A new kinetic and membrane-binding assay for phospholipase A2 (PLA2) has been developed utilizing phospholipid-coated hydrophobic styrene-divinylbenzene beads (5.2 ± 0.3 μm diameter). Phospholipids formed a stable monolayer film on styrene-divinylbenzene beads with average surface packing density of (1.3 ± 0.2) × 10^2 molecule/Å^2. Secretory PLA2 readily hydrolyzed 1-palmitoyl-2-[3H]-oleoyl-sn-glycero-3-phosphoglycerol coated on styrene-divinylbenzene beads which could be easily monitored by measuring the radioactivity of fatty acid released to solution in the presence of bovine serum albumin. For human cytosolic PLA2 with high specificity for sn-2 arachidonyl group, styrene-divinylbenzene beads coated with 1-stearoyl-2-[14C]-arachidonyl-sn-glycero-3-phosphocholine and dioleoylglycerol (7:3, mol/mol) were used as substrate. PLA2 activity was linearly proportional to the enzyme concentration in the range from 1 to 150 nM for human class II secretory PLA2, and from 1 to 20 nM for cytosolic PLA2; the specific activity was 1.6 and 1.7 μmol/min/mg, respectively. Finally, styrene-divinylbenzene beads coated with polymerized 1,2-bis[12-(lipoyloxy)dodecanoyl]-sn-glycero-3-phosphoglycerol were used to measure the membrane binding affinity of PLA2, which in conjunction with kinetic data provides important insights into how PLA2 interacts with membranes.

Phospholipase A2 (PLA2; E.C. 3.1.1.4) catalyzes the hydrolysis of the fatty acid ester in the 2-position of 3-

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4Abbreviations used: BLPC, 1,2-bis[12-(lipoyloxy)dodecanoyl]-sn-glycero-3-phosphocholine; BLPG, 1,2-bis[12-(lipoyloxy)dodecanoyl]-sn-glycero-3-phosphoglycerol; BSA, bovine serum albumin; cPLA2, human cytosolic PLA2; DOG, 1,2-dioleoylglycerol; hs-PLA2, human secretory class II PLA2; h-PLA2, human secretory PLA2; PLA2, phospholipase A2; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-sn-
sn-phospholipids, liberating fatty acid and lysophospholipid (for recent reviews, see Refs. 1, 2). Since these hydrolysis products function either as a precursor of proinflammatory metabolites or as a lipid second messenger, PLA2 is thought to play key roles in inflammation and cell signaling. Thus, the assay of PLA2 activity is an important tool for many biomedical and cellular studies. Among various PLA2 assays, the radiometric assay is the most sensitive and widely used (3). The radiometric assay follows PLA2 hydrolysis by directly measuring the release of one of hydrolysis products, typically radiolabeled fatty acid. This entails the separation of the labeled product from the reaction mixture by chromatography (4), solvent extraction (5, 6), or centrifugation (7), which is often tedious and laborious. As an alternative to these conventional radiometric assays, we have developed a PLA2 assay using radiolabeled phospholipids coated on hydrophobic beads of uniform size. It was previously shown (8) that phospholipids form a stable monomolecular film on the surface of hydrophobic beads, such as styrene-divinylbenzene (SDVB), and that phospholipid-coated beads could be used to assess the membrane binding affinity of proteins and peptides. These phospholipid-coated beads are simple to prepare, stable in aqueous dispersions, and suitable for experimental manipulations for kinetic and binding measurements. Herein, we present a new simple radiometric PLA2 assay using phospholipid-coated beads which is applicable to a wide range of PLA2s, including human secretory and cytosolic PLA2. In addition, we describe a related membrane binding assay of PLA2 using the beads coated with nonhydrolyzable phospholipids, which in conjunction with kinetic experiments provides important insights into how each PLA2 interacts with membranes.
MATERIALS AND METHODS

