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Identification of a specific region of low molecular weight phospholipases A_2 (residues 21–40) as a potential target for structure-based design of inhibitors of these enzymes

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ABSTRACT **We have identified a specific region of porcine pancreatic phospholipase A_2 (residues 21–40) which interacts with a neutralizing antibody causing a dramatic inhibition of its enzymatic activity (K_i in the order of 10^{-8} M). The binding equilibrium of the antibody–phospholipase A_2 complex is reached in <3 min at 37°C. Fab fragments are equally effective phospholipase A_2 inhibitors, as are intact IgG molecules. The inhibition is virtually complete and noncompetitive with respect to phosphatidylcholine substrate. The formation of precipitating immunocomplexes is not involved in the inhibition. The region of phospholipase A_2 (residues 21–40) recognized by this antibody includes a highly conserved sequence which contains several functionally important residues of both group I and group II phospholipases A_2. These data suggest that amino acid residues in this region of porcine pancreatic phospholipase A_2 are accessible for interaction with inhibitors such as neutralizing antibodies and that agents specifically interacting with this region may have potent phospholipase A_2 inhibitory activity. Thus, this conserved region of low molecular weight, extracellular phospholipases A_2 is a potential target for structure-based design of specific noncompetitive inhibitors of these enzymes. Since these extracellular phospholipases A_2 are suggested to play a pathogenic role in several important human diseases, the development of such pharmacologic inhibitors is of potential clinical importance.**

Intracellular phospholipases A_2 (PLA_2s; phosphatidylcholine 2-acylhydrolase, EC 3.1.1.4) are involved in numerous signal transduction pathways leading to the release of arachidonate and other lipid mediators (e.g., eicosanoids, lysophospholipids, and platelet-activating factor; see refs. 1–9 for reviews). These mediators have been suggested to regulate many cellular functions (1–9). Extracellular PLA_2s, on the other hand, are thought to be involved in phospholipid metabolism and in defense against microorganisms (1, 4). Recent investigations have focused on these enzymes as mediators of cellular injury and playing a key role in the pathogenesis of inflammatory diseases (1, 3). Secretory group II PLA_2 appears to be an acute-phase protein (10). Its synthesis and secretion are induced by interleukin 6, interleukin 1, and tumor necrosis factor in human hepatoma cells (10) and in rat astrocytes (11). More recently, this enzyme has been proposed to participate in the propagation of inflammation by potentiating lymphocyte activation (12). Extracellular PLA_2s have been suggested to play an important role in the pathogenesis of numerous diseases, including rheumatoid arthritis, autoimmune uveitis, alveolar epithelial injuries in respiratory diseases, and reperfusion damage in ischemic injuries (1–3). These PLA_2s play a central role in the pathogenesis of inflammatory hyperemia (1–3). Increased serum PLA_2 activity has been demonstrated in rheumatoid arthritis, in clinical and experimental septic and endotoxic shock, and in acute pancreatitis (1–3). Thus, it is of interest to design and develop specific pharmacologic inhibitors of extracellular PLA_2s. Ideally, such molecules should selectively neutralize PLA_2 activity in the extracellular fluids without affecting intracellular enzymes involved in signal transduction or other vital cellular functions. Here, we report the characterization of a monospecific neutralizing antibody which binds to a specific region of porcine pancreatic PLA_2, causing dramatic noncompetitive inhibition of its enzymatic activity. Epitope mapping and studies with synthetic peptides suggest that this antibody interacts with residues 21–40 of porcine pancreatic PLA_2. This highly conserved region is found in all group I and II low molecular weight PLA_2s and includes several residues involved in Ca^{2+} and substrate binding, essential for PLA_2 activity. Our data suggest that this region may be a target for structure-based drug design.

EXPERIMENTAL PROCEDURES

PLA_2 Antibody. An antiserum against pure, native porcine pancreatic PLA_2 (Boehringer Mannheim) was generated in a rabbit as described (13). The antiserum showed no crossreactivity to several proteins unrelated to PLA_2 (rabbit uteroglobin, chicken egg ovalbumin, lysozyme, bovine serum albumin, trypsin inhibitor, carbonic anhydrase, and porcine insulin chains A and B) in Western blots. Total IgG was obtained from the antiserum by ion-exchange chromatography. PLA_2-specific IgG was isolated by affinity chromatography on PLA_2-CH-Sepharose 4B (Pharmacia LKB) (14). All experiments described in this paper were performed with the same batch of IgG obtained from a single animal. $F(ab)_2$ and Fab fragments were prepared and purified by digestion with pepsin and papain, respectively, using kits from Pierce. Fab fragments were purified to near homogeneity by size-exclusion FPLC on a Superose 12 column (Pharmacia LKB) equilibrated in 0.15 M NaCl/6 mM NaH_2PO_4/1 mM K_2HPO_4, pH 7.4. Fab fragments were eluted with an apparent molecular mass of 48 kDa. IgG and Fab concentrations were estimated spectrophotometrically (15). Protein concentration in diluted samples was measured with the dye-binding assay (16) by means of a kit from Bio-Rad, using rabbit IgG as the standard.

