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## Effects of Phospholipase A<sub>2</sub> on Gastric Microsomal H<sup>+</sup>,K<sup>+</sup>-ATPase System: Role of "Boundary Lipids" and the Endogenous Activator Protein<sup>†</sup>

Jyotirmoy Nandi, Mark V. Wright, and Tushar K. Ray\*

**ABSTRACT:** Pig gastric microsomal vesicles enriched in gastric H<sup>+</sup>,K<sup>+</sup>-ATPase and K<sup>+</sup>-pNPPase were digested with bee venom phospholipase A<sub>2</sub> at 21 or 37 °C. The unattacked phospholipids were then related to the remaining enzyme activities, followed by reconstitution with microsomal phospholipids and the endogenous activator protein. Gastric K<sup>+</sup>-stimulated ATPase was nearly abolished within 10 min of phospholipase A<sub>2</sub> treatment. A substantial amount of pNPPase activity remained unaffected under identical conditions. About 80% of the microsomal phosphatidylethanolamine was attacked by phospholipase A<sub>2</sub> at both temperatures while 60 and 79% of the phosphatidylcholine was hydrolyzed at 21 and 37 °C, respectively. Analysis of the phospholipids revealed that phospholipase A<sub>2</sub> attacked only the phosphatidylcholine and phosphatidylethanolamine molecules enriched in polyunsaturated fatty acids. Microsomal H<sup>+</sup>,K<sup>+</sup>-ATPase

system inactivated by phospholipase A<sub>2</sub> at 21 °C could be largely restored by the endogenous activator alone. On the other hand, those inactivated at 37 °C needed pretreatment with phosphatidylcholine before assaying with the activator protein for maximal reconstitution; phosphatidylethanolamine was totally ineffective in restoration of the enzyme activity. Analysis of the fatty acid composition of the lyso-phosphatidylcholine following phospholipase A<sub>2</sub> treatment at 21 and 37 °C suggested involvement of some phosphatidylcholine molecules relatively enriched in saturated fatty acids and extremely poor in polyunsaturated fatty acids in gastric ATPase function. The data not only pointed out the importance of phosphatidylcholine and the endogenous activator in gastric microsomal H<sup>+</sup>,K<sup>+</sup>-ATPase reaction but also demonstrated considerable heterogeneity within the same species of microsomal phospholipids.

**P**hospholipids are known to play critical roles in the function of membrane-bound transport ATPases. Several reports (Hesketh et al., 1976; Hidalgo et al., 1976; Moore et al., 1981) suggest that both the fatty acid side chains of the phospholipids are important for the operation of the ion transport enzymes. Thus, phosphatidylcholine with unsaturated or disordered acyl side chains has recently been reported to be necessary for the optimal activity of the sarcoplasmic reticular Ca<sup>2+</sup>-stimulated ATPase (Moore et al., 1981). The authors (Moore et al., 1981) suggested that a "fluid" membrane bilayer containing unsaturated phospholipid acyl chains is critically important for the conformational changes necessary during the operation of the enzyme.

Recent reports (Sen & Ray, 1979a, 1980; Ray et al., 1980) from this laboratory suggested an important role of the membrane phosphatidylcholine in maintaining the activity and stability of the gastric microsomal H<sup>+</sup>,K<sup>+</sup>-ATPase system, which has recently been identified as the pumping mechanism for the transport of protons. Also, the fatty acid side chains of the phospholipid appeared to be critical in the gastric ATPase function (Sen & Ray, 1980).

The present study was undertaken to further investigate the lipid environment of the gastric H<sup>+</sup>,K<sup>+</sup>-ATPase, using phospholipase A<sub>2</sub> treatment at different temperatures. The ability of phospholipase A<sub>2</sub> to inactivate the microsomal H<sup>+</sup>,K<sup>+</sup>-ATPase system was related to the nature and extent of the various phospholipids hydrolyzed. Subsequent reconstitution of the phospholipase A<sub>2</sub> treated enzyme was studied under different conditions. Our data demonstrate that while the enzyme activity was abolished by phospholipase A<sub>2</sub> treatment at both 21 and 37 °C, the lipid environment was severely affected only at 37 °C. Thus, activity of the microsomal

H<sup>+</sup>,K<sup>+</sup>-ATPase inactivated by phospholipase A<sub>2</sub> at 21 °C could be largely restored by the cytosolic activator protein (Ray, 1978) in absence of any added phospholipid. Reconstitution of the enzyme treated with phospholipase A<sub>2</sub> at 37 °C necessitated the preexposure to phosphatidylcholine before assaying with the activator protein. Phosphatidylethanolamine was without any effect. Analysis of the fatty acid composition of the residual phospholipids suggested intimate involvement of some phosphatidylcholine species consisting predominantly of 16:0, 18:1, and 18:2 in the gastric ATPase function. The data also suggest that the activator factor is an extrinsic protein and is critical for the operation of the gastric H<sup>+</sup>,K<sup>+</sup>-ATPase system.

### Materials and Methods

**Materials.** Tris,<sup>1</sup> Pipes, EDTA, sucrose, ATP, pNPP, fat-free BSA, and bee venom phospholipase A<sub>2</sub> were purchased from Sigma Chemical Co., St. Louis, MO. TCA, chloroform, methanol, acetone, and acetic acid were purchased from Fisher Scientific. Silica gel H was from E. Merck, West Germany. All other reagents and chemicals used were the best grade available from the market.

**Isolation of Gastric Microsomes.** Purified microsomal membranes from pig gastric mucosa were prepared by the method of Ray (1978). Briefly, the fundic mucosa from pig was desquamated and scraped (Forte et al., 1972) to collect the oxyntic cell-enriched fractions. The mucosal cells were homogenized in 0.25 M sucrose containing 0.2 mM EDTA and 2.0 mM 1,4-piperazinediethanesulfonic acid (Pipes) buffer (pH 6.8) by using a loose-fitting pestle in a Dounce homogenizer. The homogenate was centrifuged at 8000g for 5 min. The process was repeated 3 times. All supernatants were pooled together and layered over 40 mL of 37% (w/v) sucrose

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<sup>1</sup> Abbreviations: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; Pipes, 1,4-piperazinediethanesulfonic acid; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; TLC, thin-layer chromatography.

in 84-mL screw-cap tubes and centrifuged at 100000g for 5 h in a type 35 Beckman angle rotor. The microsomal membrane band appeared at the interface of the clear soluble supernatant and 37% sucrose. The soluble supernatant was used as the source of the activator protein for K<sup>+</sup>-stimulated ATPase (Ray, 1978). The membrane bands were collected, diluted with homogenizing medium, and centrifuged at 100000g for 90 min. The pellet was resuspended in homogenizing medium at an appropriate protein concentration and used for the study. Proteins were assayed by the method of Lowry et al. (1951) with bovine serum albumin as standard.