Materials. 1,2-Dioleoylglycerol (DOG), 1-palmitoyl-2-oleyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleyl-sn-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleyl-sn-glycero-3-phosphoglycerol (PGP), 1-palmitoyl-2-oleyl-sn-glycero-3-phosphoglycerol (POPG), 1-stearoyl-2-oleyl-sn-glycero-3-phosphocholine (SAPC), 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine, 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphoglycerol, 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine were purchased from Avanti Polar Lipids (Alabaster, AL). 1-Hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphocholine (pyrene-PC) was purchased from Molecular Probes (Eugene, OR). 1,2-Bis[12-(lipoyloxy)dodecanoyl]-sn-glycero-3-phosphocholine (BLPG) and -glycerol (BLPG) were synthesized as described elsewhere (9, 10). Fatty acid-free bovine serum albumin (BSA) was from Bayer Inc. (Kankakee, IL). Styrene-divinylbenzene (SDVB) beads (5.2 ± 0.3 μm diameter) were purchased from Seradyn, Inc. (Indianapolis, IN). [9,10-3H]oleic acid (sp act 30 Ci/mmol) and [5,6,8,9,11,12,14,15-3H(N)]arachidonic acid (sp act 60 Ci/mmol) were from American Radiolabeled Chemicals (St. Louis, MO) and 1-stearoyl-2-[14C]arachidonoyl-sn-glycero-3-phosphocholine ([14C]SAPC) (sp act 55 mCi/mmol) was from Amersham (Arlington Heights, IL). Tritiated POPC ([3H]POPC) was prepared from 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine and [9,10-3H]oleic acid using rat liver microsomes as described (11). Purified [3H]POPC (20 mCi/mmol) was converted to phosphatidyl ethanolamine ([3H]POPE) and phosphatidyl glycerol ([3H]PG) by the phospholipase D-catalyzed transphosphatidylation with ethanolamine and glycerol, respectively (12). Purified [3H]POPE and [3H]-PGP had specific radioactivity of 18 and 20 mCi/mmoll, respectively.

Phospholipases A2. Recombinant monomeric Asp-49 Agkistrodon piscivorus piscivorus PLA2 (13), bovine pancreatic PLA2 (14, 15), and human secretory class II PLA2 (hs-PLA2) (16) were prepared as described elsewhere. Recombinant human cytosolic PLA2 (cPLA2) was expressed in insect cells using a baculovirus expression vector generously provided by Dr. B. Kennedy of Merck Frosst Co. and purified as described (17). Purity of protein was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Protein concentrations were determined by the bicinchoninic acid method (Pierce; Rockford, IL).

Phospholipid-coated styrene–divinylbenzene beads. The silica suspension of SDVB beads (10%) was washed with 20% NaOH solution to remove silica emulsion. Typically, the bead suspension was mixed with ca. 10 vol of 20% NaOH and the mixture was shaken gently for 1 h and then vigorously for 1 min. After centrifugation (1500g × 5 min) of the mixture, the supernatant was carefully removed by aspiration from a floating cake of beads. After repeatedly washing (3×) with 20% NaOH solution, the beads were rinsed with 7.3 (v/v) water/ethanol mixture and collected by centrifugation (1500g × 5 min). From this point on, the beads readily formed a pellet. The washing was repeated until the pH of the washed solution turned neutral. The beads were finally rinsed with 1% aqueous ethanol solution, collected by centrifugation (1500g × 5 min) and dried by lyophilization. Lyophilized beads were stored in a glass tube at room temperature. For coating beads with phospholipids, weighed beads were suspended with an appropriate volume (e.g., 5 ml for 100 mg of beads) of ethanol:hexane (1:1, v/v) in a round-bottomed flask and a proper volume of radiolabeled phospholipid solution in ethanol:hexane (1:1, v/v) was added to this suspension. After the brief vortexing, the organic solvents were removed by rotary evaporation. The dried beads were resuspended in water in a glass centrifuge tube and were subjected to the low-energy sonication in a sonicating bath; this sonication step ensures the even coating of beads with phospholipids and also removes phospholipid multilayer films. Beads evenly coated with a phospholipid monolayer should be readily dispersible in an aqueous solution. To remove excess phospholipids and uncoated beads, the suspension was centrifuged (15,000g × 5 min) at room temperature and the supernatant and floating beads were decanted. The washing procedure was repeated until the radioactivity of supernatant leveled off. At this point, the radioactivity of aqueous dispersion containing a known amount of beads was determined to calculate the surface packing density of phospholipid on beads. Phospholipid-coated beads were normally used immediately after preparation but could be stored at 4°C for at least several days in an aqueous solution. The stability of phospholipid-coated beads under different conditions was measured by determining the release of radioactivity to supernatant after incubating them for a given period of time. Used beads were collected from buffer solutions by centrifugation and recycled. Typically, lipids were removed from beads by washing with ethanol (3×) and adsorbed proteins degraded by soaking the beads in 20% NaOH for 30 min. Washed beads were rinsed in 1% aqueous ethanol and lyophilized for further use.