PLA_2 Assay. PLA_2 was assayed with a mixed micellar substrate (deoxycholate and 1-stearoyl-2-[1-^{14}C]arachidonyl-sn-glycero-3-phosphocholine, 58 mCi/mmol, Amersham; 1 Ci = 37 GBq) (13, 17) in a total volume of 50 μl. PLA_2 (5 nM) was preincubated with antibodies or Fab fragments in 10 mM Tris·HCl, pH 8.0/1 mM $CaCl_2$. Unless otherwise stated, preincubations were carried out at 37°C for 5 min. Then, 20 μl of the preincubation mixture was added to 30 μl of the

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Abbreviations: PLA_2, phospholipase A_2; PVDF, poly(vinylidene difluoride).

reaction mixture (13, 17). Reaction mixtures were incubated at 37°C for 30 sec. Controls were kept in which PLA_2 was preincubated with rabbit preimmune IgG (Pierce). In all experiments, additional controls were kept in which PLA_2 was preincubated with buffer only. For kinetic experiments, PLA_2 (5 nM) was preincubated with affinity-purified IgG, Fab fragment, or buffer as control, for 5 min at 37°C. After preincubation, PLA_2 reaction mixtures were incubated for 30 sec at 37°C in the presence of 2–90 μM phosphatidylcholine. The concentration of deoxycholate was kept fixed at 1 mM (13). Kinetic data were analyzed by means of the program ENZYME (18). To test the inhibitory activity of our antibody in the absence of detergents, we used a substrate consisting of autoclaved *Escherichia coli* metabolically labeled with [^{3}H]oleic acid (9.0 Ci/mmol, DuPont/NEN). Assays were performed as described by Haigler *et al.* (19) with minor modifications (13). In brief, PLA_2 (5 nM) was preincubated with several concentrations of preimmune or immune antibody in a total volume of 140 μl of 10 mM Tris·HCl, pH 8.0/1 mM $CaCl_2$ for 5 min at 37°C. After preincubation, PLA_2 reactions were started by adding 60 μl of the preincubation mixture to 90 μl of the reaction mixture. Reaction mixtures were incubated for 5 min at 25°C.

PLA_2 Epitope Mapping. For digestion with *Staphylococcus aureus* V8 protease (20), PLA_2 (750 μg) was precipitated with 1 ml of 10% (wt/vol) trichloroacetic acid for 1 hr on ice. The pellet was washed four times with 1 ml of ice-cold ethanol/ethyl ether (1:1, vol/vol) and suspended in 75 μl of SDS/PAGE (21) sample buffer containing 5% (vol/vol) 2-mercaptoethanol. The sample was boiled for 10 min and then 75 μg (25 μl) of V8 protease was added. Reaction mixtures were incubated at room temperature. Samples (25 μl) were withdrawn from the reaction mixtures at various times and boiled for 10 min to stop the digestion. For CNBr cleavage, PLA_2 (134 μg) was incubated in 70% formic acid with a few crystals of CNBr in a total volume of 100 μl at room temperature for 24 hr in the dark. Controls included PLA_2 incubated either with 70% formic acid alone or with water. The reaction was quenched by evaporating CNBr under vacuum in the presence of anhydrous NaOH, and the samples were lyophilized. Each sample was suspended in 134 μl of SDS/PAGE sample buffer containing 5% 2-mercaptoethanol. Peptides produced by proteolytic digestion or by CNBr cleavage were analyzed by modified SDS/PAGE (22) in 0.75-mm-thick 15% polyacrylamide gels (10 × 8 cm). Peptide bands were then transferred to poly(vinylidene difluoride) (PVDF) membranes (Millipore) in 25 mM Caps/10% methanol buffer, pH 11.0, at room temperature, for 5 min at 350 mA constant current (23). We obtained reproducible transfer of oligopeptides to PVDF membranes by omitting preequilibration of gels in transfer buffer. Blots were either stained with Coomassie blue or immunostained with affinity-purified PLA_2 antibody. Immunostaining was performed by means of an immunogold silver staining kit (Janssen) according to the manufacturer's instructions. Protein bands excised from Coomassie-stained blots were then used for microsequencing.