**Preparation of Partially Purified Activator Protein.** The soluble supernatant of the pig gastric cells was dialyzed exhaustively against 2 mM Pipes buffer (pH 6.8) containing 0.2 mM dithiothreitol at 0–4 °C for 48 h with two changes. The dialyzed supernatant was then carefully titrated with 1 N acetic acid to pH 4.8 at 4 °C. The precipitate was collected by centrifugation, dissolved in an appropriate volume of the fresh dialyzing buffer as above, and used as the source of the activator for the K<sup>+</sup>-stimulated ATPase (Sen & Ray, 1980).

**Digestion of Gastric Microsomes with Phospholipase A<sub>2</sub>.** Phospholipase A<sub>2</sub> from bee venom was the highest purity grade commercially available (Sigma Chemical Co.). SDS gel electrophoresis of the phospholipase A<sub>2</sub> gave a single band of about 22 000 daltons. Consistent with the report of others (Saccomani et al., 1979), the bee venom enzyme was found to be specific for the 2-position of phosphatidylcholine and phosphatidylethanolamine. Also, with the help of a highly sensitive method (Wiesner & Troll, 1982) using a fluorescent-labeled [2-methoxy-2,4-diphenyl-3(2H)-furanone] casein as substrate, no protease activity could be detected under conditions of our digestion in the phospholipase A<sub>2</sub> preparation against a trypsin standard. The latter observation is consistent with the fact that no change in SDS gel pattern of the microsomes could be identified following phospholipase A<sub>2</sub> treatment.

Purified gastric microsomes (1 mg) were treated with 130 IU of phospholipase A<sub>2</sub> (sp act. 1800 IU/mg of protein) at 21 or 37 °C in 1 mL of a medium consisting of 250 mM sucrose, 2 mM CaCl<sub>2</sub>, and 20 mM Tris-HCl buffer (pH 7.4) for different time periods. The reactions were stopped by diluting the incubation medium 30-fold with ice-cold 250 mM sucrose–0.2 mM EDTA–20 mM Tris buffer (pH 7.4). After centrifugation for 60 min at 150000g, the pellet was washed once in the calcium-free buffer by suspension and centrifugation. The pellet was resuspended in an appropriate volume of the calcium-free sucrose–EDTA–Tris buffer (pH 7.4) as above and assayed for K<sup>+</sup>-stimulated ATPase and (*p*-nitrophenyl)phosphatase (pNPPase) activities. In some studies, the phospholipase A<sub>2</sub> digested membranes were washed with the calcium-free medium as above containing 1% fat-free BSA with a view to bind the released fatty acids. Control membranes were treated under identical conditions in the absence of any phospholipase A<sub>2</sub>. The possibility of the phospholipase preparation having any contaminant inhibitory to the gastric ATPase system was also checked by appropriate experiments. Thus, the membranes were exposed to phospholipase in sucrose–Tris–EDTA medium as above without any Ca<sup>2+</sup> and immediately diluted 30-fold with the same ice-cold buffer. Following high-speed centrifugation, the pellet was washed once again by resuspension and centrifugation and checked for the enzyme activities. No difference in enzyme activities was observed before and after such treatment.

**Assay of ATPase and pNPPase.** The ATPase was assayed as previously described (Sen & Ray, 1980). The incubation

mixture contained, in a total volume of 1 mL, 50 μmol of Pipes (pH 6.8), about 50 μg of the activator protein, 1 μmol of MgCl<sub>2</sub>, 2 μmol of Tris–ATP, and 10 μg of membrane protein in the presence or absence of 5 μmol of KCl. After 10-min preincubation at 37 °C, the reactions were started with 2 μmol of Tris–ATP and incubated for 15 min. The reactions were stopped by adding 1 mL of 12% (w/v) trichloroacetic acid. The P<sub>i</sub> was assayed by the procedure of Sanui (1974). The K<sup>+</sup>-stimulated ATPase activity was calculated by subtracting the basal rate (with Mg<sup>2+</sup> as the only cation) from the rate of hydrolysis of ATP in presence of both Mg<sup>2+</sup> and K<sup>+</sup>.

The pNPPase was assayed at 37 °C in 1 mL of a medium consisting of 50 μmol of Tris–HCl (pH 7.4), 2 μmol of MgCl<sub>2</sub>, 5 μmol of pNPP, and 10 μg of membrane protein in presence and absence of the activator protein with and without 20 μmol of KCl. After 20-min incubation, the reactions were stopped by 1 N NaOH. After a brief centrifugation, the supernatant was read at 410 nm.

**Reconstitution of Phospholipase A<sub>2</sub> Treated Enzymes.** Assay of the enzymes in presence of the endogenous activator was found to be essential for near complete restoration of the phospholipase A<sub>2</sub> inactivated enzymes. Contrary to the microsomal ATPase system treated with phospholipase A<sub>2</sub> at 21 °C, those treated at 37 °C needed prior exposure to phosphatidylcholine before assaying with the activator for maximal activity. Various phospholipids (12 mg/mL) isolated from gastric microsomes were dispersed in 10 mM Pipes buffer (pH 6.8) containing 0.5 mM EDTA and 0.2 mM dithiothreitol by sonication for 10 min at 37 °C as described previously (Sen & Ray, 1980). Equal volumes of the phospholipid suspension and the phospholipase A<sub>2</sub> treated microsomes were mixed and incubated for 15 min at 37 °C with frequent vortexing under nitrogen atmosphere. The incubation mixture was then diluted 8-fold with ice-cold 250 mM sucrose–20 mM Pipes–2 mM EDTA buffer (pH 6.8). Aliquots (0.1 mL) were immediately assayed for the enzyme activities in the presence and absence of the activator protein. The control membranes were run in parallel all throughout the procedure and assayed for the enzymes.

**Extraction of Lipids.** The lipids were extracted from the control and phospholipase A<sub>2</sub> treated microsomes by the method of Bligh & Dyer (1959). A nitrogen atmosphere was maintained during extraction to prevent serial oxidation. Lipid phosphorus was assayed by the Bartlett (1959) procedure.