Kinetic measurements. In a typical kinetic experiment, ca. 100 mg of phospholipid-coated beads were dispersed in 5 ml of an assay buffer solution (10 mM Hepes, pH 8.0 containing 10 mM CaCl2 and 25 μM BSA) to make the bulk concentration of phospholipid in the range of 50 to 100 μM. One hundred-microliter aliquots of the dispersion were transferred to 1.5-ml microcentrifuge tubes and the hydrolysis was initiated by adding PLA2 to a final concentration of 1 to 50 nM. The solutions were mixed immediately and incubated at room temperature for a given period (e.g., 5 min) and
the reaction was finally quenched by adding 10 μl of trifluoroacetic acid to the mixtures. The beads were pelleted using a microcentrifuge (15,000g × 15 s) and 50-μl aliquots of the supernatants were transferred to scintillation vials and mixed with 4 ml of scintillation liquid and the radioactivity was counted using a scintillation counter. For time-dependent measurements, aliquots of the reaction mixture were taken at a regular interval and the radioactivity of supernatants was measured. For both measurements, the radioactivity of aliquots from the reaction mixture minus enzyme was measured under the same conditions and subtracted from the enzyme kinetic data for the background correction. The background value at time zero was typically less than 6% of total radioactivity on beads and did not increase appreciably with time in the absence of enzyme. For kinetic analyses, corrected radioactivity values were converted into phospholipid concentrations using the known specific radioactivity of phospholipid.

