Biochimica et Biophysica Acta, 649 (1981) 1–12
Elsevier/North-Holland Biomedical Press

BBA 79437

STUDIES ON ($K^+ + H^+$)-ATPase

IV. EFFECTS OF PHOSPHOLIPASE C TREATMENT

J.J. SCHRIJEN, A. OMACHI *, W.A.H.M. VAN GRONINGEN-LUYBEN, J.J.H.H.M. DE PONT and S.L. BONTING

Department of Biochemistry, University of Nijmegen, P.O. Box 9101, 6500 HB Nijmegen (The Netherlands)

(Received March 31st, 1981)

Key words: ($K^+ + H^+$)-ATPase; Gastric secretion; Phospholipase C; Phospholipid

(1) The total phospholipid content of a gradient purified ($K^+ + H^+$)-ATPase preparation from pig gastric mucosa is 105 μmol per 100 mg protein, and consists of 29% sphingomyelin, 29% phosphatidylcholine, 28% phosphatidylethanolamine, 10% phosphatidylserine and 4% phosphatidylinositol. The cholesterol content corresponds to 50 μmol per 100 mg protein. (2) Treatment with phospholipase C (from *Clostridium welchii* and *Bacillus cereus*) results in an immediate decrease of the phosphate content. Up to 50% of the phospholipids are hydrolyzed by each phospholipase C preparation alone, without further hydrolysis by increased phospholipase concentration or prolonged incubation time. Combined treatment with the two phospholipase C preparations, sequentially or simultaneously, hydrolyzes up to 65% of the phospholipids. (3) The ($K^+ + H^+$)-ATPase and K^+ stimulated *p*-nitrophenylphosphatase activities are decreased proportionally with the total phospholipid content, indicating that these enzyme activities are dependent on phospholipids. (4) Phospholipase C treatment does not change optimal pH, K_m value for ATP and temperature dependence of the gastric ($K^+ + H^+$)-ATPase, but slightly decreases the K_a value for K^+. (5) Phospholipase C treatment lowers the Ado*PP*[NH]*P* binding and phosphorylation capacities, suggesting that inactivation occurs primarily on the substrate binding level. (6) Most of the results can be understood by assuming that hydrolysis of the phospholipids by phospholipase C leads to aggregation of the membrane protein molecules and complete inactivation of the aggregated ATPase molecules.

Introduction

A vesicular membrane fraction, which contains an Mg^{2+}-dependent, K^+-stimulated ATPase, can be isolated from the mucosa of the gastric corpus and fundus of various species [1–3]. The vesicles are capable of proton uptake in exchange for K^+ upon addition of ATP in the presence of Mg^{2+} and K^+. The enzyme shows a remarkable resemblance in its reaction mechanism to the transport ATPases ($Na^+ + K^+$)-ATPase and ($Ca^{2+} + Mg^{2+}$)-ATPase and is considered to be a ($K^+ + H^+$)-ATPase transport system, which may be involved in gastric acid secretion.

A K^+ activated *p*-nitrophenylphosphatase activity is co-purified during isolation of the ($K^+ + H^+$)-ATPase containing membranes by isopycnic gradient centrifugation and free flow electrophoresis [4]. This activity, which was actually discovered before the ($K^+ + H^+$)-ATPase activity, appears to be sensitive to phospholipase C treatment and acetone extraction [5,6]. Since the K^+-stimulated ATPase and phosphatase activities are now thought to represent a single enzyme [1–4], it can be assumed that both activities are dependent on lipids. This lipid dependence may be further supported by the temperature

* Present address: Department of Physiology and Biophysics, College of Medicine, University of Illinois, Chicago, IL 60680, U.S.A.

Abbreviations: EGTA, ethyleneglycol-bis-(aminoethyl ether)-*N,N'*-tetraacetic acid; Ado*PP*[NH]*P*, adenylyl imidodiphosphate.