Protein Microsequencing and Amino Acid Analysis. N-terminal sequence analysis and amino acid analysis of PLA_2 fragments were performed by Harvard Microchemistry, Cambridge, MA, with an Applied Biosystems 477A gas-phase sequenator (24, 25).

Synthesis of Oligopeptides. Peptides corresponding to PLA_2 residues 1–16, 21–40, 46–70, and 109–124 were synthesized and purified by Peptide Technology (Washington, DC). Cysteine residues were replaced with serine in peptide 46–70 to prevent oxidation of cysteine and formation of disulfide bonds. Peptides were purified by reverse-phase HPLC on a C_{18} column. Purity was determined by reverse-phase HPLC and amino acid analysis. Lyophilized peptides were stored desiccated at −20°C.

Competition Experiments. Synthetic peptides were dissolved at 5 mM in 100 mM Tris·HCl, pH 8.0/10 mM $CaCl_2$. These solutions were heated at 56°C for 10 min and then diluted to working concentration with the same buffer preheated at 37°C. IgG was then incubated with PLA_2 at 37°C for 10 min in the presence of peptides or of an identical volume of buffer. PLA_2 activity was determined by the *E. coli* assay (13, 17).

Effect of Ca^{2+} Concentration on PLA_2 Inhibition by Antibody. We preincubated PLA_2 (5 nM) with IgG (120 nM) in the absence of Ca^{2+} and in the presence of 0.05, 0.1, 0.5, 1, 2, 4, and 6 mM Ca^{2+}. For each Ca^{2+} concentration, assays were run in triplicate. Controls were run simultaneously in which PLA_2 was preincubated with each concentration of Ca^{2+} without IgG. PLA_2, diluted in 100 mM Tris·HCl, pH 8.0, without Ca^{2+}, was added to tubes containing Ca^{2+} and antibody dissolved in the same buffer. After 5 min at 37°C, each preincubation mixture was assayed for PLA_2 activity in the mixed micellar assay. The final concentration of PLA_2 in the assay was 2 nM, and the antibody concentration was 50 nM. The final Ca^{2+} concentration varied from 1 to 3.4 mM due to the contribution of Ca^{2+} from the preincubation mixture.

RESULTS AND DISCUSSION

Inhibition of Porcine Pancreatic PLA_2 Activity by Affinity-Purified Rabbit IgG. The concentration-dependent inhibition of PLA_2 activity in a mixed micellar assay obtained by preincubating PLA_2 with affinity-purified PLA_2 antibody is shown in Fig. 1*A*. The curve reached a plateau around 50 nM antibody, with a maximum inhibitory effect of about 89%. Preimmune rabbit IgG did not show any significant concentration-dependent effect on the activity of the enzyme. Similar results were obtained when metabolically labeled *E. coli* membranes were used as substrate of PLA_2 (Fig. 1*B*). In this case, a plateau was reached around 100 nM antibody, and virtually complete inhibition of PLA_2 activity was obtained. Both curves appear near-hyperbolic and not sigmoid in shape. Double-reciprocal plots obtained from these data were linear (data not shown) with an apparent IC_{50} of 6.7 nM when the mixed micellar substrate was used and of 27.2 nM with *E. coli* membranes. This difference in the estimated IC_{50} values may be due to experimental error or to the different assay conditions used with the two substrates (presence of bovine serum albumin in the *E. coli* assay, different reaction time, etc.). The micellar assay allows measurements under conditions of initial velocity (hydrolysis of <1% substrate) whereas the *E. coli* assay does not. It is also possible that in the presence of deoxycholate the affinity of PLA_2 for inhibitory IgG in our preparation may be higher. Radial immunodiffusion experiments showed no precipitin arcs with a wide range of antibody concentrations (1.6 μM to 10 nM) (data not shown). Additionally, we could not detect any alterations in the electrophoretic mobility of PLA_2 or the appearance of any degradation products by SDS/PAGE or Western blot after 2 hr of incubation of PLA_2 with a 10-fold molar excess of IgG at 37°C. This ruled out the possibility that the observed inhibition may be due to protease activities contaminating the antibody or that the antibody itself may have proteolytic activity.