Binding measurements. The binding of hs-PLA2 to phospholipid-coated beads was measured using BLPG (or BLPC) polymerized on SDVB surfaces. BLPG molecules were coated on SDVB beads as described above and were allowed to polymerize in the presence of 10 mM dithiothreitol at 37°C for 24 h. The beads were then washed with water (2×) and with the binding assay buffer (10 mM Tris–HCl, pH 7.4, 10 mM CaCl2 with different concentrations of NaCl) (2×). For the determination of total phospholipid concentration coated on beads, BLPG was doped with 0.5 mol% of radiolabeled POPG and coated on the known amount of beads. Aliquots of washed bead dispersion were taken and their radioactivity was measured. For binding measurements, ca. 100 mg of polymerized BLPG-coated beads was resuspended in 5 ml of the binding assay buffer (bulk phospholipid concentration ≈60 μM). Two hundred-microliter aliquots of this dispersion were transferred to 1.5-ml microcentrifuge tubes and incubated with 0 to 5 μM of enzyme for 30 min at 37°C. After beads were pelleted (15,000g × 15 s), the concentration of free enzyme ([E]f) in each supernatant was determined by measuring the enzyme activity toward pyrene-PG/BLPG polymerized mixed liposomes as described elsewhere (10, 15). Typically, the reaction (at 37°C) was initiated by adding 2- to 200-μl aliquots of the supernatant to 2 ml of 10 mM Tris–HCl buffer, pH 7.4, containing pyrene-PG (0.1 μM)/BLPG (9.9 μM) polymerized mixed liposomes, 2 μM BSA, 0.16 M NaCl, and 10 mM CaCl2. The progress of hydrolysis was monitored as an increase in fluorescence emission at 380 nm using a Hitachi F4500 fluorescence spectrometer with the excitation wavelength set at 345 nm. The pseudo-first-order rate constant (≈(Kcat/KM)app; [total enzyme]) was calculated from the nonlinear least-squares analysis of reaction progress curve. Total enzyme concentration was calculated using the apparent second order constant, (Kcat/KM)app, which was separately determined for hs-PLA2 under the same conditions; it was then converted to [E]f based on the dilution factor. The bound PL2 concentration ([E]b) was plotted as a function of [E]f, and values of n and Kd were determined by the nonlinear least-squares analysis of the [E]b vs [E]f plot using the equation [E]b = ([PL]0/n)/(1 + Kd[Es]), where [PL]0 represents total phospholipid concentration. This equation assumes that each enzyme molecule binds independently to a site on the bead surface composed of n phospholipids and with dissociation constant of Kd.

RESULTS

Phospholipase A2 hydrolysis of phospholipids coated on styrene–divinylbenzene beads. It was previously shown that phospholipids form a stable monomolecular film on the surface of hydrophobic beads, such as SDVB bead (8). Also, the surface packing density of phospholipid could be adjusted by varying the phospholipid-to-bead ratio (w/w). We first determined the surface packing density of the phospholipids on SDVB beads under our experimental conditions by measuring the radioactivity of known amounts of phospholipid-coated beads. When SDVB beads of 5.2 ± 0.3 μm diameter (density = 1.05 g/cm³) were coated with [3H]POPG, an equilibrium packing density value of (1.3 ± 0.2) × 10⁻² molecule/Å² (i.e., ca. 80 Å²/molecule) was consistently achieved. POPG has the nominal cross-sectional area of 60 Å²/molecule when maximally packed at the air–water interface, whereas the area for the most loosely packed confluent POPG monolayer attainable at the air–water interface is ca. 160 Å²/molecule (18). Thus, POPG molecules on SDVB beads have relatively compact surface packing density under our experimental conditions. Then, we measured the stability of the phospholipid-coated beads under different conditions. It was shown (8) that egg phosphatidylcholines coated on SDVB beads were resistant to hydrolysis in the pH range 2.0 to 9.0 and stable for 30 days when stored at 4°C and at pH 7.4. We found that POPG, POPE, and POPE coated on SDVB beads were stable for at least 3 days at room temperature in 10 mM Tris–HCl buffer, pH 7.4, containing different concentrations of NaCl (0, 0.16, 0.5, and 1 M). Also, CaCl2 up to 10 mM had no effect on the stability of phospholipid-coated beads.