0005-2736/81/0000–0000/$02.50

dependence of the proton uptake by gastric vesicles, which shows a transition temperature of 22°C in an Arrhenius plot [7]. For the $(K^+ + H^+)$-ATPase activity a transition temperature of 28°C has recently been obtained by Sachs et al. [8]. On the other hand, Lee et al. [29] report that only the valinomycin-stimulated fraction of the $(K^+ + H^+)$-ATPase activity in gastric vesicles shows a transition temperature of 14°C, while the ionophore insensitive K^+-stimulated activity shows no transition point, as has also been found by Sachs et al. [8] for the polarization signal of diphenylhexatriene, a probe of bulk lipid viscosity.

The reports on the phospholipid composition of vesicles from pig gastric mucosa by Sen and Ray [9–11] and by Saccomani and co-workers [12,13] show considerable differences, especially with regard to the total phospholipid contents (184 vs. 625 mg per g protein, respectively) and the relative amounts of phosphatidylserine (1 vs. 11%, respectively) and phosphatidylethanolamine (40 vs. 22%, respectively). These two groups have used different methods for the investigation of protein-lipid interactions. Saccomani et al. [12] treat the vesicles with phospholipase A_2 and obtain 75% inhibition of the $(K^+ + H^+)$-ATPase activity, when 50% of the phospholipids is hydrolyzed. Sen and Ray [10,11] extract phospholipids with 15% ethanol and find complete inactivation of the enzyme when approx. 6% of the total phospholipids is removed after incubation of the vesicles at 37°C for 60 s. Surprisingly, these authors claim that lipids from the immediate environment of the enzyme molecules are preferentially extracted under these circumstances.

The discrepancies in these findings led us to reinvestigate the phospholipid composition of the $(K^+ + H^+)$-ATPase-containing membrane preparation from pig gastric mucosa. In addition, we report the effects of phospholipase C treatment on the phospholipid content and on the $(K^+ + H^+)$-ATPase and K^+-stimulated *p*-nitrophenylphosphatase activities of these membranes in an attempt to explore possible lipid-protein interactions.

Materials and Methods

$(K^+ + H^+)$-ATPase preparation

A $(K^+ + H^+)$-ATPase containing membrane fraction is isolated from pig gastric mucosa as previously described [14]. After distribution of the microsomal fraction over a linear sucrose gradient, fractions with a density of 1.11–1.13 are diluted and centrifuged at $100\,000 \times g_{av}$ for 1 h. The resulting pellets are routinely resuspended in unbuffered 0.25 M sucrose to a protein concentration of 1 mg per ml, protein being determined according to Lowry et al. as previously described [19]. This membrane suspension is either frozen at −20°C in small portions, or it is dropwise added to liquid nitrogen and the resulting beads stored at −20°C. The final preparations have a specific $(K^+ + H^+)$-ATPase activity of 90–140 μmol P_i/h per mg^{-1} protein, which remains constant during storage for at least six months.

The $(K^+ + H^+)$-ATPase containing gastric membrane preparation does not consist of ion-tight vesicles, since valinomycin under isotonic conditions does not stimulate the enzyme activity.

Phospholipase preparations

Phospholipase C from Bacillus cereus. The enzyme was isolated according to a modified method of Otnaess et al. [15] by Mr. P. Jansen of this laboratory. The enzyme has a specific activity of 975 units per mg protein, is free of proteolytic activity, and can be stored at −20°C in 50% (v/v) glycerol for over a year without loss of activity. It is used in a solution containing 400 units per ml.

Phospholipase C from Clostridium welchii. The enzyme was obtained as type I from Sigma (St. Louis, MO, U.S.A.) with a specific activity of 19 units per mg protein. This phospholipase C preparation also appears to be free of proteolytic activity, as shown by incubation at pH 7.0 with azoalbumin and by sodium dodecylsulfate polyacrylamide gel electrophoresis of the gastric $(K^+ + H^+)$-ATPase after prolonged incubation with the phospholipase C preparation under the experimental conditions specified below. It is used as a solution of 2 mg phospholipase preparation per ml Stahl medium [16], which consists of 4 mM histidine, 0.6 mM $CaCl_2$, 0.5 mM Na_2ATP, 0.4 mg/ml bovine serum albumin, 25% (v/v) dimethylsulfoxide and 0.1 mM $ZnCl_2$ (pH 7.4). One unit of phospholipase activity corresponds in both cases to 1 μmol substrate hydrolyzed per min at pH 7 and 37°C.