Inhibitory Effect of Fab and $F(ab)_2$ Fragments on PLA_2 Activity. FPLC-purified Fab fragments inhibited PLA_2 activity in a concentration-dependent way in a mixed micellar assay (Fig. 1*C*). The dose–response curve was hyperbolic and very similar to that obtained with intact IgG (Fig. 1*C*). These data suggest that interaction of one antigen-binding site with one molecule of PLA_2 is sufficient to cause inhibition of enzyme activity. A double-reciprocal plot obtained from these data (Fig. 1*C Inset*) gave an apparent IC_{50} of 27.7 nM

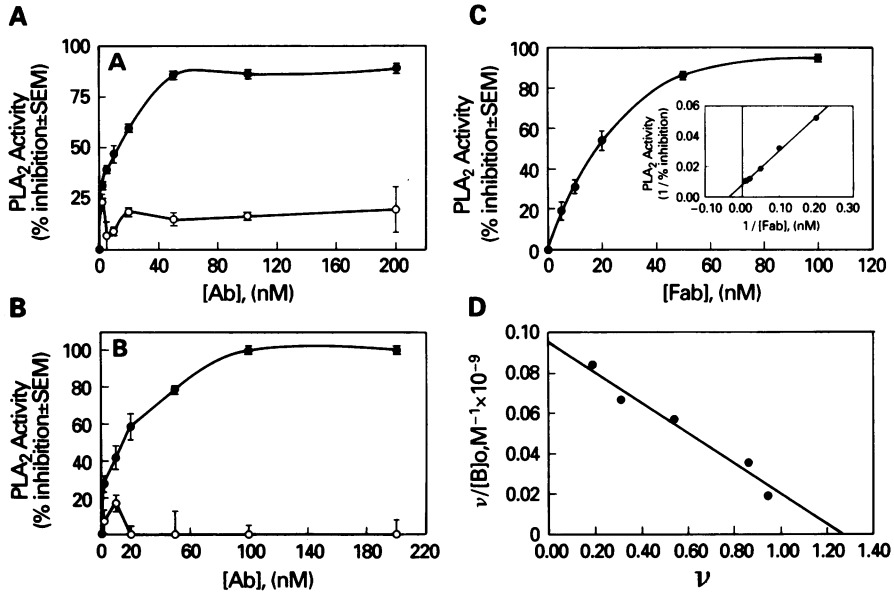

FIG. 1. Inhibition of porcine pancreatic PLA_2 by affinity-purified anti-PLA_2 IgG and Fab fragments. (*A* and *B*) Affinity-purified anti-PLA_2 IgG (●) and rabbit preimmune IgG (○) were tested for PLA_2 inhibitory activity in the mixed micellar assay (*A*) and the *E. coli* assay (*B*). Antibody concentrations ([Ab]) are final concentrations in the assay mixture. (*C*) Dose–response curve of PLA_2 inhibition by FPLC-purified Fab fragment in the mixed micellar assay. Fab concentrations are final concentrations in the assay mixture. (*Inset*) Double-reciprocal plot obtained from these data. (*D*) Scatchard analysis of the Fab dose–response curve in *C*. $[B]_o$ = molar concentration of free ligand (Fab); ν = fractional saturation (moles of Fab bound per mole of PLA_2). In all experiments each point represents the average of at least three determinations, each performed in duplicate.

for the purified Fab. Virtually identical results were obtained with $F(ab)_2$ fragments (data not shown). Scatchard analysis was performed on the data from Fig. 1*C* by assuming that PLA_2 molecules bound to neutralizing Fab are enzymatically inactive. Thus, the observed percent inhibition of enzyme activity is a good estimate of the percentage of PLA_2 molecules which are bound to neutralizing Fab at each antibody concentration. This plot (Fig. 1*D*) is linear (r = 0.98), with a calculated K_d of 13.24 ± 1.49 nM and a calculated n of 1.26 sites per molecule. A similar analysis of the data obtained with intact IgG yielded a calculated K_d of 12.38 ± 4.72 nM, with a calculated n of 0.84 site per PLA_2 molecule (data not shown). These data are consistent with the hypothesis that neutralizing Fab recognizes only one class of binding sites in PLA_2. When time of preincubation with affinity-purified antibody (28 nM) was varied, the maximum inhibitory effect was reached after 3 min of preincubation at 37°C and it remained approximately constant up to 10 min (data not shown). This suggests that a relatively rapid binding equilibrium is responsible for the observed inhibition of enzyme activity and that formation of large, precipitating immunocomplexes is not necessary for the inhibition. We then examined the time course of PLA_2 reaction (20 sec to 4 min) after 5 min of preincubation with antibody at 28 or 56 nM. In both cases the maximum inhibitory effect was observed within 30 sec of reaction (data not shown). After 60 sec the percent inhibition tended to decrease slightly and remained constant thereafter up to 4 min. This slight decrease might be due to a change of the antigen–antibody binding equilibrium as a result of the 2.5-fold dilution of the preincubation mixture when it is added to the substrate.