Given the stability and the well-defined surface property of phospholipids coated on SDVB beads, we explored a possibility to use them for PL2 assay. Most commercially available radiolabeled phospholipids contain a label in their sn-2 fatty acyl group which can be liberated by PL2 hydrolysis. The liberated fatty acids will remain on hydrophobic bead surfaces due to their
KIM ET AL.112 Because BSA removes the fatty acid from bead surfaces, more hydrophobic bead surfaces will be exposed, which will in turn provide high-affinity binding sites for BSA molecules some of which contain fatty acids in their binding pockets. As a result, the nonspecific adsorption of BSA to hydrophobic bead surfaces will reduce the effective concentration of fatty acid released to the assay buffer. It should be noted, however, that despite this limitation a consistent extent (25%) of the removal of oleic acid from SDVB bead surfaces was achieved when 25 μM BSA was allowed to interact with SDVB beads coated with 20 to 200 μM of oleic acid. Based on these results, the time course of the hydrolysis of [3H]POPG by different PLA2s were measured with 25 μM BSA and with 70 μM of phospholipids. As shown in Fig. 2, secretory PLA2s, including A. p. piscivorus PLA2, bovine pancreatic PLA2, and hs-PLA2, readily hydrolyzed [3H]POPG molecules coated on SDVB beads. Anionic POPG was used as substrate because of the anionic phospholipid preference of these secretory PLA2s (9, 10). The relative activity of these PLA2s determined from the initial slopes of progress curves roughly reflected their relative activity toward anionic liposomes (10). For A. p. piscivorus PLA2 and hs-PLA2, the total amount of oleic acid released to the assay buffer (e.g., after 60 min) corresponded to ca. 25% of total POPG molecules coated on SDVB beads. This was not due to the incomplete hydrolysis of POPG because the addition of fresh enzyme after the completion of initial hydrolysis did not appreciably increase the released amount of oleic acid.

low solubility in water. Since the SDVB beads are readily separated from the supernatant by centrifugation, it is possible to monitor PLA2 hydrolysis of radiolabeled phospholipids coated on the beads if the liberated fatty acid can be released to solution. We previously showed that BSA could selectively bind and quantitatively remove fatty acids and lysophospholipids, but not phospholipids, from liposome surfaces (9, 10). We tested if BSA could also extract fatty acids from bead surfaces. As shown in Fig. 1, BSA in the concentration range of 2.5 to 100 μM did not appreciably remove POPG (70 μM) from SDVB beads. Neither did BSA remove POPC and POPE under the same conditions (data not shown). In contrast, BSA in the same concentration range was able to remove radiolabeled oleic acid from SDVB beads. When SDVB beads were coated with different mixtures of [9,10-3H]oleic acid, 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphoglycerol, and POPG, BSA extracted oleic acid from bead surfaces in a concentration-dependent manner (Fig. 1). At 25 to 50 μM, BSA spontaneously removed approximately 25% of oleic acid from bead surfaces in the presence or absence of 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphoglycerol and POPG. With higher concentrations of BSA, a slight decrease in the released amount of oleic acid was observed. These findings can be explained in terms of the ability of BSA to avidly and irreversibly bind to hydrophobic bead surfaces (8). Because BSA removes the fatty acid from bead surfaces, more hydrophobic bead surfaces will be exposed, which will in turn provide high-affinity binding sites for BSA molecules some of which contain fatty acids in their binding pockets. As a result, the nonspecific adsorption of BSA to hydrophobic bead surfaces will reduce the effective concentration of fatty acid released to the assay buffer. It should be noted, however, that despite this limitation a consistent extent (25%) of the removal of oleic acid from SDVB bead surfaces was achieved when 25 μM BSA was allowed to interact with SDVB beads coated with 20 to 200 μM of oleic acid. Based on these results, the time course of the hydrolysis of [3H]POPG by different PLA2s were measured with 25 μM BSA and with 70 μM of phospholipids. As shown in Fig. 2, secretory PLA2s, including A. p. piscivorus PLA2, bovine pancreatic PLA2, and hs-PLA2, readily hydrolyzed [3H]POPG molecules coated on SDVB beads. Anionic POPG was used as substrate because of the anionic phospholipid preference of these secretory PLA2s (9, 10). The relative activity of these PLA2s determined from the initial slopes of progress curves roughly reflected their relative activity toward anionic liposomes (10). For A. p. piscivorus PLA2 and hs-PLA2, the total amount of oleic acid released to the assay buffer (e.g., after 60 min) corresponded to ca. 25% of total POPG molecules coated on SDVB beads. This was not due to the incomplete hydrolysis of POPG because the addition of fresh enzyme after the completion of initial hydrolysis did not appreciably increase the released amount of oleic acid.
PHOSPHOLIPASE A2 KINETIC AND BINDING ASSAY

