 10-μm particle size, 300-Å pore size), with a gradient of 40–100% B of 0.1% TFA in 60% CH_3CN to 0.1% TFA-H_2O in 60 min. The fractions corresponding to the major peak were combined and rechromatographed under identical conditions. The overall yield was 15–25%. The integrity of the peptides obtained was verified by analytical reverse-phase chromatography, amino acid analysis, and sequencing.

Results

Heparin Inhibits B-PLA_2 but Not UB-PLA_2. Heparin is a potent inhibitor of PLA_2 activity (Diccianni et al., 1990). The interaction of heparin with the isoform of PLA_2 that can bind to heparin-Affi-Gel, B-PLA_2 (Diccianni et al., 1991), decreased its catalytic activity by greater than 90% with an IC_{50} of 8 μg/mL (Figure 1A). The minor isoform of PLA_2, designated UB-PLA_2 and identified (Diccianni et al., 1991) as iso-pig PLA_2, which does not bind heparin, was not inhibited by heparin at concentrations as high as 1 mg/mL. The extent of heparin inhibition of B-PLA_2 is optimum under these conditions and diminishes with increasing ionic strength (Diccianni et al., 1990).

B-PLA_2 Interacts with Heparin in Solution. The binding of B-PLA_2 to heparin immobilized Affi-Gel (Diccianni et al., 1991) indicates its affinity for heparin. To determine whether heparin also can interact with PLA_2 in solution, a fluorescence assay was used. ANS has proven to be a useful probe of changes in PLA_2 structure (Pieterson et al., 1974). When bound to either PLA_2 isoform, ANS had a fluorescence maximum between 470 and 480 nm (Figure 2). The ANS fluorescence intensity was somewhat higher when the probe was associated with B-PLA_2 compared to UB-PLA_2. This difference was calcium ion dependent, since the spectra were identical in the absence of added calcium ion (data not shown). The addition of heparin decreased ANS fluorescence markedly when the probe was associated with B-PLA_2 (Figure 2B). The

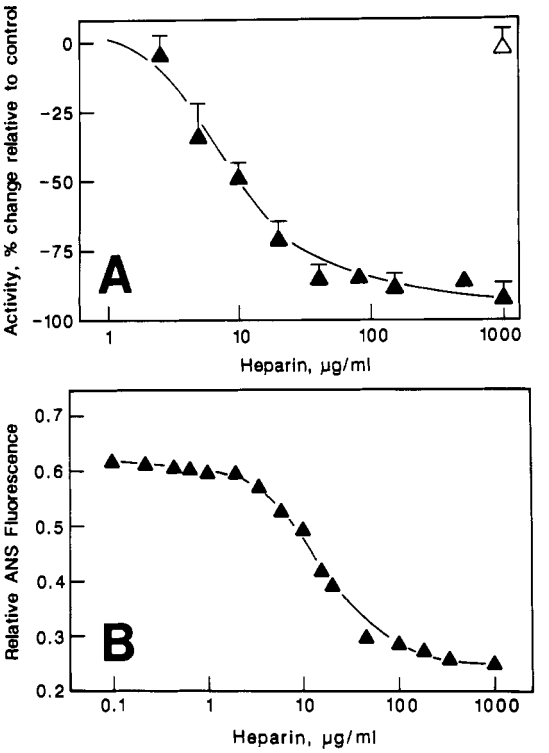

FIGURE 1: B-PLA_2 inhibition by heparin. The extent of inhibition is correlated with the reduction of ANS fluorescence. (A) B-PLA_2 (▲, 80 μg/ml) and UB-PLA_2 (△) were assayed, with the detergent assay described under Materials and Methods. The data represent the average of triplicate determinations. The IC_{50} was determined by nonlinear least-squares fit analysis. (B) B-PLA_2 (86 μg/mL) was mixed with ANS (60 μM) in 50 mM HEPES, pH 7, containing 0.1 mM EGTA and 1 mM $CaCl_2$. Heparin was added as indicated. The fluorescence intensity, with excitation at 390 nm, was measured at 480 nm and corrected for background fluorescence of ANS alone.

fluorescence of ANS associated with UB-PLA_2 was little perturbed by heparin (Figure 2A).