Treatment of $(K^+ + H^+)$-ATPase with phospholipases

The procedure is essentially the same as described

previously by De Pont et al. [17] for $(Na^+ + K^+)$-ATPase. The $(K^+ + H^+)$-ATPase preparation is sedimented by centrifugation at 100 000 × *g* to remove sucrose, resuspended in Stahl medium at a final protein concentration of 1.5 mg per ml and preincubated at 30°C. To 2 ml $(K^+ + H^+)$-ATPase preparation is added 200 μl phospholipase C (*Cl. welchii)* solution at time 0. After mixing, the tubes are incubated at 30°C and the reaction is stopped at suitable times by the transfer of 300-μl aliquots to 1 ml ice-cold 0.1 M EGTA adjusted with Tris to pH 7.4. The membranes are collected by centrifugation, are washed once and are then resuspended in 0.25 M sucrose. $(K^+ + H^+)$-ATPase and K^+-stimulated *p*-nitrophenylphosphatase activities are determined immediately. Protein determinations and phospholipid analysis are carried out later. Treatment with phospholipase C (*B. cereus*) is carried out in the same way, except that 10 μl phospholipase C preparation is added to 2 ml $(K^+ + H^+)$-ATPase suspension. For controls 200 μl Stahl medium or 10 μl 50% (v/v) glycerol is used for the phospholipase C preparations from *Cl. welchii* and *B. cereus*, respectively.

When $(K^+ + H^+)$-ATPase is incubated with both phospholipases in succession, the enzyme is first incubated for 10 min at 30°C with phospholipase C (*Cl. welchii*). Then phospholipase C (*B. cereus*) is added and the incubation is continued for another 10 min., followed by addition of 0.1 M EGTA-Tris (pH 7.4) and the treatment described above.

The phospholipase C treated membrane preparations can be stored in 0.25 M sucrose overnight at −20°C without loss of $(K^+ + H^+)$-ATPase activity. In the text 'treated membranes' refers to preparations treated with both phospholipases in succession, while 'control membranes' refers to preparations carried through the same incubation procedure in the absence of phospholipases.

Prior to determination of parameters like Ado*PP*[NH]*P*-binding, phosphorylation level and K_m for ATP, residual ATP is removed from the membranes in the following way. The membranes are resuspended in 0.25 M sucrose and are incubated for 30 min at 37°C in the K^+-containing assay medium [14] in the presence of 0.25 M sucrose but without ATP. After centrifugation and washing by pelleting (45 min 100 000 × *g*), the membranes are resuspended in 0.25 M sucrose for determination of the phosphorylation level and the K_m for ATP, or in 50 mM imidazole-HCl (pH 7.0) for determination of the Ado*PP*[NH]*P* binding.

Lipid analysis

Total lipids are extracted from approx. 400 μg protein in 120 μl 0.25 M sucrose with 3 ml chloroform/methanol (2 : 1, v/v) by vigorous shaking during 30 min under nitrogen at room temperature [18]. After centrifugation for 10 min at 6000 × *g*, the supernatant is collected and the extraction of the sediment is repeated twice. Subsequently, the pooled extracts (approx. 10 ml) are repeatedly washed with 2 ml of 0.1 M KCl according to Palmer [19]. After centrifugation the lower layer is concentrated by evaporation in vacuum. The concentrated extract is taken to dryness in a stream of nitrogen and the residue is dissolved in $CHCl_3/CH_3OH$ (1 : 1, v/v).