**Separation, Identification, and Quantitation of Phospholipids.** The phospholipids were separated by two-dimensional TLC (silica gel H, Sigma) according to the procedure of Rouser et al. (1967). The solvent systems used were as follows: first dimension, chloroform–methanol–concentrated ammonia (65:35:5 v/v); second dimension, chloroform–methanol–acetic acid–water (50:10:20:10:5 v/v). The two-dimensional TLC plates were charred following the procedure of Rouser et al. (1967). The spots were marked, scraped off, transferred into microkjeldahl flasks, and digested with 0.9 mL of perchloric acid. Appropriate blank spots were run in parallel. After digestion, the inorganic phosphate was estimated colorimetrically (Bartlett, 1959).

Bulk quantities of the gastric microsomal phosphatidylcholine and phosphatidylethanolamine necessary for the reconstitution studies were isolated in purified form by preparative thin-layer chromatography (silica gel H) in chloroform–methanol–water (65:25:4, v/v) and chloroform–methanol–acetic acid–water (25:15:4:2 v/v) solvent systems as described previously (Sen & Ray, 1979a,b). Characterization of the phospholipids was made by comparing the R<sub>f</sub> values with

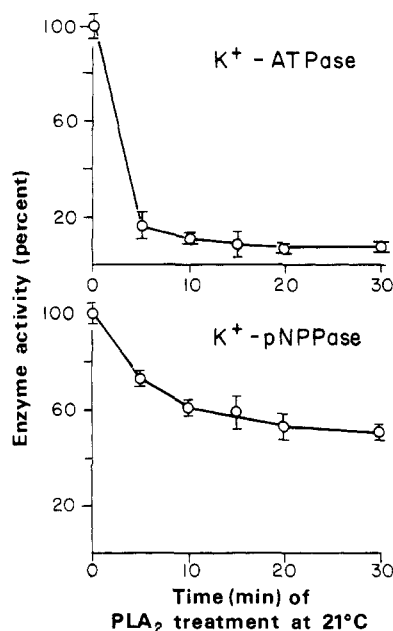


FIGURE 1: Effects of digestion of pig gastric microsomes with phospholipase  $A_2$  at 21 °C for increasing length of time on gastric  $H^+, K^+$ -ATPase and pNPPase activity. The control membranes (time zero) were run in parallel under conditions identical with those of the treated membranes (see Materials and Methods) without any phospholipase  $A_2$ . The activity [ $\mu\text{mol}/(\text{mg}\cdot\text{h})$ ] of the  $K^+$ -stimulated ATPase and pNPPase of the control membranes was  $52 \pm 5$  and  $25 \pm 4$ , respectively. Details of phospholipase  $A_2$  treatment and enzyme assays are given under Materials and Methods. Data are mean  $\pm$  SD ( $n = 6$ ).

authentic standards and by using specific spray reagents such as ninhydrin for amino group containing lipids, Dragendorff reagent (Wagner et al., 1961) for choline-containing lipids, and molybdenum blue (Dittmer & Lester, 1964) for phospholipids in general.

**Assay of Fatty Acids.** Fatty acids were obtained from individual phospholipids after mild alkaline hydrolysis followed by acidification with 1 N acetic acid (Kates, 1972). The fatty acids thus obtained were esterified with diazomethane (Metcalf & Schmitz, 1961). After evaporation of the solvent, the fatty acid composition was determined by gas chromatography as previously described (Sen & Ray, 1979a,b).

## Results

**Effects of Phospholipase  $A_2$  Treatment on Gastric Microsomal  $H^+, K^+$ -ATPase System.** Both gastric  $K^+$ -stimulated ATPase and pNPPase activities were reduced by phospholipase  $A_2$  digestion at 21 (Figure 1) and 37 °C (Figure 2). However, the  $H^+, K^+$ -ATPase activity was far more sensitive to phospholipase  $A_2$  inactivation than  $K^+$ -pNPPase (Figures 1 and 2) at both temperatures. Thus, the  $H^+, K^+$ -ATPase activity was reduced by about 90 and 95% at 21 and 37 °C, respectively, at 10 min and remained the same thereafter while the  $K^+$ -pNPPase activity was inactivated to the extent of only 40 and 50% under similar conditions and was reduced further to about 55 and 25% of the original activity after 20 min of phospholipase  $A_2$  digestion.

Activity of phospholipase  $A_2$  is known to be dependent on the presence of a high concentration of  $\text{Ca}^{2+}$ , which is inhibitory to the  $H^+, K^+$ -ATPase system (Ray, 1978). Therefore, it was necessary to ensure that the membranes were free from interfering concentrations of  $\text{Ca}^{2+}$  after the phospholipase  $A_2$  reaction. This was achieved by repeated washing by centrifugation following dilution of the phospholipase  $A_2$  reaction medium containing 2  $\mu\text{mol}$  of  $\text{Ca}^{2+}$  with 20 mL of a calci-

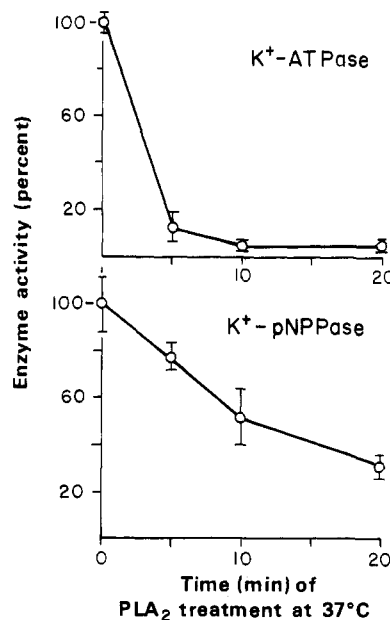


FIGURE 2: Effects of treatment of gastric microsomes with phospholipase  $A_2$  at 37 °C as a function of time on microsomal  $H^+, K^+$ -ATPase and pNPPase activity. The enzyme activities of the control membranes (zero time) were the same as given in Figure 1. Details of the phospholipase  $A_2$  digestion and enzyme assays are given under Materials and Methods. Data are mean  $\pm$  SD ( $n = 5$ ).

um-free Tris buffer (pH 7.4) containing about 6  $\mu\text{mol}$  of EDTA (see Materials and Methods). The control membranes treated with high  $[\text{Ca}^{2+}]$  as those of the phospholipase  $A_2$  treated groups showed full activity (Figures 4 and 5, control) following the EDTA washing as above, thus ensuring effective removal of  $\text{Ca}^{2+}$ . Furthermore, the extent of inhibition of the membrane-bound enzymes washed in the BSA-containing medium following phospholipase  $A_2$  digestion was nearly identical (unpublished data) with those shown in Figures 1 and 2.