The change in ANS fluorescence is not due to a heparin–calcium ion interaction since heparin at concentrations that inhibit the activity of B-PLA_2 by >95% does not chelate calcium ion (Diccianni et al., 1991). It is equally unlikely that a direct interaction between ANS and heparin occurred since heparin did not change the fluorescence intensity or the maximum wavelength of fluorescence of ANS alone. Moreover, ovalbumin, a protein that does not bind heparin, bound ANS but the ANS fluorescence was not altered by heparin (Figure 2C). Unfortunately, the weak binding of ANS to PLA_2 (Pieterson et al., 1974) did not allow us to discern whether heparin decreased ANS fluorescence by changing the environment of bound ANS or by decreasing the amount of ANS associated with B-PLA_2.

The decrease in fluorescence intensity of ANS/B-PLA_2 depended on the amount of heparin added (Figure 1B). The concentration of heparin that decreased ANS fluorescence by 50% was calculated to be 8 μg/mL when the concentration of B-PLA_2 was 86 μg/mL, in excellent agreement with the IC_{50} obtained for inhibition of catalytic activity at the same enzyme concentration (Figure 1A). Furthermore, the IC_{50} of heparin depended directly on the PLA_2 concentration. As the concentration of B-PLA_2 was increased from 20 to 40 to 80 μg/mL, the IC_{50} increased from 1.3 ± 1.1 to 2.6 ± 1.1 to 5.7 ± 1.1 μg/mL.

Finding that the decrease in ANS fluorescence is a sensitive indicator of the interaction of B-PLA_2 with heparin, we compared the ability of GAGs to inhibit B-PLA_2 activity and to reduce ANS fluorescence (Figure 3). The GAGs were

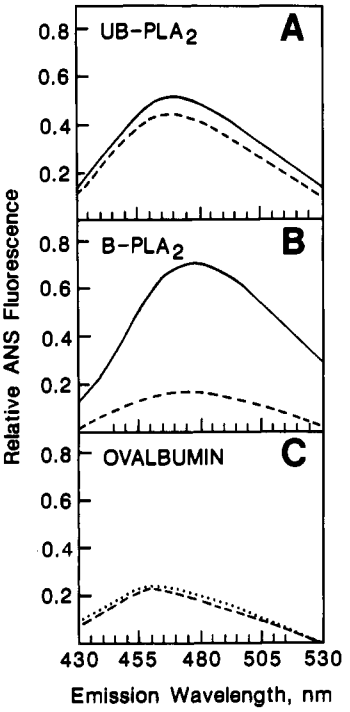

FIGURE 2: Heparin alteration of the fluorescence of ANS associated with B-PLA_2. PLA_2 was fractionated by heparin-Affi-Gel as described under Materials and Methods. B-PLA_2 or UB-PLA_2 (80 μg/mL) or ovalbumin (300 μg/mL) was mixed with ANS (60 μM) in 50 mM HEPES, pH 7, containing 0.1 mM EGTA and 0.1 mM $CaCl_2$ (PLA_2) or 1 mM $CaCl_2$ (ovalbumin). The solid lines represent ANS fluorescence in the absence of heparin. The dashed lines represent ANS fluorescence in the presence of 50 μg/mL of heparin. Spectra are corrected for background (ANS, no protein) fluorescence.

compared at a concentration of 25 μg/mL. This concentration of heparin inhibited by 60%, allowing assessment of whether a particular GAG was more or less inhibitory than heparin. Low molecular weight (LMW) heparin and unfractionated heparin had similar inhibitory potencies and were the most effective inhibitors among those GAGs tested. The pattern of inhibition was LMW heparin = heparin > hexasaccharide = CS-C = CS-A > de-*N*-sulfated heparin > heparan sulfate > hyaluronic acid. The inhibition of activity vs decrease in ANS fluorescence data indicate a good correlation ($r = 0.85$) between the ability of a given GAG to inhibit catalytic activity and to decrease ANS/B-PLA_2 fluorescence.

Heparin Influences the Structure of B-PLA_2. ANS binds to PLA_2 in the vicinity of the active site (Oda et al., 1986; van Eijk et al., 1984). The heparin-induced change in ANS/B-PLA_2 fluorescence suggests that heparin influences the active site. The effect of heparin on the chemical modification of an active site residue was tested by using BPB, which inactives PLA_2 by modifying His-48 (Volwerk et al., 1974). Heparin enhanced the rate of BPB inactivation of B-PLA_2 with an IC_{50} (Figure 4) of about 5 μg/mL, in agreement with the IC_{50} obtained for heparin-induced inhibition of catalytic activity and decrease in ANS fluorescence. The inactivation of UB-PLA_2 by BPB was not influenced by heparin (data not shown).