Thin-layer chromatography of lipids is conducted on glass plates (20 × 20 cm) coated with silica gel (0.3 mm) and preactivated for 1 h at 110°C [20]. Lipid extracts (0.4 μmol phospholipid) are applied as single spots, and are evaporated in a stream of nitrogen. The chromatogram is developed using $CHCl_3/CH_3OH$/14 M ammonia/H_2O (90 : 54 : 5.5 : 5.5, v/v) in the first and $CHCl_3/CH_3OH$/acetic acid/H_2O (90 : 45 : 12 : 1, v/v) in the second dimension. The spots are visualized by staining with iodine vapour, and are then scraped off and transferred to test tubes for phosphate determination. Phosphate blanks are prepared from silica gel outside the spots. The phosphate determination is carried out as described by Broekhuyse [20]. The relative standard error for this determination varies from 5 to 10%, depending on the amount of phospholipid, whilst the limit of detection is 1% of the amount of total phospholipid applied to the plate.

The cholesterol content of a lipid extract is estimated with the cholesterol esterase-cholesterol oxidase colorimetric method ('CHOD-PAP', Boehringer, Mannheim, F.R.G.) from a calibration curve prepared with pure cholesterol.

Analytical methods

$(K^+ + H^+)$-ATPase and K^+-stimulated *p*-nitrophenylphosphatase activities and their dependence on pH and K^+ concentration are determined as previously described [14]. The temperature dependence

of $(K^+ + H^+)$-ATPase in control and treated membranes is determined by incubating them at temperatures between 10 and 40°C for suitable lengths of time. Results are presented in the form of Arrhenius plots.

The high affinity K_m for ATP is determined in the ATPase assay media [14] in the presence of 15, 20, 30, 50 and 100 μM [γ-^{32}P]ATP. The reaction is started by adding 10 μl membrane suspension (0.75 and 2.41 mg protein per ml 0.25 M sucrose for control and treated membranes, respectively) to 100 μl incubation medium. After incubation for 30 s at 37°C in triplicate, the reaction is stopped by addition of 200 μl ice-cold 8.6% (w/v) trichloroacetic acid. Blanks without enzyme are run to determine the extent of [γ-^{32}P]ATP hydrolysis. After addition of 200 μl molybdate-$FeSO_4$ reagent and standing for 30 min at room temperature, the reduced phosphomolybdate complex is extracted into 400 μl isobutanol by vigorous mixing [21]. Finally, 150-μl aliquots of the isobutanol layer are added to 4 ml Pico-fluor ™15 (Packard Becker, BV, Groningen, The Netherlands), and ^{32}P is counted in a liquid scintillation analyzer.

Phosphorylation by ATP and binding of Ado*PP*[NH]*P* are determined as previously described [22,23].

Materials

Phosphatidylcholine is isolated from egg-yolk according to Pangborn [24]. Phosphatidylserine, grade I from bovine spinal cord, monosodium salt is obtained from Lipid Products (South Nutfield, U.K.). All nucleotides are purchased from Boehringer (Mannheim, F.R.G.). Silicagel 60 HR is obtained from Merck (Darmstadt, F.R.G.) and magnesium silicate from Woelm (Eschwege, F.R.G.). All other chemicals are from Merck (Darmstadt, F.R.G.) and are of analytical grade.

Results

Phospholipid content and composition

The gradient purified $(K^+ + H^+)$-ATPase preparation, isolated from pig gastric mucosa, contains 105 (S.E. = 3; n = 26) mol phospholipid per 100 000 g protein. At an average phospholipid molecular weight of 750, this corresponds to 79 mg of phospholipid per 100 mg protein, which is close to the value of 62.4 reported by Saccomani et al. [12], but is more than four times the value of 18.4 of Sen and Ray [9]. The cholesterol content of the gastric membranes is 19.5 (S.E. = 0.8; n = 5) mg per 100 mg protein, which is 1.5-times greater than the value reported by the latter authors [9] and corresponds to 50 mol cholesterol per 100 000 g protein, or a molar cholesterol/phospholipid ratio of 0.48.

In our lipid extraction procedure more than 98% of the phospholipids is extracted. Table I gives the phospholipid composition of the $(K^+ + H^+)$-ATPase preparation, which agrees with that reported by others for gastric membranes [12,25] and other

TABLE I

PHOSPHOLIPID COMPOSITION OF A $(K^+ + H^+)$-ATPase PREPARATION FROM PIG GASTRIC MUCOSA

The results are presented as means with standard errors. The number of experiments is given in parentheses.