Steady-State Kinetics of PLA_2 Inhibition by Affinity-Purified IgG and FPLC-Purified Fab. Michaelis–Menten kinetics of IgG- or Fab-treated and untreated PLA_2 were studied with the mixed micellar assay (13). Fig. 2 *A* and *B* show the hyperbolic and double-reciprocal plots of PLA_2 kinetics after preincubation with affinity-purified IgG and FPLC-purified Fab, respectively. The two models of inhibition which best fit the data were (*i*) pure classic noncompetitive and (*ii*) pure mixed noncompetitive. In the pure classic noncompetitive model, the inhibitor has identical K_i values for free enzyme (E) and the enzyme–substrate complex (ES). In the pure mixed noncompetitive model, the two K_i values for E and ES are different. Our data fit model *i* slightly better than model *ii*, but the difference is not statistically significant. Model *i* was used in the preparation of Fig. 2. The calculated K_i values were 16.6 ± 2.8 nM for intact antibodies and 32.2 ± 4.16 nM for Fab fragments. The value obtained for Fab fragments is almost exactly double the value for intact antibodies. If inhibitor concentrations were expressed in moles of antigen-binding sites per liter, the two K_i values would be virtually identical within the experimental error. In addition, the apparent K_i values calculated from kinetic experiments are in good agreement with the apparent K_i values obtained from inhibitor dose–response curves and with apparent K_d values calculated by Scatchard analysis. This suggests that the assumption used to construct the Scatchard plot (i.e., that antibody-bound PLA_2 molecules can be considered enzymatically inactive) is correct. All in all, the dose–response, time-course, and kinetic data strongly support the hypothesis that inhibition of PLA_2 activity results from the formation of soluble immunocomplexes containing one molecule of IgG or Fab bound to one molecule of enzyme and that this inhibition is noncompetitive with respect to the phospholipid substrate.

Epitope Mapping Studies. The proteolytic fragments obtained by digesting porcine pancreatic PLA_2 with *S. aureus* V8 endopeptidase or CNBr were separated by modified SDS/PAGE (22) and antibody binding to these fragments was detected by Western blot. Two identical blots were prepared for each experiment. One of them was stained with affinity-purified PLA_2 antibody while the other was stained with Coomassie blue. Coomassie-stained bands that corresponded to immunoreactive bands in matching blots were excised and subjected to microsequencing. Fig. 3 *A* and *B* show the results of V8 endopeptidase mapping. The smallest immunoreactive peptide is at 4–5 kDa. Another band of lower apparent molecular mass was stained by Coomassie blue but was not recognized by the antibody. This latter band pre-

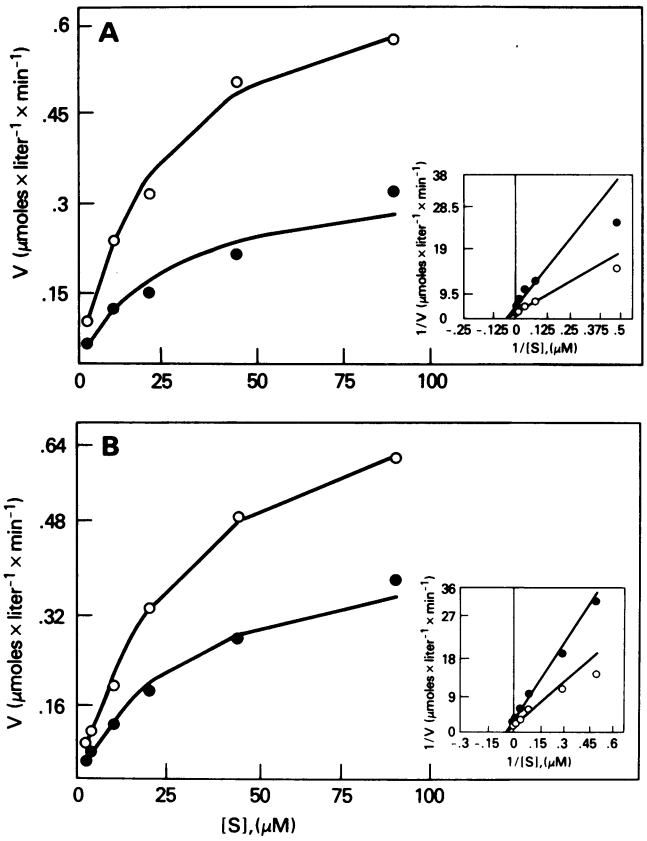

FIG. 2. Kinetic analysis of PLA_2 inhibition by affinity-purified IgG and FPLC-purified Fab. (*A*) Hyperbolic plots of reaction velocity of PLA_2 preincubated with buffer (○) or with affinity-purified IgG (●). (*Inset*) Double-reciprocal plots from the same data. (*B*) Hyperbolic plots of reaction velocity of PLA_2 preincubated with buffer (○) or with FPLC-purified Fab (●). (*Inset*) Double-reciprocal plots from the same data. Each point represents the average of at least three determinations, each performed in duplicate. Standard errors were <10%.