**Effects of Phospholipase  $A_2$  Digestion on Integrity of Microsomal Vesicles.** Vesicular nature of the gastric microsomes as evidenced from the electron microscopic pictures (not shown) was totally lost after 20 min of phospholipase  $A_2$  digestion both at 37 and at 21 °C. Such disruption in vesicular integrity could be related to the massive conversion of the major microsomal phospholipids such as phosphatidylethanolamine and phosphatidylcholine to their respective lyso compounds (Figure 3).

**Composition of Gastric Microsomal Phospholipids before and after Phospholipase  $A_2$  Digestion.** Quantitation of the microsomal phospholipids revealed that phospholipase  $A_2$  attacked only the phosphatidylcholine (PC) and phosphatidylethanolamine (PE) without affecting sphingomyelin, phosphatidylinositol, and phosphatidylserine (Table I) at both temperatures. About 80% of the phosphatidylethanolamine is converted to the lyso compound at both 21 and 37 °C after 20 min of phospholipase  $A_2$  digestion. Under similar conditions, about 60 and 80% of the phosphatidylcholine is hydrolyzed at 21 and 37 °C, respectively. Similar to phospholipase  $A_2$ , the effects of phospholipase C digestion of gastric microsomes has also been reported (Olaissou et al., 1982) to hydrolyze about 80% of the total PC and PE without affecting the other major lipids. It should be noted that the phospholipid content of the control gastric microsomal vesicles (Table I) is closely similar to those reported by Saccomani et al. (1979) but somewhat lower than that reported by Schrijen et al. (1981) and significantly higher than those previously reported

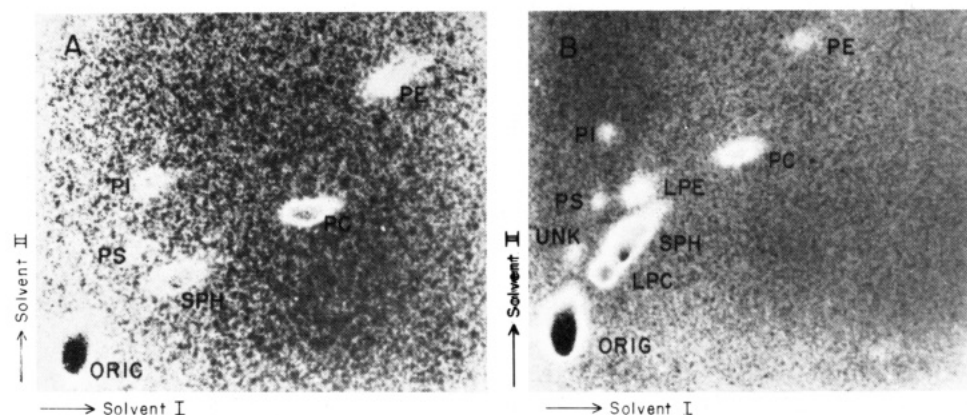


FIGURE 3: Two-dimensional thin-layer chromatogram of phospholipids derived from control and phospholipase A<sub>2</sub> treated microsomes: (first dimension) solvent I, chloroform-methanol-concentrated NH<sub>3</sub> (65:35:5 v/v); (second dimension) solvent II, chloroform-methanol-acetone-acetic acid-water (50:10:20:10:5 v/v). After the chromatogram was dried in fume hood, the plate was sprayed lightly with sulfuric acid-dichromate reagent (Rouser et al., 1967) as a fine mist and heated in a forced-draft oven at 180 °C for 60 min: (a) control; (b) phospholipase A<sub>2</sub> treated microsomes. The abbreviations used are the same as those in Table I. ORIG and UNK stands for origin and unidentified, respectively. Note that the unidentified spot was absent in the control membrane lipids (see Figure 4A).

Table I: Phospholipid Composition of Purified Pig Gastric Microsomes before and after Digestion with Phospholipase A<sub>2</sub> at 21 and 37 °C<sup>a</sup>

phospholipids	micromoles of phospholipids/100 mg of membrane protein		
	control	phospholipase A <sub>2</sub> treated at	
		21 °C	37 °C
total	80.1 ± 1.06	80.3 ± 0.9	80.5 ± 1.1
PE	19.2 ± 1.0	4.9 ± 0.8	4.0 ± 0.4
PC	24.2 ± 1.0	9.8 ± 1.0	5.2 ± 0.5
Sph	23.6 ± 0.4	23.2 ± 0.3	23.5 ± 0.6
PI	4.4 ± 0.1	4.5 ± 0.1	4.3 ± 0.1
PS	5.1 ± 0.4	5.8 ± 0.3	5.6 ± 0.4
LPE	0	14.0 ± 1.4	14.0 ± 1.3
LPC	0	17.8 ± 0.6	22.7 ± 0.6

<sup>a</sup> Treatment with phospholipase A<sub>2</sub> (130 IU) was carried out for 20 min at the indicated temperatures. The reactions were stopped by 20-fold dilution with ice-cold sucrose-Pipes-EDTA buffer (pH 6.8) followed by immediate centrifugation at 150000g for 60 min. The control (untreated) and the phospholipase A<sub>2</sub> treated membrane pellets were then extracted with CHCl<sub>3</sub>-CH<sub>2</sub>OH, and the individual phospholipids were estimated after separation on a two-dimensional TLC system as described under Materials and Methods. Abbreviations: PE, phosphatidylethanolamine; PC, phosphatidylcholine, Sph, sphingomyelin; PI, phosphatidylinositol; PS, phosphatidylserine; LPE, lysophosphatidylethanolamine; LPC, lysophosphatidylcholine. Data are mean ± SD (n = 4).

Table II: Fatty Acid Composition of the Membrane-Associated Phosphatidylethanolamine and/or Lysophosphatidylethanolamine after Phospholipase A<sub>2</sub> Digestion at 21 and 37 °C<sup>a</sup>

fatty acids	fatty acids (% of total)			
	untreated PE	phospholipase A <sub>2</sub> treated at 21 or 37 °C		
		PE (21 °C)	LPE (21 °C)	LPE (37 °C)
12:0	4.6 ± 0.04	2.5 ± 0.4	nd	8.56 ± 0.1
14:0	0.5 ± 0.12	1.1 ± 0.05	3.0 ± 0.3	14.6 ± 0.4
15:0	1.0 ± 0.07	0.95 ± 0.1	2.2 ± 0.3	7.7 ± 0.1
16:0	15.1 ± 0.3	30.2 ± 2.1	32.1 ± 0.1	34.0 ± 0.6
16:1	1.1 ± 0.1	nd	nd	nd
17:0	0.7 ± 0.1	1.5 ± 0.2	nd	nd
18:0	13.3 ± 0.13	36.5 ± 2.6	39.9 ± 0.36	23.3 ± 0.5
18:1	25.6 ± 0.7	25.7 ± 2.2	22.1 ± 0.2	11.2 ± 0.3
18:2	22.7 ± 1.0	nd ±	nd	nd
20:4	15.3 ± 1.4	nd	nd	nd