Phospholipid	Phospholipid contents	
	in % of total phospholipid content	in μmol phospholipid per 100 mg protein
Total	≡100%	105 ± 3 (26) [a]
Sphingomyelin	29.3 ± 1.9 (10)	30.8
Phosphatidylcholine	28.9 ± 0.8 (10)	30.3
Phosphatidylethanolamine	27.6 ± 0.9 (10)	29.0
Phosphatidylserine	10.5 ± 1.1 (10)	11.0
Phosphatidylinositol	4.3 ± 0.4 (10)	4.5

[a] Derived from determination of the phosphate content of membranes, assuming that all phosphate is lipid bound.

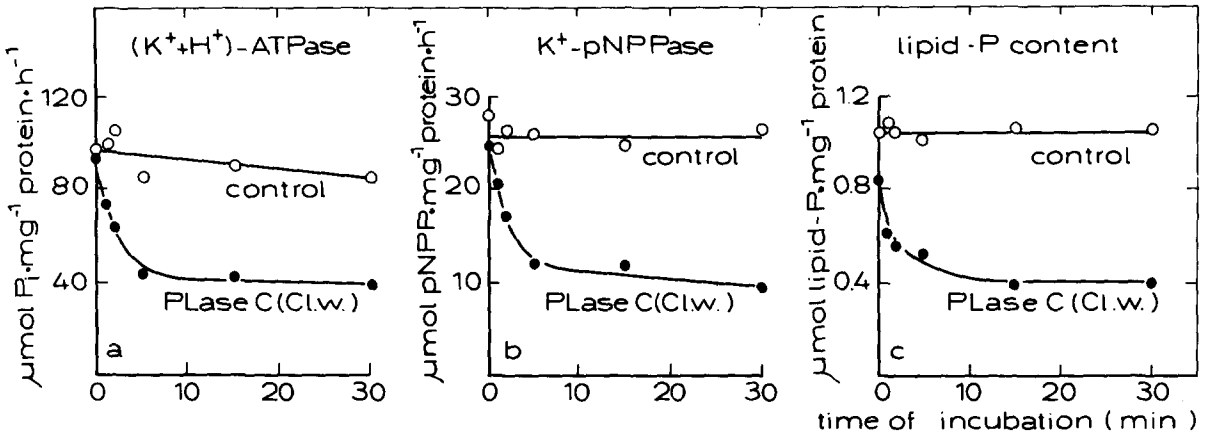

Fig. 1. Effect of incubation with phospholipase C (PLase C) from *Clostridium welchii* on $(K^+ + H^+)$-ATPase, K^+-stimulated *p*-nitrophenylphosphatase (K^+-pNPPase) and lipid phosphorus content of gastric membranes as a function of incubation time. Membranes are incubated at 30°C with (•) or without (o) phospholipase C as described under Materials and Methods. A typical experiment out of three is shown.

plasma membranes [17,26]. The relatively high sphingomyelin and cholesterol contents are common to most plasma membrane preparations.

Effects of phospholipase C treatment on phospholipid content and enzyme activities

Incubation of gastric membranes with phospholipase C from either *Cl. welchii* or *B. cereus* rapidly decreases the phospholipid content (Figs. 1c and 2b). The $(K^+ + H^+)$-ATPase and K^+-stimulated *p*-nitrophenylphosphatase activities are lowered in parallel with the phospholipid content (Figs. 1 and 2). Table II summarizes the results of all experiments in which $(K^+ + H^+)$-ATPase preparations are incubated with these phospholipases. These findings indicate that phospholipids are required for optimal enzyme activity.

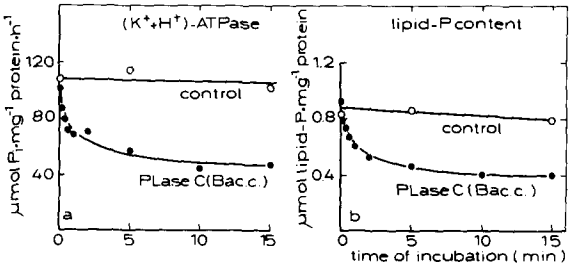

Fig. 2. Effect of incubation with phospholipase C (PLase C) from *Bacillus cereus* on $(K^+ + H^+)$-ATPase and lipid phosphorus content of gastric membranes as a function of incubation time. Membranes are incubated at 30°C with (•) or without (o) phospholipase C as indicated under Materials and Methods. A typical experiment out of three is shown.