sumably corresponds to a mixture of fragments 47–71 and 93–114, which differ by only two residues in length and most likely cannot be resolved electrophoretically. The N-terminal sequence (15 residues) of the immunoreactive fragment was determined and found to be identical to that of the intact PLA_2. Thus, the immunoreactive fragment includes residues 1–40 (theoretical molecular mass, about 4.8 kDa) or residues 1–46 (theoretical molecular mass, about 5.5 kDa). This fragment includes residues forming two functionally important regions of PLA_2: the N-terminal α-helix and part of the "Ca^{2+}-binding loop" (4, 26). The results of CNBr mapping are shown in Fig. 3 *C* and *D*. CNBr can cleave porcine pancreatic PLA_2 at positions 8 and 20, generating three fragments including residues 1–8, 9–20, and 21–124. Two main immunoreactive bands, of apparent molecular masses 14.5 and 16 kDa, respectively, were observed in a Western blot of CNBr-digested PLA_2. The lower band is identical in electrophoretic mobility to intact PLA_2 and it corresponds to undigested enzyme. Its amount was insufficient for N-terminal sequence determination. The band with slower electrophoretic mobility was identified by N-terminal sequencing (13 residues) and corresponds to fragment 21–124. This band showed a paradoxical shift in electrophoretic mobility; the loss of the hydrophobic residues in the N-terminal α-helix may reduce the ability of fragment 21–124 to bind SDS and, thus, alter the charge/mass ratio of the SDS–protein complex compared with intact PLA_2. This observation indicates that loss of residues 1–20 does not appreciably affect the recognition of PLA_2 fragment 21–124 by our antibody in a Western blot. These results supported the hypothesis that the region including residues 21–40 (or 21–46) contains the main epitope(s) of PLA_2 recognized by our antibody. In addition, preincubating the enzyme with deoxycholate micelles before adding anti-PLA_2 IgG did not affect the PLA_2-inhibitory activity of the antibody (data not shown). This rules out an interaction of the antibody with the positively charged side chains in the C-terminal region which are of paramount importance for the binding of PLA_2 to negatively charged interfaces (27). When affinity-purified IgG (1 ml of a 160 nM solution) was preadsorbed at 4°C overnight with porcine pancreatic PLA_2 (12.5 μg) bound to a PVDF membrane, the PLA_2 inhibitory activity of the IgG was completely abolished (data not shown).

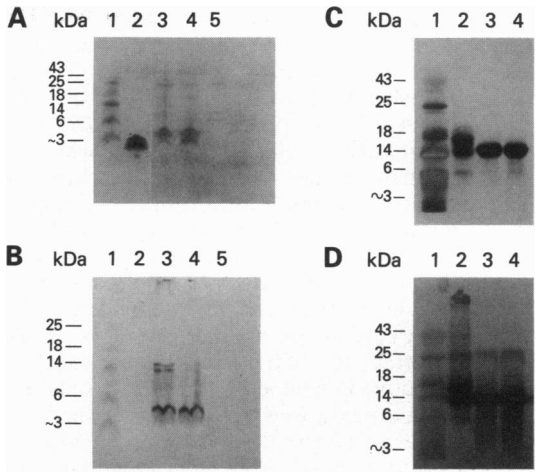

FIG. 3. Peptide mapping. (*A* and *B*) *S. aureus* V8 protease mapping. Duplicate PVDF membranes were stained with Coomassie blue (*A*) or immunostained with affinity-purified anti-PLA_2 IgG (*B*). Lanes: 1, prestained molecular size standards (BRL); 2, bradykinin (10 μg); 3, digestion mixture after 2 hr of incubation; 4, overnight digestion: 5, V8 protease alone incubated overnight (5 μg); (*C* and *D*) CNBr cleavage of PLA_2. Duplicate PVDF membranes were Coomassie blue-stained (*C*) or immunostained (*D*). Lanes: 1, prestained molecular size standards (BRL); 2, CNBr-digested PLA_2 (10 μg); 3, PLA_2 (10 μg) incubated with water; 4, PLA_2 (10 μg) incubated with formic acid. The minor low molecular mass bands, present in all three samples, are probably degradation products. The ≈25-kDa band, also present in all three samples, is observed in our hands in overloaded SDS/polyacrylamide gels and Western blots of porcine pancreatic PLA_2. This band may represent aggregates of PLA_2 (presumably dimers) whose formation is induced by negatively charged detergents (4).