<sup>a</sup> PE and LPE stand for phosphatidylethanolamine and lysophosphatidylethanolamine, respectively. Untreated PE was the phosphatidylethanolamine isolated from control (without any phospholipase digestion) microsomes; hence, it represents total gastric microsomal PE. No lysophosphatidylethanolamine could be detected in control microsomes (Table I). nd stands for not detectable. Details on the phospholipase A<sub>2</sub> treatment, separation of phospholipids, and quantitation of the phospholipids and fatty acids are given under Materials and Methods. Data are mean ± SD (n = 4).

from our laboratory (Sen & Ray, 1979a,b). The low previous values have been traced to the use of a faulty digestion condition, leading to incomplete hydrolysis of the phospholipids to inorganic phosphate (Ray et al., 1983).

**Characterization of Fatty Acids of Phospholipase A<sub>2</sub> Attacked Phospholipids.** Consistent with our earlier report (Sen & Ray, 1979a,b), the fatty acid composition of both phosphatidylethanolamine (Table II) and phosphatidylcholine (Table III) of untreated control microsomes shows predominance of 16:0, 18:0, 18:1, 18:2, and 20:4. It is interesting that some of the minor fatty acids like 12:0, 14:0, and 15:0 were enriched to variable extent in lysophosphatidylethanolamine (Table II) and lysophosphatidylcholine (Table III) after phospholipase A<sub>2</sub> digestion at 21 and 37 °C. One noticeable aspect of the phospholipase A<sub>2</sub> attacked phosphatidylethanolamine molecules is that the lyso compounds were totally devoid of the polyunsaturated fatty acids like 18:2 and 20:4 (Table II), suggesting that these fatty acids are located ex-

clusively at the 2-position. The residual phosphatidylethanolamine molecules remaining unattacked by phospholipase A<sub>2</sub> at 21 °C also demonstrated absence of unsaturated fatty acids (Table II). These data in Table I suggest that there are at least two classes of phosphatidylethanolamine in gastric microsomes. Furthermore, bee venom phospholipase A<sub>2</sub> attacks more specifically those phosphatidylethanolamine molecules containing polyunsaturated fatty acids at the 2-position.

Unlike lysophosphatidylethanolamine (Table II), the lysophosphatidylcholine molecules associated with gastric microsomes treated with phospholipase A<sub>2</sub> at 21 and 37 °C show striking similarity in their fatty acid composition (Table III), suggesting that the same class of phosphatidylcholine molecules are attacked at both temperatures. The fatty acid composition of the residual phosphatidylcholine is also significantly different than the lyso compounds, suggesting at least two classes of this phospholipid in gastric microsomes. Predominance of

Table III: Fatty Acid Composition of the Membrane-Associated Phosphatidylcholine and/or Lysophosphatidylcholine following Phospholipase A<sub>2</sub> Digestion at 21 or 37 °C<sup>a</sup>

	fatty acids (% of total)			
	phospholipase A <sub>2</sub> treated at 21 or 37 °C			
	untreated PC	PC (21 °C)	LPC (21 °C)	LPC (37 °C)
12:0	1.0 ± 0.4	5.5 ± 0.3	11.8 ± 0.2	10.7 ± 0.2
14:0	1.6 ± 0.4	1.2 ± 0.2	12.7 ± 0.3	13.1 ± 0.2
15:0	1.8 ± 0.1	1.6 ± 0.5	5.7 ± 0.3	5.7 ± 0.2
16:0	36.3 ± 1.6	34.8 ± 2.2	42.3 ± 1.9	47.2 ± 1.0
16:1	0.5 ± 0.01	3.0 ± 0.4	1.6 ± 0.1	3.1 ± 0.6
17:0	0.4 ± 0.01	1.0 ± 0.1	0.5 ± 0.01	nd
18:0	9.4 ± 0.16	21.4 ± 1.0	12.5 ± 0.8	10.3 ± 0.4
18:1	18.5 ± 0.6	14.1 ± 0.7	5.6 ± 0.3	4.5 ± 0.5
18:2	26.5 ± 3.4	5.7 ± 0.6	trace	trace
20:4	6.1 ± 0.1	nd	nd	nd

<sup>a</sup> PC and LPC stand for phosphatidylcholine and lysophosphatidylcholine, respectively. Untreated PC was the phosphatidylcholine isolated from the control (without phospholipase A<sub>2</sub>) microsomes; hence, it represents total gastric microsomal PC. No lysophosphatidylcholine could be detected in control microsomes (Table I). nd stands for not detectable. The microsomes were digested under conditions identical with those in Table II. Details are given under Materials and Methods. Data are mean ± SD (n = 4).

saturated fatty acids in lysophosphatidylcholine (Table III) suggests that similar to its effect on phosphatidylethanolamine (Table II), phospholipase A<sub>2</sub> attacks more specifically those phosphatidylcholine molecules containing polyunsaturated fatty acids at the 2-position.

**Reconstitution of Phospholipase A<sub>2</sub> Treated Gastric Microsomal H<sup>+</sup>,K<sup>+</sup>-ATPase System.** The residual activities of the K<sup>+</sup>-stimulated ATPase and pNPPase associated with gastric microsomes treated with phospholipase A<sub>2</sub> at 21 °C for 20 min could be restored to the extent of over 80% of the original activity by assaying the enzymes in presence of the endogenous activator protein (Figure 4). The phospholipase A<sub>2</sub> digested membranes washed with fat-free BSA also showed similar activation by the activator protein (unpublished data). Phosphatidylcholine (Figure 4) or phosphatidylethanolamine (not shown) did not have any effect on the activator-mediated reconstitution under any conditions tested such as concentration, time of exposure with and without sonication, temperature, etc. Under the conditions of the phospholipase A<sub>2</sub> treatment (Figure 4), about 60% of the phosphatidylcholine and 75% of the phosphatidylethanolamine were hydrolyzed (Table I).