To determine if heparin also influences the amino-terminus of PLA_2, we measured the response of the intrinsic fluorescence of the PLA_2 isoforms to heparin. When PLA_2 is excited at 295 nm, the fluorescence primarily reflects the contribution of the single Trp at position 3 in the amino-terminus (van Dam-Mieras et al., 1975). Heparin decreased B-PLA_2 fluorescence by 15% and induced a blue spectral shift of 8 nm (Figure 5B). The IC_{50} of both effects was determined to be about 5 μg/mL, in good agreement with the IC_{50} for heparin's

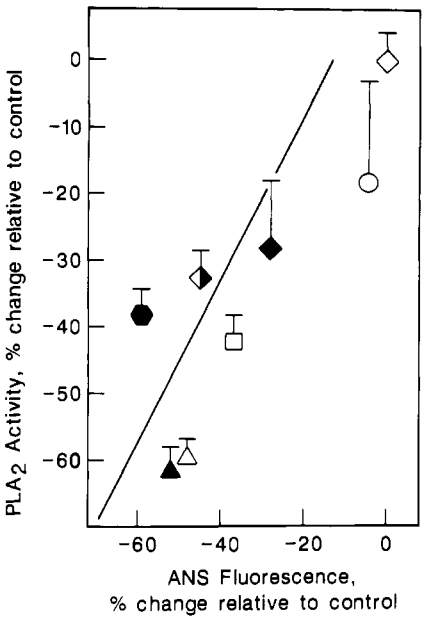

FIGURE 3: Correlation between glycosaminoglycan-induced decreases in B-PLA_2 catalytic activity and ANS fluorescence. B-PLA_2 activity was determined with the detergent assay described under Materials and Methods. ANS fluorescence intensity was determined at 480 nm in the presence of 1 mM EGTA and 0.1 mM $CaCl_2$ as described under Materials and Methods. All GAG concentrations were 25 μg/mL. Symbols: (◇) control, no GAG; (▲) heparin; (△) low molecular weight heparin; (□) hexasaccharide; (◈) de-N-SO_4 heparin; (◆) heparan sulfate; (○) hylauronic acid; (●) chondroitin-6-sulfate.

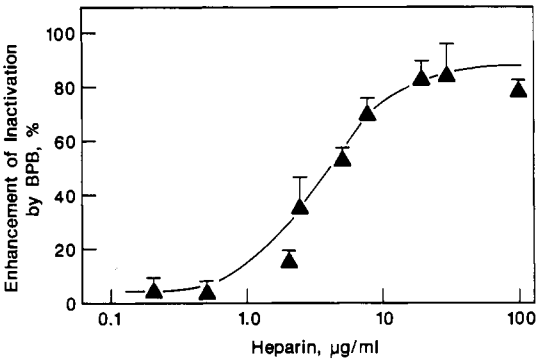

FIGURE 4: Dose response of heparin enhancement of BPB-inactivation of B-PLA_2. Heparin was incubated with PLA_2 at a 4:1 (mol/mol) ratio. Inactivation conditions and the modified PLA_2-assay are described under Materials and Methods.

effects on catalytic activity, ANS/B-PLA_2 fluorescence, and BPB inactivation. The decrease in fluorescence intensity and the wavelength shift induced by heparin were both reversed by 100 mM NaCl (not shown). In contrast, heparin decreased the fluorescence intensity of UB-PLA_2 by only 7% (Figure 5A). The small decrease in the fluorescence intensity may be due to light scattering. The important difference between B- and UB-PLA_2 was the absence of a heparin-induced shift in the optimum wavelength of UB-PLA_2 fluorescence. Heparin itself exhibited no intrinsic fluorescence. Moreover, heparin had no influence on the intrinsic fluorescence properties of ovalbumin (not shown).