Studies with Synthetic Peptides. Synthetic peptides representing PLA_2 residues 1–16, 21–40, 46–70, and 109–124 were tested for their ability to compete with intact PLA_2 when incubated with PLA_2 antibody (Table 1). When these peptides were incubated with PLA_2 antibody (125 nM) in the

Table 1. Synthetic peptides used in competition experiments

Amino acid sequence	PLA_2 residues	Effect in abolishing PLA_2 inhibition by antibody
ALWQFRSMTKCAIPGS	1–16	–
DFNNYGCYCGLGGSGTPVDE	21–40	+
ETHDNSYRDAKNLDSSKFLVDNPYT*	46–70	–
APYNKEHKNLDTKKYC	109–124	–

All peptides were tested in triplicate at a final concentration of 250 μM.
*Cysteine (C) residues were replaced with serine (S) in this peptide.

presence of 5 nM PLA_2, peptide 21–40 competed with intact PLA_2, causing a concentration-dependent reduction of the inhibitory activity of the antibody. Peptide 21–40 at 250 μM completely abolished the inhibitory activity of the antibody. Peptide 21–40 at 25 and 125 μM caused 26% and 66% reduction of inhibitory activity, respectively, while concentrations lower than 25 μM were ineffective. Freezing and thawing of peptide solutions (5 mM) resulted in a partial loss of activity. Peptides 1–16, 46–70, and 109–124 did not significantly affect the inhibitory activity of the antibody. It should be noted that (*i*) peptide 21–40 required preheating at 56°C for 10 min in order to compete with PLA_2; (*ii*) when incubated with PLA_2 in the absence of antibody, peptide 21–40 did not affect the activity of the enzyme; (*iii*) this peptide was effective in competing with PLA_2 only in the *E. coli* assay. These observations indicate that (*a*) heat-induced noncovalent interactions (possibly intermolecular) or the formation of disulfide bonds in peptide 21–40 may stabilize the antigenic conformation of this peptide; (*b*) the effect of the peptide is not due to direct stimulation of PLA_2 activity; and (*c*) deoxycholate either disrupts the antigen–antibody complexes formed by this peptide or affects the structure of the peptide itself, rendering it incapable of binding the antibody in the mixed micellar assay. Finally, since peptide 21–40 contains several residues which are part of the Ca^{2+}-binding loop of PLA_2, we investigated the possibility that Ca^{2+} concentration may affect PLA_2 inhibition by our antibody. We preincubated PLA_2 with IgG in the absence or presence of various concentrations of Ca^{2+}. The percent inhibition with 50 nM IgG increased significantly with increasing Ca^{2+} (from 64 ± 0.99 in the absence of Ca^{2+} to 77 ± 1.47 in the presence of 1 mM Ca^{2+}). Above 1 mM Ca^{2+}, the percent inhibition remained approximately constant up to 6 mM (data not shown). The published K_d of the Ca^{2+}-binding loop in porcine pancreatic PLA_2 is 2–4 mM (4). This observation might indicate that our neutralizing antibody has a higher affinity for Ca^{2+}-bound PLA_2 than for Ca^{2+}-free enzyme.

In conclusion, our data provide strong evidence that the epitope(s) recognized by our inhibitory antibodies resides in the conserved region of PLA_2 between positions 21 and 40. This region is also highly conserved in mammalian group II PLA_2s, which have been suggested to be involved in several important human diseases (28). X-ray crystallographic data on recombinant human synovial PLA_2 have confirmed that the structure of this enzyme is similar to that of the pancreatic and snake venom PLA_2s (29). In particular, the Ca^{2+}-binding loop is almost identical to that of the bovine pancreatic PLA_2 (29). The present study identifies a specific region of porcine pancreatic PLA_2 which interacts with a neutralizing antibody, leading to a virtually complete inhibition of the activity of this enzyme. These data suggest that the conserved "core" region of PLA_2 including most of the Ca^{2+}-binding loop and the exposed residues at the edge of the active site may be a potential target for developing selective noncompetitive inhibitors of extracellular PLA_2s. This information, in combination with the structural data on human synovial PLA_2 (29), might be useful for the rational design of specific inhibitors of this enzyme. Our observations also imply that recombinant monoclonal IgG, Fab, or Fv fragments directed against the appropriate region(s) of PLA_2, synthetic oligopeptides, or peptidomimetics derived from the complementarity-determining regions of such monoclonal immunoglobulins (30) may serve as model PLA_2 inhibitors in pharmacological studies. One possible application of such inhibitors may be the neutralization of circulating PLA_2 in septic shock.