In contrast, DOG enhanced the cPLA2 activity toward \([^{14}C]\)SAPC in a concentration-dependent manner (Fig. 3). Also, the percentage of released radioactivity increased with the increase in DOG content, from ca. 40% for the SAPC:DOG (9:1) mixture to 66% for the SAPC:DOG (6:4) mixture. The latter increase was largely due to higher fatty acid extraction efficiency of BSA in the presence of DOG because it was shown that the removal of tritiated arachidonic acid from bead surfaces increased, to a comparable extent, when the mole fraction of DOG in the arachidonic acid:DOG mixture was increased from 0.1 to 0.4 (data not shown). Although higher concentrations of DOG further enhanced the cPLA2 activity, the SAPC:DOG (7:3) mixture was selected for routine cPLA2 assay due to the difficulty encountered in dispersing beads coated with higher concentrations of DOG in an aqueous solution. This presumably derives from high hydrophobicity of DOG which in turn makes the surface of coated bead particles more hydrophobic.

**FIG. 3.**
cPLA2-catalyzed hydrolysis of \([^{14}C]\)SAPC:DOG mixture coated on styrene-divinyl benzene beads. The beads were coated with \([^{14}C]\)SAPC (●), \([^{14}C]\)SAPC:DOG (9:1, molar ratio) (○), \([^{14}C]\)SAPC:DOG (8:2) (▲), \([^{14}C]\)SAPC:DOG (7:3) (△), and \([^{14}C]\)SAPC:DOG (6:4) (■). The assay buffer was 10 mM Hepes, pH 8.0, containing 10 mM CaCl2 and 25 μg BSA. Total lipid concentration coated on beads was 70 μM and the cPLA2 concentration was 20 nM.