When the membranes are incubated sequentially with both phospholipases, i.e. adding phospholipase C (*B. cereus*) after 10 min incubation with phospholipase C (*Cl. welchii*), the phospholipid content and the $(K^+ + H^+)$-ATPase activity are further decreased (Fig. 3 and Table II). The same pattern is obtained when the phospholipases are added in reverse sequence. A second addition of the same phospholipase C instead of the other phospholipase C does not result in a further decrease (Fig. 3). Addition of both phospholipases C together results in the same low final level as when they are added sequentially. Neither the use of larger amounts of phospholipase, nor an additional sequential treatment with the two enzymes further diminish the phospholipid content or the ATPase activity. These observations suggest that substrate (phospholipid) accessibility for one phospholipase C is not increased by the action of the other.

Effect of phospholipase C treatment on phospholipid composition

Table III shows the effects of incubation with the

TABLE II

EFFECTS OF TREATMENT OF GASTRIC MEMBRANES WITH PHOSPHOLIPASE C FROM TWO DIFFERENT SOURCES, SEPARATELY OR SEQUENTIALLY, ON LIPID PHOSPHORUS CONTENT AND ($K^+ + H^+$)-ATPase ACTIVITY

The results are presented as means of percent residual phospholipid content of enzyme activity after 10 min incubation at 30°C; *n* is the number of determinations. Experimental procedures are described in Fig. 1.

Treatment	Percent of control value		
	Phospholipase C (*B. cereus*) (*n* = 6)	Phospholipase C (*Cl. welchii*) (*n* = 9)	Both phospholipase C sequentially (*n* = 8)
Lipid phosphorus	51 ± 1.1	50 ± 1.8	35 ± 1.1
($K^+ + H^+$)-ATPase	43 ± 1.9	45 ± 1.5	28 ± 1.8

two phospholipases C on the phospholipid composition of the gastric membranes. Phospholipase C (*B. cereus*) hydrolyzes phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine, but not phosphatidylinositol or sphingomyelin (column 3). This substrate preference is qualitatively similar to that found with mixed lipid suspensions from photoreceptor membranes [27] and with phospholipids from different sources in Triton X-100/phospholipid mixed micelles [28], which are hydrolyzed at different rates.

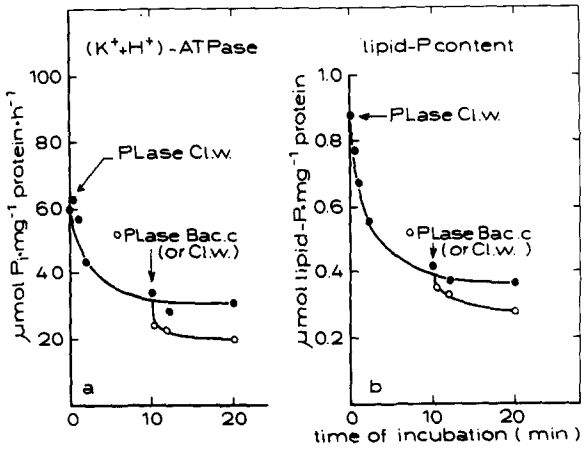

Fig. 3. Effect of sequential treatment with two phospholipases C (PLase C) on ($K^+ + H^+$)-ATPase and lipid phosphorus content as a function of incubation time. Membranes are first incubated at 30°C with phospholipase C (*Cl. welchii*) as described under Materials and Methods. After 10 minutes, either phospholipase C (*B. cereus*) (○) or a second addition of PLase C (*Cl. welchii*) (●) is administered. A typical experiment out of two is shown.