1. Pruzanski, W. & Vadas, P. (1990) *Adv. Exp. Med. Biol.* **279**, 239–251.
2. Vadas, P. & Pruzanski, W. (1986) *Lab. Invest.* **55**, 391–404.
3. Mukherjee, A. B., Cordella-Miele, E. & Miele, L. (1992) *DNA Cell Biol.* **11**, 233–243.
4. Waite, M. (1987) *The Phospholipases: Handbook of Lipid Research* (Plenum, New York).
5. O'Flaherty, J. T. & Wykle, R. L. (1983) *Clin. Rev. Allergy* **1**, 353–367.
6. Axelrod, J., Burch, R. M. & Jelsema, C. L. (1988) *Trends Neurol. Sci.* **11**, 117–123.
7. Burch, R. M. (1990) *Adv. Exp. Med. Biol.* **279**, 185–195.
8. Cordella-Miele, E., Miele, L. & Mukherjee, A. B. (1993) in *New Perspectives in Endocrinology* (Raven, New York), Vol. 99, pp. 95–107.
9. Mayer, R. J. & Marshall, L. A. (1993) *FASEB J.* **7**, 339–348.
10. Crowl, R., Stoller, T. J., Conroy, R. R. & Stoner, C. (1991) *J. Biol. Chem.* **266**, 2647–2651.
11. Oka, S. & Arita, H. (1991) *J. Biol. Chem.* **266**, 9956–9960.
12. Asaoka, Y., Yoshida, K., Sasaki, Y., Nishizuka, Y., Murakami, M., Kudo, I. & Inoue, K. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 716–719.
13. Cordella-Miele, E., Miele, L. & Mukherjee, A. B. (1990) *J. Biol. Chem.* **265**, 17180–17188.
14. Goding, J. W. (1976) *J. Immunol. Methods* **13**, 215–226.
15. Mandy, W. J. & Nisonoff, A. (1963) *J. Biol. Chem.* **238**, 206–211.
16. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254.
17. Facchiano, A., Cordella-Miele, E., Miele, L. & Mukherjee, A. B. (1991) *Life Sci.* **48**, 453–464.
18. Lutz, R. A., Bull, C. & Rodbard, D. (1986) *Enzyme* **36**, 197–206.
19. Haigler, H. T., Schlaepfer, D. D. & Burgess, W. H. (1987) *J. Biol. Chem.* **262**, 6921–6930.
20. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
21. Carrey, E. A. (1989) in *Protein Structure: A Practical Approach*, ed. Creighton, T. E. (IRL, Oxford), pp. 117–144.
22. Schagger, H. & von Jagow, G. (1987) *Anal. Biochem.* **166**, 368–379.
23. Matsudaira, P. T. (1987) *A Practical Guide to Protein and Peptide Purification for Microsequencing* (Academic, New York).
24. Hewick, R. M., Hunkapiller, M. W., Hood, L. E. & Dreyer, W. J. (1981) *J. Biol. Chem.* **256**, 7990–7997.
25. Andrews, P. C. & Dixon, J. E. (1987) *Anal. Biochem.* **161**, 524–528.
26. Dijkstra, B. W., Kalk, K. H., Hol, W. G. J. & Drenth, J. (1981) *J. Mol. Biol.* **147**, 97–123.
27. Kuipers, O. P., van den Bergh, C. J., Verheij, H. M. & de Haas, G. H. (1990) *Adv. Exp. Med. Biol.* **279**, 65–84.
28. Crowl, R., Stoner, C., Stoller, T., Pan, Y. C. & Conroy, R. (1990) *Adv. Exp. Med. Biol.* **279**, 173–184.
29. Wery, J. P., Schevitz, R. W., Clawson, D. K., Bobbitt, J. L., Dow, E. R., Gamboa, G., Goodson, T., Jr., Hermann, R. B., Kramer, R. M., McClure, D. B., Mihelich, E. D., Putnamm, J. E., Sharp, J. D., Stark, D. H., Teater, C., Warrick, M. W. & Jones, N. D. (1991) *Nature (London)* **352**, 79–82.
30. Williams, W. V., Moss, D. A., Kieber-Emmons, T., Cohen, J. A., Myers, J. N., Weiner, D. B. & Green, M. I. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 5537–5541.