Heparin Inhibits B-PLA_2's Ability To Catalyze Hydrolysis of Micellar but Not Monomeric Phospholipids. If the conformational change at the active site accounts for heparin inhibition of B-PLA_2 catalytic activity, the activity of B-PLA_2 toward both monomeric and micellar substrates should be inhibited by heparin. The water soluble substrate L-diheptanoylphosphatidylcholine (di-C7-PC), which exists in

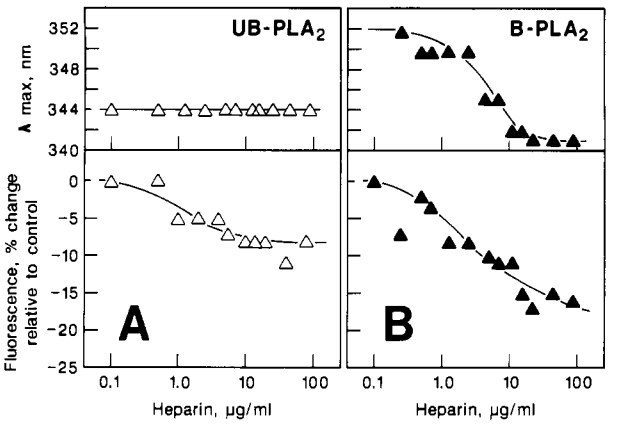

FIGURE 5: Heparin reduces the fluorescence intensity of both B- and UB-PLA_2 but causes a wavelength shift only in B-PLA_2 fluorescence. Heparin was titrated into a 1.5-mL reaction cuvette at 37 °C containing 50 mM HEPES (pH 7), 0.1 mM EGTA, 0.1 mM $CaCl_2$, and 86 μg/mL B- or UB-PLA_2. The excitation wavelength was 295 nm, and fluorescence intensitites were measured at the wavelength of maximum intensity.

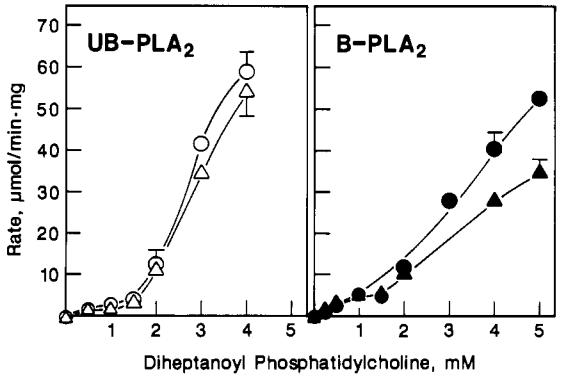

FIGURE 6: Rate of PLA_2-catalyzed hydrolysis of monomeric and micellar di-C7-PC. Rates were measured at 37 °C in 2 mM HEPES, (pH 7), 0.1% BSA, and 100 mM NaCl, with (△,▲) or without (○,●) heparin (200 μg/mL). Rates are given in μmol/(min·mg). The absence of an error bar indicates that the SD was less than the symbol size.

the monomeric state below and as micelles above its cmc of ~1.5 mM, was used to test this prediction. Heparin did not alter the cmc of di-C7-PC (not shown). Figure 6 demonstrates that UB-PLA_2 and B-PLA_2 had similar catalytic activities toward di-C7-PC monomers and that both isoforms exhibited interfacial activation at ~1.5 mM di-C7-PC. Heparin inhibited the activity of B-PLA_2, but only toward micellar di-C7-PC. This inhibition, although less pronounced than that which occurred when detergent micelles were used as a substrate (Figure 1A), was reproducible and statistically meaningful. The reduced magnitude of the heparin inhibition relative to that obtained with detergent micelles was due to the relatively high ionic strength (100 mM NaCl) required for the monomer–micelle transition in the absence of detergent. In contrast, the hydrolysis of monomeric substrate catalyzed by B-PLA_2 was insensitive to heparin. Furthermore, heparin had no significant influence on the activity of UB-PLA_2 toward di-C7-PC below or above the cmc.

Heparin Prevents Binding of B-PLA_2 to Phospholipid Micelles. Since heparin inhibited B-PLA_2 activity toward organized phospholipid substrates, we asked whether heparin prevents the interaction of B-PLA_2 with micelles. Binding of B-PLA_2 to organized phospholipids causes a conformational change in Trp-3 of PLA_2 (van Dam-Mieras et al., 1975; de Araujo et al., 1979; Hille et al., 1981). Utilizing fluorescence