Since phospholipase A<sub>2</sub> did not have any detectable proteolytic activity when measured by a highly sensitive fluorescent technique (Wiesner & Troll, 1982) under conditions identical with those used for the inactivation of the gastric ATPase system (Figure 4), we can rule out any proteolytic inactivation (Ray & Sen, 1981) of the activator. However, phospholipase A<sub>2</sub> can directly inactivate the activator activity if the activator protein has a critical phospholipase A<sub>2</sub> sensitive component. Alternatively, phospholipase A<sub>2</sub> treatment may simply cause the release of the membrane-associated activator into the bulk medium without inactivating it. At present, we cannot distinguish between these two possibilities. In either case, however, the activator protein is expected to be released from the membrane phase. In a preliminary experiment, we observed that about 5% of the total membrane proteins with a heterogeneous protein composition was released from the membrane during 20 min of phospholipase A<sub>2</sub> digestion at 21 °C (unpublished data). Comparison of the SDS gel patterns

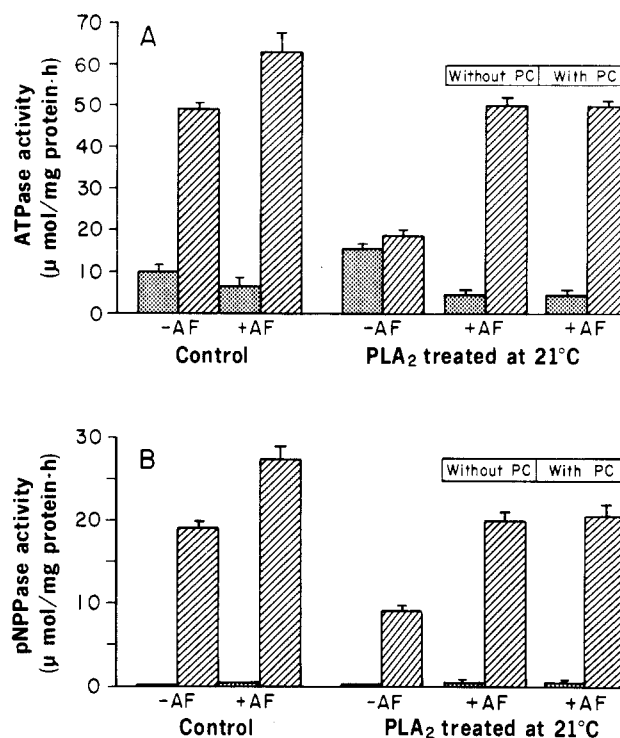


FIGURE 4: Reactivation of phospholipase A<sub>2</sub> treated gastric microsomal H<sup>+</sup>,K<sup>+</sup>-ATPase and K<sup>+</sup>-pNPPase by endogenous activator. Microsomes were treated with 130 IU of phospholipase A<sub>2</sub> at 21 °C for 20 min. About 100 μg of the partially purified activator protein was used for each assay. Details for the phospholipase A<sub>2</sub> treatment, the preparation of the partially purified activator, and the enzyme assays are given under Materials and Methods. (A) ATPase activity in the absence (open bars) and presence (hatched bars) of 5 mM K<sup>+</sup>; (B) pNPPase activity in the absence (open bars) and presence (hatched bars) of 20 mM K<sup>+</sup>. Data are mean ± SD (n = 8).

of the control and phospholipase A<sub>2</sub> treated membranes with those of the phospholipase A<sub>2</sub> extract and crude activator fraction suggested that one of the protein bands having molecular weights of 56 000, 65 000, or 79 000 was the activator. Since about 80% of the K<sup>+</sup>-ATPase is inactivated within 5 min of phospholipase A<sub>2</sub> digestion (Figure 1), it is expected that most of the activator will be preferentially released during such a short period. Thus, an approach using a brief phospholipase A<sub>2</sub> digestion may be helpful toward characterization of the activator in future.

Contrary to the gastric ATPase system inactivated at 21 °C (Figure 4), those treated at 37 °C need prior treatment with phosphatidylcholine for maximal reconstitution with the activator protein (Figure 5). About 80% of the K<sup>+</sup>-ATPase and 96% of the K<sup>+</sup>-pNPPase were restored by phosphatidylcholine plus activator compared to about 33 and 60%, respectively, in the presence of the activator alone (Figure 5). Phosphatidylethanolamine was totally ineffective as a substitute for phosphatidylcholine in the reconstitution of the gastric ATPase system (data not shown). Such inability of phosphatidylethanolamine may be due to the degradation to lysolipids by some phospholipase A<sub>2</sub> being retained by the membranes even after extensive washing. However, this seems to be highly unlikely due to the following reasons: Absence of Ca<sup>2+</sup> in the reconstitution and ATPase assay medium would keep the phospholipase A<sub>2</sub>, which is dependent on high Ca<sup>2+</sup>, virtually inactive. Due to our use of a large excess of phosphatidylethanolamine during reconstitution, any degradative loss arising from such residual phospholipase A<sub>2</sub> activity would not appreciably influence the reactivation. Another possibility is that phosphatidylethanolamine may bind poorly to the