Phospholipase C from *Cl. welchii* hydrolyzes phosphatidylcholine, phosphatidylethanolamine and sphingomyelin, but not phosphatidylinositol or phosphatidylserine under our experimental conditions (column 4). This resembles qualitatively the findings of De Pont et al. [17] for a highly purified ($Na^+ + K^+$)-ATPase preparation from rabbit kidney outer medulla.

The effect of sequential treatment with the two phospholipases on the phospholipid composition of the gastric membranes is shown in column 5 of Table III. The effects of the two phospholipases are approximately additive, when compared to the data obtained with each enzyme alone. The apparent increase in phosphatidylinositol content is probably due to an analytical error magnified by the large decrease in total phospholipid content.

Effects of sequential phospholipase C treatment on enzyme parameters

After treatment with both phospholipases C, the release of P_i from ATP through the action of ($K^+ + H^+$)-ATPase remains linear with the incubation time (not shown). The optimal pH for the ($K^+ + H^+$)-ATPase and *p*-nitrophenylphosphatase activities is 7.0 (Table IV), which is similar to the pH-optimum of the ($K^+ + H^+$)-ATPase activity in untreated gradient purified membranes (Fig. 3 in Ref. 14).

The apparent affinity for K^+ of ($K^+ + H^+$)-ATPase in treated membranes is somewhat increased (Fig. 4): the K_a value in treated membranes is 1.51 mM (S.E. = 0.15; *n* = 3) vs. 2.53 mM (S.E. = 0.02; *n* = 3) for untreated controls. The latter value closely

TABLE III

EFFECTS OF TREATMENT WITH PHOSPHOLIPASE C FROM TWO DIFFERENT SOURCES ON THE PHOSPHOLIPID COMPOSITION OF GASTRIC ($K^+ + H^+$)-ATPase PREPARATION

Incubation is for 10 min at 30°C for each phospholipase. Experimental procedures are described in Materials and Methods. Results are expressed as percent of total phospholipids in the untreated preparation (Table I), calculated by means of the lipid phosphorus percentages from Table II; *n* is the number of experiments.

	Untreated membranes (Table I)	Membranes treated with *B. cereus* (*n* = 3)	Membranes treated with *Cl. welchii* (*n* = 4)	Membranes sequentially treated with both phospholipases C (*n* = 3)
Sphingomyelin	29.3	27.0 ± 2.9	17.2 ± 0.9	14.5 ± 0.5
Phosphatidylcholine	28.9	8.6 ± 3.8	6.0 ± 0.7	3.5 ± 0.4
Phosphatidylethanolamine	27.6	5.9 ± 0.2	10.0 ± 1.2	3.7 ± 0.8
Phosphatidylserine	10.5	4.5 ± 0.9	11.3 ± 1.2	5.6 ± 0.6
Phosphatidylinositol	4.3	5.1 ± 0.2	5.4 ± 1.0	7.8 ± 0.9
		51.1	49.9	35.1

approximates the K_a value of 2.7 mM reported previously [14], showing that the incubation procedures as such do not affect this parameter. The lower K_a value reported by Forte et al. [1] may be due to the higher pH or lower ATP concentration employed by them [8,29].

Treatment with both phospholipase C preparations does not affect the K_m for ATP (Table IV), but lowers V. It will be noted that the V in these experiments is much lower than observed for untreated membranes in the normal assay medium. This is due to the fact that the enzyme has two ATP sites, a low affinity site with high V and a high affinity site with low V [8]. At the low ATP concentrations used in these experiments only the high affinity site with low V is active. The K_m value of 73 μM for the high

TABLE IV

EFFECT OF SEQUENTIAL TREATMENT WITH PHOSPHOLIPASE C PREPARATIONS FROM TWO DIFFERENT SOURCES ON ENZYME PARAMETERS OF GASTRIC ($K^+ + H^+$)-ATPase PREPARATION

Enzyme parameters are determined as described under Materials and Methods; the number of determinations is given in parentheses. The standard error for two experiments is calculated as 0.63 × the range, according to the approximation method of Davies and Pearson [30].