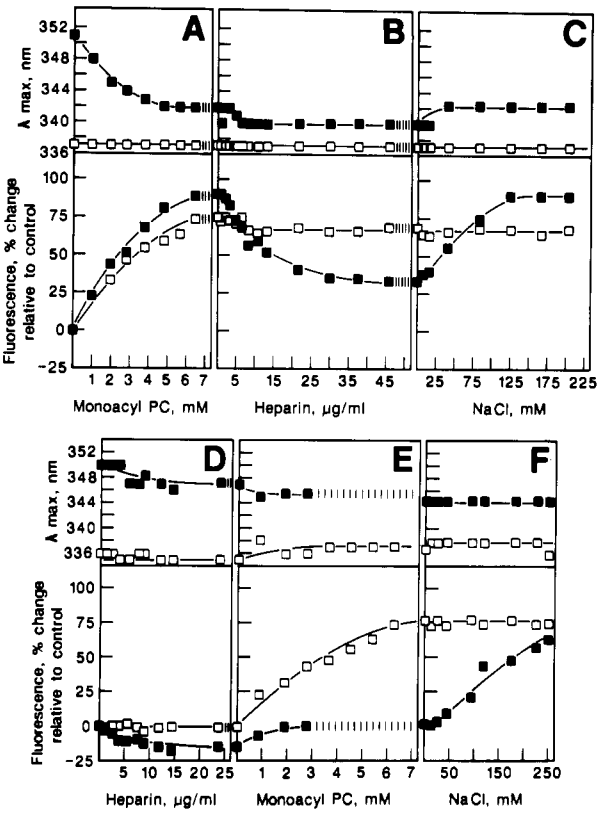

FIGURE 7: Heparin both reverses (A–C) and prevents (D–F) the binding of B-PLA_2, but not UB-PLA_2, to micelles of monoacyl-PC. Binding of UB-PLA_2 (□) or B-PLA_2 (■) to monoacyl-PC was measured by the change in fluorescence intensity and wavelength maximum of Trp-3. Experiments were performed at 37 °C in 50 mM HEPES (pH 7), 1 mM EGTA, 1.0 mM $CaCl_2$, and 80 μg/mL PLA_2 in a 1.5-mL sample volume. Stock solutions of monoacyl-PC, heparin, and NaCl were added directly to the cuvette as indicated. The excitation wavelength was 295 nm; the fluorescence intensity was measured at the wavelength of maximum intensity. The experiments were performed in a continuous fashion from A to C and from D to F. The order of addition of heparin and monoacyl-PC in A–C and D–F was different.

to monitor Trp-3, we assessed binding of PLA_2 to micelles of the substrate analogue monoacylphosphatidylcholine (monoacyl-PC). Monoacyl-PC is not a substrate for PLA_2, so that binding of the enzyme to phospholipid micelles can be assessed in the absence of phospholipid hydrolysis (Volwerk et al., 1974). The question was addressed in two ways: (1) by allowing the enzyme to bind to micelles and subsequently determining if heparin can reverse binding (Figure 7A–C), and (2) by determining if heparin can prevent the enzyme–micelle interaction (Figure 7D–F). In Figure 7, B-PLA_2 is represented by closed symbols and UB-PLA_2 by open symbols.

The fluorescence of both PLA_2 isoforms increased in the presence of monoacyl-PC micelles (Figure 7A, lower panel). There was a blue-shift of 9 nm in the wavelength of maximum fluorescence of B-PLA_2 but not UB-PLA_2 (Figure 7A, upper panel). With B-PLA_2, heparin partially reversed the monoacyl-PC-induced increase in B-PLA_2 fluorescence without affecting the wavelength of maximum fluorescence (Figure 7B). Under identical conditions, heparin did not influence the fluorescence properties of UB-PLA_2/micelle complexes. NaCl, when added after heparin, reversed the heparin effect on B-PLA_2/monoacyl-PC fluorescence without altering the fluorescence wavelength significantly (Figure 7C).

The increase in B-PLA_2 fluorescence intensity that occurred in response to monoacyl-PC was prevented by including heparin in the reaction mixture prior to monoacyl-PC addition

(Figure 7D,E). Heparin itself, in the absence of monoacyl-PC, caused a small decrease in B-PLA_2 fluorescence and a blue-shift in wavelength (Figure 7D). In the presence of heparin, monoacyl-PC induced no further decrease in the maximum wavelength of fluorescence of B-PLA_2 (Figure 7E, top panel). The addition of NaCl to a mixture of PLA_2/heparin/monoacyl-PC relieved the heparin/B-PLA_2 interaction and restored the B-PLA_2/monoacyl-PC interaction, as determined by the increase in fluorescence intensity (Figure 7F, lower panel). Preincubation of heparin with UB-PLA_2 had no effect on the ability of monoacyl-PC to induce a change in intensity of fluorescence of this isoform (Figure 7D–F).