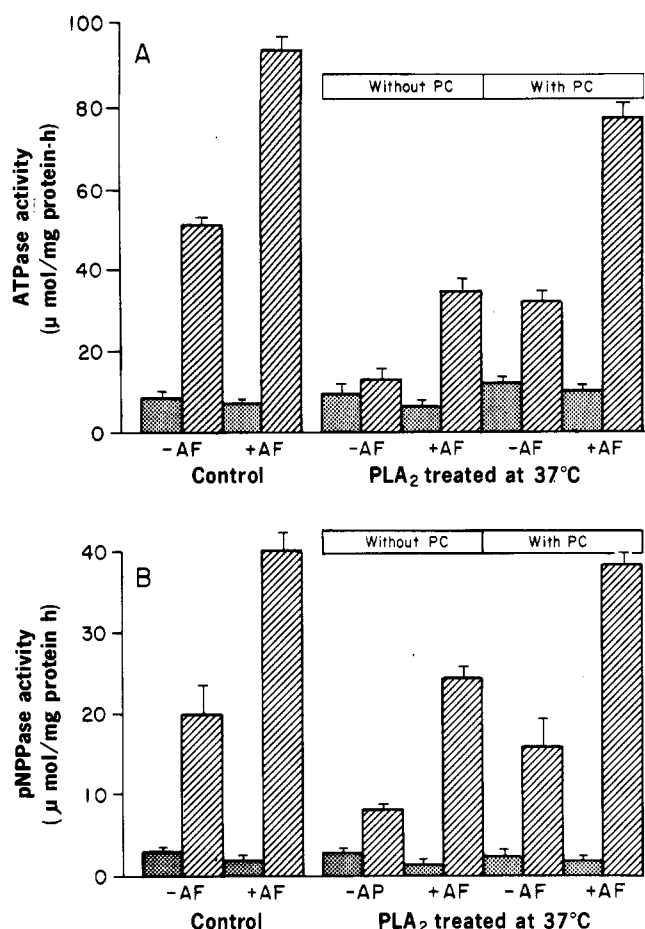


FIGURE 5: Reconstitution of gastric microsomal H<sup>+</sup>,K<sup>+</sup>-ATPase and K<sup>+</sup>-pNPPase inactivated by phospholipase A<sub>2</sub> treatment at 37 °C. Microsomes were digested with phospholipase A<sub>2</sub> (130 IU) for 20 min at 37 °C. Reconstitution of the inactivated enzymes was carried out by mixture with phosphatidylcholine (protein to phospholipid ratio on a weight basis 1:12), followed by assay in presence of 100 μg of the activator protein. Details on the reconstitution by phosphatidylcholine, activator protein preparation, and enzyme assays are given under Materials and Methods. (A) ATPase activity in the absence (open bars) and presence (hatched bars) of 5 mM K<sup>+</sup>; (B) pNPPase activity in the absence (open bars) and presence (hatched bars) of 20 mM K<sup>+</sup>. Data are mean ± SD (*n* = 8).

membranes and, hence, would be ineffective in the reconstitution. However, no significant change in phosphatidylethanolamine content of the phospholipase A<sub>2</sub> treated membranes at 21 and 37 °C (Table I) and restoration of the enzyme activity of the former membrane by the activator alone (Figure 4) would argue against such possibility.

Even though the hydrolysis of phosphatidylethanolamine remained almost unaltered at 21 and 37 °C (Table I), a significantly higher portion (about 20%) of the membrane phosphatidylcholine was attacked by phospholipase A<sub>2</sub> at 37 °C compared to that at 21 °C (Table III). If one assumes that all the products of phospholipase A<sub>2</sub> hydrolysis are retained by the microsomes, the amount of free fatty acids and lysolipids introduced into the ATPase assay medium by the digested (37 °C) membranes would be about 3.5 μM, which is too low a concentration to have any direct effect on the activity and stability of the enzyme (Im & Blakeman, 1982). Hence, the observed inhibition of gastric H<sup>+</sup>,K<sup>+</sup>-ATPase by phospholipase A<sub>2</sub> treatment appears to be unrelated to the hydrolyzed products of the membrane phospholipids.

#### Discussion

Phospholipase A<sub>2</sub> has been used by several laboratories (Saccomani et al., 1979; Krebs, 1982) to understand the

structural and functional organization of various phospholipids in biological membranes. The substrate specificity observed with phospholipase A<sub>2</sub> in this study is consistent with the reports that the enzyme from various sources reveals remarkable substrate specificity (Marinetti, 1964; Mollby & Wadstrom, 1973; Adamich & Dennis, 1978); hence, it is useful as a valuable tool for probing the gastric membrane structure and function. The bee venom phospholipase A<sub>2</sub> used in the present study was found to attack only the gastric microsomal phosphatidylethanolamine and phosphatidylcholine (Figure 3, Table I) at both 21 and 37 °C without having any effect on other microsomal lipids. Even after prolonged digestion at 37 °C when the vesicular integrity was totally lost (see Results), a significant part (about 20%) of both phosphatidylethanolamine and phosphatidylcholine remained unattacked by phospholipase A<sub>2</sub>. Analysis of the fatty acid composition of the ethanolamine- and choline-containing phospho- and lysophospholipids (Tables II and III) reveal some interesting features of the microsomes. The fatty acid compositions of the lysophosphatidylcholine obtained at 21 and 37 °C are nearly identical (Table III), suggesting that one species of microsomal phosphatidylcholine is attacked by phospholipase A<sub>2</sub> under those conditions. In the case of phosphatidylethanolamine, however, it appears (Table II) that at least two classes of the microsomal species are attacked by phospholipase A<sub>2</sub> since the fatty acid compositions of the lysophosphatidylethanolamine at 21 and 37 °C are significantly different. Gastric microsomal phosphatidylcholine (Table III) and phosphatidylethanolamine (Table II) remaining after phospholipase A<sub>2</sub> digestion at 21 °C become enriched in 18:0 but are depleted (Table II) or highly reduced (Table III) in polyunsaturated fatty acids like 18:2 and 20:4 compared to the phospholipase A<sub>2</sub> untreated control membranes. These latter observations demonstrate that phospholipase A<sub>2</sub> attacks primarily the microsomal phosphatidylethanolamine- and phosphatidylcholine-containing polyunsaturated fatty acids, which are located exclusively at the 2-position of phosphatidylethanolamine and to a large extent in phosphatidylcholine. Also, 25.5% of the total phosphatidylethanolamine and 40.5% (Table I) of the phosphatidylcholine, which are not attacked by phospholipase A<sub>2</sub> at 21 °C either lack in (Table II) or are very poor in (Table II) polyunsaturated fatty acids. The data, as discussed above, clearly demonstrate that there are more than one class of both phosphatidylcholine and phosphatidylethanolamine in gastric microsomes and bee venom phospholipase A<sub>2</sub> selectively attacks those species containing polyunsaturated fatty acids at the 2-position. It is noteworthy in this connection that different domains of phosphatidylcholine containing different fatty acids have recently been demonstrated to exist in human erythrocyte (Shukla & Hanahan, 1982).

Gastric microsomal H<sup>+</sup>,K<sup>+</sup>-ATPase activity is nearly abolished within 10 min of phospholipase A<sub>2</sub> treatment at both 21 (Figure 1) and 37 °C (Figure 2). However, under the same conditions, the K<sup>+</sup>-pNPPase activity is highly resistant to phospholipase A<sub>2</sub> inactivation and is reduced by only 50 and 75% after 20-min digestion at 21 (Figure 1) and 37 °C (Figure 2), respectively. Similar differential effects of phospholipase A<sub>2</sub> on gastric H<sup>+</sup>,K<sup>+</sup>-ATPase and pNPPase were also observed by Saccomani et al. (1979). However, the reasons for such differential effects are unknown. It is noteworthy that gastric H<sup>+</sup>,K<sup>+</sup>-ATPase and pNPPase, which have been suggested to be a manifestation of the same enzymic activity (Forte et al., 1976), also show differential sensitivity to a number of different agents (Forte et al., 1981; Nandi et al., 1983).