Taken together, these results establish that SDVB beads coated with different radiolabeled phospholipids can be used as substrate for various PLA2 species and that the hydrolysis can be quantitatively monitored by measuring the radioactivity of supernatant in the presence of BSA. Having shown this, we then determined the correlation between the activity and enzyme concentration to establish the feasibility of this method as a routine PLA2 assay. Specifically, we measured the activity of two human enzymes, hs-PLA2 and cPLA2, toward \([^{3}H]\)POPG- and \([^{14}C]\)SAPC:DOG (7:3)-coated SDVB beads, respectively, as a function of enzyme concentration. Because the radioactivity of released product is readily converted into the actual concentration from the known specific radioactivity of \([^{3}H]\)POPG and \([^{14}C]\)SAPC, the activity of PLA2 (μmol/min) can be directly determined from the initial slope of reaction progress curve. The effect of enzyme concentration on the activity of hs-PLA2 and cPLA2 is shown in Fig. 4. For hs-PLA2, the activity was directly proportional to the concentration of hs-PLA2 in the range of 1 to 50 nM in 100 μl assay buffer (1.4 to 70 ng) and the specific activity was 1.6 μmol/min/mg. This value is comparable to that determined using anionic polymerized mixed liposomes (10). The activity of cPLA2 was proportional to the concentration of enzyme in the range of 0.5 to 20 nM in 100 μl assay buffer (4.3 to 170 ng) and the specific activity was 1.7 μmol/min/mg which is also comparable to the specific activity of cPLA2 determined using SAPC/DOG mixed vesicles (22, 23). For a rapid assay during enzyme purification, the activity can be determined with reasonable accuracy by measuring the increase in product concentration at a given time (e.g., 5 min).
beads were incubated with 10 mM dithiothreitol at 37°C for 24 h. The average surface packing density of BLPG on SDVB was $(1.3 \pm 0.3) \times 10^{-2}$ molecule/Å², which is essentially the same as that of POPG and SAPC. We then measured the PLA2 activity toward BLPG-coated SDVB beads before and after polymerization to test if the polymerized BLPG molecules on SDVB beads were resistant to PLA2 hydrolysis. When monitored using a pH stat at pH 7.4, the activity of hs-PLA2 toward nonpolymerized BLPG (0.1 mM) coated on SDVB beads (sp act: 2.5 μmol/min/mg) was comparable to that of POPG coated on the same beads. In contrast, no hydrolysis was detectable when polymerized BLPG (and BLPC) coated on SDVB beads were practically resistant to PLA2 hydrolysis, we then measured the binding of hs-PLA2 to the polymerized BLPG- and BLPC-coated beads at different ionic strength. The binding isotherms are illustrated Fig. 5 and the $n$ and $K_d$ values determined from curve fittings are listed in Table 1. Based on the surface area of putative interfacial binding surface of hs-PLA2, one expects that a PLA2 molecule will bind up to 40 phospholipid molecules (25). Our $n$ values indicate that ca. 20 molecules of phospholipids are in contact with the enzyme,
which is in reasonable agreement with the calculated value. Furthermore, the $K_d$ values demonstrate that hs-PLA$_2$ has high specificity for anionic surfaces as reported previously (26–28); at 0.16 M NaCl, it binds anionic BLPG surfaces ca. 380 times more tightly than electrically neutral BLPC surfaces. Also, high dependency of the BLPG binding on the ionic strength of medium demonstrates largely electrostatic nature of binding; when NaCl concentration increased from 0.16 to 1 M, the binding decreased 440-fold. In contrast, the lower-affinity binding of hs-PLA$_2$ to polymerized BLPC-coated beads was essentially insensitive to the change in ionic strength. As a result, the binding affinity for polymerized BLPG-coated beads at 1 M NaCl was comparable to that for polymerized BLPC-coated beads at 0.16 M NaCl. Presumably, the binding under these circumstances is mainly driven by hydrophobic interactions which have been shown to make a considerable contribution to overall interfacial binding of several secretory PLA$_2$s (for instance see Ref. 15). Taken together, these results demonstrate that polymerized phospholipids on SDVB beads can be used to conveniently and accurately measure the membrane binding affinity of PLA$_2$s which in conjunction with kinetic data will provide important insights into how they interact with membranes.

**DISCUSSION**

This report describes a new kinetic and membrane-binding assay for PLA$_2$ using phospholipid-coated hydrophobic beads. Radiometric assays are most widely used for PLA$_2$s mainly due to the high sensitivity. However, conventional radiometric PLA$_2$ assays are often laborious and time-consuming because they rely on the separation of radiolabeled product(s) from the reaction mixture by either chromatography (4) or solvent extraction (5, 6). An alternative assay using radiolabeled Escherichia coli membranes is faster than these methods but suffers from a lack of control on the phospholipid composition of E. coli membranes which restricts its general applicability (7). The radiometric kinetic assay using phospholipid-coated SDVB beads offers the sensitivity comparable to other radiometric assays. The detection limit is ca. 1 ng for hs-PLA$_2$ and 4 ng for cPLA$_2$. These numbers are lower estimates of sensitivity for two reasons. First, BSA has a limited ability to remove a hydrolysis product, fatty acid, from the bead surface. Although BSA was used in this study for mainly economical reasons, more efficient removal of fatty acid could be achieved by employing, e.g., various fatty-acid-binding proteins. Second, [3H]POPG and [14C]SAPC were diluted with corresponding unlabeled phospholipids to coat a large amount of beads in this study. In principle, one can coat a smaller amount of beads with undiluted radiolabeled phospholipids to detect a smaller amount of PLA$_2$.