The Amino-Terminus of B-PLA_2 Is Responsible for Heparin Inhibition. Three results point to an interaction between heparin and the amino-terminus of B-PLA_2, as we previously suggested (Diccianni et al., 1991). Heparin can alter the intrinsic fluorescence of Trp-3 in the amino-terminus of B-PLA_2 but not UB-PLA_2. Heparin can inhibit the B-PLA_2-catalyzed hydrolysis of micellar but not monomeric PC substrates. Heparin can prevent the direct interaction of B-PLA_2 but not UB-PLA_2 with micelles. It is generally accepted (van Dam-Mieras et al., 1975; Hille et al., 1981; Peers et al., 1987) that the amino-terminus of B-PLA_2 binds to the lipid interface of micellar substrates. To test the hypothesis that the amino-terminus of B-PLA_2 mediates heparin inhibition, a synthetic peptide corresponding to the amino-terminal 26 residues of B-PLA_2 was synthesized and tested for its ability to relieve GAG inhibition of B-PLA_2, with histone as a positive control (Diccianni et al., 1990). The hexasaccharide of heparin (25 μg/mL) inhibited B-PLA_2 by about 75% (enzyme activity reduced to 26.6 ± 5.8% of control), and this inhibition was relieved completely by histone (260 μg/mL). The B-PLA_2 amino-terminal peptide (200 μg/mL) also significantly relieved inhibition: in the presence of hexasaccharide plus the peptide, the activity of the enzyme was 71.5 ± 5.9% of control.

If the activity of the B-PLA_2 amino-terminus is relevant to the heparin effect on PLA_2, the peptide corresponding to the amino-terminal 26 residues of the non-heparin-inhibitable isoform UB-PLA_2 should be inactive. In fact, this was the result (Figure 8). The UB-PLA_2 peptide did not relieve heparin inhibition of PLA_2 at any concentration tested. The B-PLA_2 amino-terminal peptide showed a dose-dependent relief of heparin inhibition. Neither peptide had significant influence on the catalytic activity of PLA_2 in the absence of heparin. The relative water insolubility of the synthetic peptides necessitated the use of an assay modified to include DMSO. Heparin and the heparin hexasaccharide had equivalent inhibitory potentcies in this modified assay system (data not shown).

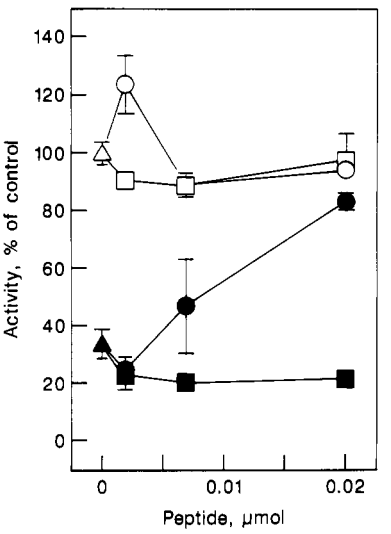

FIGURE 8: Synthetic peptide corresponding to the amino-terminus of B-PLA_2 but not UB-PLA_2 relieves heparin inhibition of PLA_2. The conditions of the assay are described under Materials and Methods. PLA_2 was assayed in the absence (open symbols) or presence (closed symbols) of heparin (50 μg/mL) with additions of (○, ●) increasing concentrations of the B-PLA_2 amino-terminal synthetic peptide or (□, ■) the UB-PLA_2 amino-terminal synthetic peptide.

Discussion

Heparin inhibition of the catalytic activity of B-PLA_2, the isoform of the porcine pancreatic enzyme that can bind to heparin-Affi-Gel, is a consequence of the direct interaction between B-PLA_2 and heparin. This interaction is indicated by a heparin-induced change in the fluorescence properties of ANS associated with B-PLA_2 and in the intrinsic fluorescence of B-PLA_2. Heparin causes no comparable changes in the fluorescence of ANS/UB-PLA_2 complexes or of UB-PLA_2 alone. The IC_{50} for heparin-induced reduction of ANS/B-PLA_2 fluorescence intensity is equivalent to that for inhibition of catalytic activity in the detergent-phospholipid micelle assay system, indicating that the interaction between heparin and B-PLA_2 accounts for inhibition.