Studies on the reactivation of the microsomal enzymes treated with phospholipase A<sub>2</sub> at 21 and 37 °C offers some insight into the lipid-protein association of the enzymes. Thus, ability of the cytosolic activator protein alone to restore the phospholipase A<sub>2</sub> inactivated preparation at 21 °C (Figure 4) suggests that under such conditions the phospholipase only removes the enzyme-associated activator protein without affecting the phospholipids intimately associated with the gastric H<sup>+</sup>,K<sup>+</sup>-ATPase complex. Similar effects of the activator protein have recently been demonstrated with trypsin- (Ray & Sen, 1981) and ethanol- (Sen & Ray, 1980; Ray et al., 1980) inactivated gastric microsomal H<sup>+</sup>,K<sup>+</sup>-ATPase system. The data (presented in this paper) suggest that the membrane-bound activator is an extrinsic protein that needs some phospholipids such as phosphatidylethanolamine or phosphatidylcholine for its association with the gastric H<sup>+</sup>,K<sup>+</sup>-ATPase complex in an appropriate manner. The phospholipase A<sub>2</sub> treated enzyme at 37 °C, however, needs supplementation with phosphatidylcholine before it could be fully reactivated by the activator protein (Figure 5). Phosphatidylethanolamine was without any effect in the reconstitution process (see Results). The data (Figure 5) strongly suggest that phosphatidylcholine provides the appropriate lipid environment for the gastric microsomal H<sup>+</sup>,K<sup>+</sup>-ATPase and for the association of the enzyme with the activator protein. Furthermore, some species of phosphatidylcholine containing a high level of saturated fatty acids and poor in polyunsaturated fatty acids are primarily involved in providing the phospholipid microenvironment of the microsomal H<sup>+</sup>,K<sup>+</sup>-ATPase system (Table III, compare the composition of residual phosphatidylcholine and lysophosphatidylcholine at 21 and 37 °C). These data with phospholipase A<sub>2</sub> treatment at 37 °C are in general agreement with the results of our earlier studies on the effects of removal of microsomal lipids by mild ethanol treatment (Sen & Ray, 1980).

Although about 60% of the phosphatidylcholine (Table I) is removed by phospholipase A<sub>2</sub> at 21 °C, the lipid environment of the gastric ATPase is not appreciably affected as demonstrated by nondependency of the enzyme on phospholipids for reactivation (Figure 5). Removal of 20% more phosphatidylcholine by phospholipase A<sub>2</sub> at 37 °C (Table I) makes the enzyme dependent on exogenous phosphatidylcholine for its activity (Figure 6), suggesting that a portion of this phosphatidylcholine is coming from the gastric H<sup>+</sup>,K<sup>+</sup>-ATPase environment within the microsomal membrane. Unavailability of the phosphatidylcholine molecules intimately associated with the gastric microsomal H<sup>+</sup>,K<sup>+</sup>-ATPase complex to phospholipase A<sub>2</sub> attack at 21 °C suggests that those molecules are protected by the enzyme protein by way of some kind of tight hydrophobic interaction of the protein with the surrounding lipids (boundary lipids). At physiological temperature (37 °C), however, the force of such hydrophobic interaction is likely to be greatly reduced due to the lipid phase transition providing greater freedom of movement of various conformational states of the enzyme for optimal activity. Therefore, at 37 °C, the lipid molecules within the microenvironment of the gastric ATPase would be expected to be more vulnerable to phospholipase A<sub>2</sub> attack than at 21 °C. Previous studies (Sen & Ray, 1980) with mild ethanol treatment of gastric microsomes also suggested greater fluidity of the boundary lipids of the microsomal ATPase at 37 °C compared to lower temperatures. Thus, it appears that the boundary lipids of gastric H<sup>+</sup>,K<sup>+</sup>-ATPase within the microsomal membrane are mobile at 37 °C but are relatively immobile at 21 °C.

Fluidity of any portion of a membrane bilayer would depend largely on the fatty acid composition of the regional phospholipids as well as the cholesterol content. Our previous studies (Sen & Ray, 1980) demonstrate higher efficacy of the microsomal phosphatidylcholine in restoring the ethanol-inactivated gastric ATPase than the synthetic ones tested. Studies with synthetic phosphatidylcholine also demonstrated effectiveness of the molecules containing saturated and monounsaturated fatty acids but ineffectiveness of the ones having polyunsaturated fatty acids. Our present data suggesting lack of arachidonic acid and predominance of saturated and monounsaturated fatty acids in the gastric ATPase associated phosphatidylcholine molecules are in agreement with the previous finding (Sen & Ray, 1980). Thus, a class of gastric microsomal phosphatidylcholine molecules, having a general fatty acid makeup as above, appear to provide an appropriate lipid environment for efficient operation of the enzyme at physiological temperature.

Assuming that all of 20% of the microsomal phosphatidylcholine discussed previously (see above) is associated with the H<sup>+</sup>,K<sup>+</sup>-ATPase function and about 20% of the total microsomal proteins (Forte et al., 1980) are contributed by the gastric ATPase molecules, one can get an approximate idea about the stoichiometry of the phospholipid associated with the enzyme system. Although there are some disagreements in the reported molecular weight of the gastric ATPase (Saccomani et al., 1981; Peters et al., 1982), a value of 440 000 does not appear unreasonable. On the basis of the preceding information, the calculated value for the ATPase-associated phosphatidylcholine turns out to be about 100 molecules/molecule of the enzyme system. Interestingly, such a value is similar to that (61 mol/mol of the enzyme) reported for the purified Na<sup>+</sup>,K<sup>+</sup>-ATPase (Hastings & Reynolds, 1979) of shark rectal gland.

Contrary to the present report, Saccomani et al. (1979) demonstrated that the gastric H<sup>+</sup>,K<sup>+</sup>-ATPase inactivated by phospholipase A<sub>2</sub> treatment at room temperature (21 °C) needed either phosphatidylcholine or phosphatidylethanolamine for reactivation. These authors (Saccomani et al., 1979) used 60 min of digestion compared to 20 min with the same amount (130 IU) of phospholipase A<sub>2</sub> in the present study for the reconstitution experiments. Such prolonged digestion may be the primary basis of the observed differences between these two reports.

In conclusion, the present study reemphasizes the usefulness of phospholipases as probes for exploring the structure-function relationship of biomembranes. Instead of digesting the biomembranes at one particular temperature, comparative studies of various membrane parameters after digestion in more than one temperature appear to be capable of generating more specific information. Further studies of similar nature, as discussed in this paper, with other phospholipases like phospholipases C and D may help us to understand better some of the issues raised in this paper, including lipid-activator protein association, lipid environment of gastric H<sup>+</sup>,K<sup>+</sup>-ATPase, and the heterogeneity and segregation of various membrane lipids. Works are in progress to understand these aspects.

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**Registry No.** ATPase, 9000-83-3; pNPPase, 9073-68-1; phospholipase A<sub>2</sub>, 9001-84-7.



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