Compared to the conventional methods, the bead assay has several significant experimental advantages. First, phospholipid-coated SDVB beads are simple to prepare, easy to handle, and stable in a proper buffer solution. In particular, the easy and rapid separation of reaction product from the reaction mixture by low-force centrifugation makes it ideally suited for the rapid enzyme assay during the enzyme purification. Second, the bead assay allows the flexibility in designing an optimal assay condition for a specific PLA$_2$ species. In this study, SDVB beads of 5.2 μm diameter were coated with a monomolecular film of phosphatidylglycerol or a mixture of phosphatidylcholine and diacylglycerol to achieve the average surface packing density of $1.3 \times 10^{-2}$ molecule/Å$^2$. In principle, it is possible to use any combination of bead size and chemical nature and packing density of phospholipid to prepare phospholipid-coated beads best suited for a particular PLA$_2$. For instance, the assay using SDVB beads coated with [14C]SAPC-DOG (7:3) turns out to be particularly useful for cPLA$_2$ for which only a limited number of assay methods are available. Unlike other phospholipid aggregates, the physical property of which greatly depends on the nature and composition of phospholipids, phospholipids coated on beads would be in essentially the same physical state due to the presence of bead surfaces that support the monomolecular film of phospholipid. Thus, one can systematically analyze the effect of surface charge and phospholipid packing density on the activity of a particular PLA$_2$ by varying the composition and amount of coated phospholipids. Finally, phospholipid-coated beads can be used to quantitatively assess the membrane binding affinity of PLA$_2$. Although beads coated with any nonhydrolyzable phospholipids could serve this role, polymerized BLPG (and BLPC)-coated beads were used in this study due to their availability. When compared to binding measurements using BLPG polymerized liposomes

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**TABLE 1**

The Binding of hs-PLA$_2$ to Polymerized Phospholipid-Coated SDVB Beads at Different Ionic Strength$^a$

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Salt concentration</th>
<th>$n$</th>
<th>$K_d$ (m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLPG</td>
<td>0.16 M NaCl</td>
<td>18 ± 6</td>
<td>$(2.2 \pm 1.0) \times 10^{-10}$</td>
</tr>
<tr>
<td></td>
<td>0.5 M NaCl</td>
<td>21 ± 6</td>
<td>$(3.4 \pm 1.5) \times 10^{-9}$</td>
</tr>
<tr>
<td></td>
<td>1 M NaCl</td>
<td>23 ± 4</td>
<td>$(9.6 \pm 2.5) \times 10^{-8}$</td>
</tr>
<tr>
<td>BLPC</td>
<td>0.16 M NaCl</td>
<td>20 ± 6</td>
<td>$(8.3 \pm 3.0) \times 10^{-6}$</td>
</tr>
<tr>
<td></td>
<td>0.5 M NaCl</td>
<td>18 ± 5</td>
<td>$(1.3 \pm 0.5) \times 10^{-7}$</td>
</tr>
<tr>
<td></td>
<td>1 M NaCl</td>
<td>23 ± 5</td>
<td>$(1.5 \pm 0.5) \times 10^{-7}$</td>
</tr>
</tbody>
</table>

$^a$ See Materials and Methods for experimental conditions and methods to calculate dissociation constants. Values of $n$ and $K_d$ represent (best values ± standard deviations) determined from the nonlinear least-squares analysis.
which have been extensively used for the same purpose (9, 10, 13–15), the binding measurements using polymerized BLPG-coated beads are not only much simpler and faster but also more accurate due to complete and rapid separation of the membrane-bound enzyme from the free form. In summary, phospholipid-coated hydrophobic beads are successfully used for assaying the activity and the membrane-binding affinity of various forms of PLA₂S including hs-PLA₂ and dPLA₂. Due to its well-defined surface properties and the experimental convenience that it offers, this novel model membrane system should serve as an alternative kinetic and binding assay method to the conventional radiometric assays and binding methods.

ACKNOWLEDGMENTS

This work was supported by grants from National Institutes of Health (GM52598 and GM53987) and Arthritis Foundation (Biomedical Science Grant). W.C. thanks Dr. G. Retzinger for his valuable suggestions on the use of phospholipid-coated beads.

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