Heparin binding to B-PLA_2 does not require calcium ion (Diccianni et al., 1991), placing the enzyme in the calcium ion independent class of heparin-binding proteins (Hirose et al., 1986; Jackson et al., 1991). Inspection of the enzyme's amino acid sequence indicates that it lacks a linear consensus sequence proposed by Cardin and Weintraub (1989) to be important in heparin binding. Therefore conformational determinants may create a heparin-binding domain, and precedent exists for conformational dependence of heparin binding in other proteins (Gettins & Wooten, 1987). A hexasaccharide of heparin satisfies the structural requirements for interaction with the binding domain of B-PLA_2. Heparin is a selective, rather than a specific, PLA_2 inhibitor. While other GAGs can bind to B-PLA_2, as indicated by their ability to decrease ANS fluorescence in the ANS/B-PLA_2 complex, they are less active than heparin, particularly when present at concentrations ≤25 μg/mL. The importance of the B-PLA_2/GAG interaction is substantiated by the direct relationship between the extents of reduction in ANS fluorescence intensity and in catalytic activity induced by those GAGs tested.

An understanding of the mechanism by which heparin inhibits B-PLA_2 may provide insight into possible modes of physiological regulation and into the development of specific inhibitors of the enzyme as well. On the basis of our results, the critical consequence of the heparin/B-PLA_2 interaction is loss of micelle binding capacity. Using the fluorescence properties of Trp-3 of the enzyme to monitor PLA_2/micelle interaction, we have demonstrated that heparin prevents binding of B-PLA_2, but not UB-PLA_2, to monoacyl-PC micelles and can dissociate B-PLA_2 from micelles once it has bound. Increasing the ionic strength of the solution, which prevents the interaction of B-PLA_2 with heparin-Affi-Gel (Diccianni et al., 1991), alleviates heparin's ability to prevent the B-PLA_2/micelle interaction. The sensitivity of the B-PLA_2/heparin interaction to ionic strength accounts for the somewhat diminished inhibitory effect of heparin on micelle hydrolysis due to the presence of 100 mM NaCl, as reported in Figure 7. Modest ionic strength, e.g., 100 mM NaCl, is required to promote the binding of PLA_2 to substrate di-C7-PC micelles (Dawson, 1966).

Our finding that heparin prevents PLA_2 from binding to micelles is in contrast to the report by Peers et al. (1987) that heparin does not prevent the interaction of PLA_2 with or-

ganized lipid substrates. On the basis of our results, the concentration of heparin used in their study (50 units/mL or ~300 μg/mL) should have been sufficient to prevent binding. A possible reason for the apparent discrepancy between this work and that of Peers et al. (1987) is a structural difference between bacterial membranes, the substrate in their studies, and the detergent–phospholipid or phospholipid micelles used in this study.

Conformational changes occur in at least two regions of B-PLA_2, the active site and the amino-terminus, as a result of its binding to heparin. A conformational change at the active site is indicated by the heparin-induced change in ANS fluorescence and by the heparin-induced enhancement of the rate of BPB inactivation of B-PLA_2. A conformational change in the amino-terminus is indicated by the heparin-induced change in the fluorescence properties of Trp-3. Since porcine pancreatic PLA_2 contains a single Trp, there is no ambiguity in the location of the residue experiencing a change in environment in response to heparin.

Active site and amino-terminal conformational changes, as well as the change in catalytic activity, have very similar dependencies on heparin concentration. However, in explaining the decrease in PLA_2 catalytic activity, the conformational change in the amino-terminus appears to be more important than the change in the active site. In fact, heparin binding does not destroy the active site of B-PLA_2; its activity toward monomeric phospholipids is unaffected by heparin. Only the activity toward micellar phospholipids, as diacylphospholipid micelles or as detergent-diacylphospholipid micelles, is inhibited by heparin. The prediction that the amino-terminal conformational change is the functionally significant change is consistent with the known contribution of the amino-terminus to the interfacial recognition site (IRS) (van Dam-Mieras et al., 1975; Hille et al., 1981; Dijkstra et al., 1984).

On the basis of our data, heparin prevents binding of B-PLA_2 to lipid interfaces by altering interaction of the IRS with phospholipids. Two mechanisms may be considered. Heparin may bind directly to the amino-terminus, blocking the IRS by steric hindrance. Alternatively, the conformation of the IRS may be altered by the binding of heparin to another site on B-PLA_2. Several lines of evidence support the proposal that the amino-terminal IRS participates directly in heparin binding and that heparin blocks access of the enzyme to micelles. Differences in primary sequences of the amino-termini of B- and UB-PLA_2 are consistent with conformational differences in the IRS of the two enzymes. Three of the four amino acid differences between B- and UB-PLA_2 occur in the amino-terminus (Diccianni et al., 1991), and the predictions of percent α-helix in the amino-terminal 30 residues differ significantly: 53% for that domain of B-PLA_2 compared to 33% for that of UB-PLA_2 (Diccianni et al., 1991). This study indicates that the environment of Trp-3 is different in the two isoforms, lending strength to the prediction that the amino-terminal conformations are different. Trp-3 appears to exist in a more hydrophobic environment (λ_{max} of 344 nm) in UB-PLA_2 compared to B-PLA_2 (λ_{max} of 352 nm). In addition, the amino-terminus of B-PLA_2 is altered by heparin in a manner suggestive of its contact with heparin. Heparin decreases the maximum wavelength of Trp-3 fluorescence by 8 nm, consistent with transfer of Trp to a more hydrophobic environment (Eftink & Chiron, 1976), with an IC_{50} for heparin equivalent to that for inhibition of hydrolysis of micellar phospholipid. Associated with this blue-shift is a decrease in the fluorescence intensity of Trp-3, possibly a result of charge quenching by heparin. Finally, and most definitively, like the heparin-binding protein histone, a peptide comprised of the 26 amino-terminal residues of B-PLA_2 can relieve heparin inhibition of PLA_2 activity against detergent–phospholipid micelles. The peptide corresponding to the amino-terminus of UB-PLA_2 is inactive.

These considerations lead to the conclusion that heparin inhibits B-PLA_2 by binding to the enzyme at the IRS and blocking its access to micellar substrates. This work thus defines a new distinction between phospholipases A_2 that either do or do not bind to heparin. These classes may provide a useful regulatory distinction among phospholipases that may apply not only to PLA_2 enzymes but also to other types of phospholipases. A regulatory role is suggested by the maintenance of B-PLA_2's ability to catalyze the hydrolysis of monomeric substrates in the presence of heparin. How this might translate into substrate preferences for lipids in membranes is not yet clear and is an active area of investigation. Decreasing activity toward organized lipids, e.g., membranes, with concomitant increasing activity toward monomeric lipids may be important in G-protein-dependent activation of protein kinase C and the regulation of cellular proliferation. This concept is supported by data which indicate that heparin inhibits cell proliferation as a consequence of a protein kinase C dependent mechanism (Wright et al., 1989). Furthermore, lipolytic enzymes in the gut may be responsive to heparin regulation (Bosner, et al., 1988). These intriguing results suggest that the role of heparin in physiology may be extended to a direct regulatory role on enzymes critical to the response of cells to stimuli.

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Light-Chain-Independent Binding of Adaptors, AP180, and Auxilin to Clathrin

Robert Lindner and Ernst Ungewickell*
Max-Planck-Institut für Biochemie, D-8033 Martinsried, Federal Republic of Germany
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ABSTRACT: Binding of coated vesicle assembly proteins to clathrin causes it to assemble into regular coat structures. The assembly protein fraction of bovine brain coated vesicles comprises AP180, auxilin, and HA1 and HA2 adaptors. Clathrin heavy chains, separated from their light chains, polymerize with unimpaired efficiency when assembly proteins are added. The reassembled coats were purified by sucrose gradient centrifugation and examined for composition by SDS–PAGE and immunoblotting. We found that all four major coat proteins are incorporated in the presence and absence of light chains. Moreover, each of the purified coat proteins is able to associate directly with clathrin heavy chains in preassembled cages as efficiently as with intact clathrin. We conclude that light chains are not essential for the interaction of AP180, auxilin, and HA1 and HA2 with clathrin.

Clathrin-coated membranes are the vehicles of receptor-mediated endocytosis and are responsible for routing lysosomal proteins from the trans-Golgi network to prelysosomal compartments (Goldstein et al., 1985; Brodsky, 1988; Morris et al., 1989). Cells capable of regulated exocytotic activities utilize clathrin also for the formation of secretory vesicles (Orci et al., 1984) and the retrieval of their membrane after exocytosis (Heuser, 1989). Known peripheral membrane components of clathrin-coated vesicles from bovine brain are the pinwheel-shaped protein clathrin, composed of three heavy and three light chains (Ungewickell & Branton, 1981; Kirchhausen & Harrison, 1981), the adaptor complexes HA1 and HA2, AP180, and auxilin (Morris et al., 1989; Ahle & Ungewickell, 1990). Clathrin represents the structural unit of the polygonal

* To whom correspondence should be addressed.

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