	Control membranes	Phospholipase C treated membranes
K_m for ATP (μM)	73 ± 5 (2)	74 ± 3 (3)
V (μmol ^{32}P per mg protein per h)	28 ± 6 (2)	4.7 ± 0.5 (3) [a]
K_D for Ado*PP*[NH]*P* (μM)	51 ± 2 (6)	42 ± 4 (6)
Maximal Ado*PP*[NH]*P* binding (nmol per mg protein)	1.2 ± 0.1 (6)	0.47 ± 0.05 (6) [a]
Optimal pH ($K^+ + H^+$)-ATPase	7.0 [b]	7.0
Optimal pH *p*-nitrophenylphosphatase	7.0 [b]	7.0
Transition temperature (°C)	26.7 ± 0.4 (4)	26.9 ± 1.9 (4)
Activation energy (kcal · mol^{-1})		
Below transition temperature	26.9 ± 0.3 (4)	25.6 ± 0.7 (4)
Above transition temperature	16.0 ± 1.8 (4)	16.3 ± 1.8 (4)

[a] Significantly different from control value.

[b] These values are obtained from Ref. 14 for native gastric membranes.

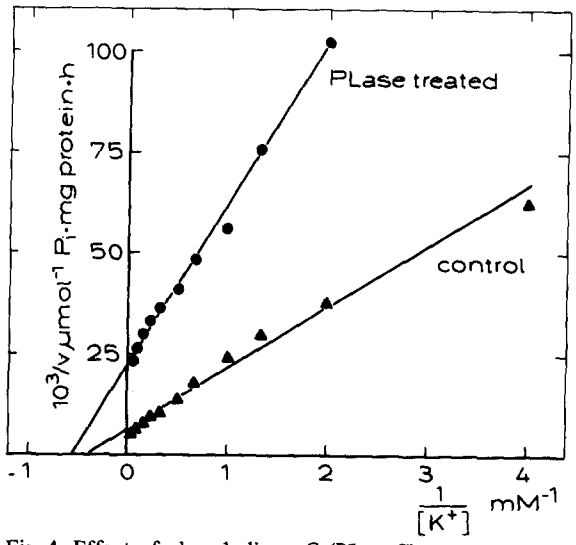

Fig. 4. Effect of phospholipase C (PLase C) treatment on the activation of $(K^+ + H^+)$-ATPase by K^+. Gastric membranes, sequentially treated with the two phospholipases C (•) and control membranes, treated in the same way without phospholipase addition (▲) are assayed at varying K^+ concentrations, as described under Materials and Methods. Results are expressed as Lineweaver-Burk plots. A typical experiment out of three is shown.

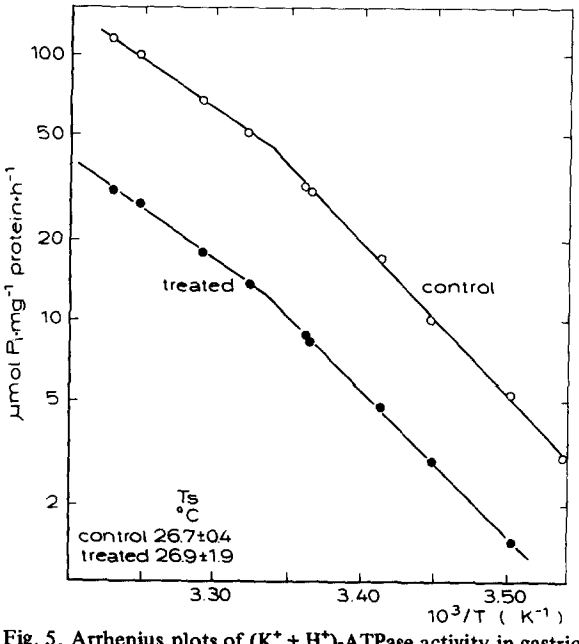

Fig. 5. Arrhenius plots of $(K^+ + H^+)$-ATPase activity in gastric membranes. $(K^+ + H^+)$-ATPase preparations with (•) or without (o) sequential phospholipase C treatment are incubated at different temperatures as described under Materials and Methods. A typical experiment out of four is shown.

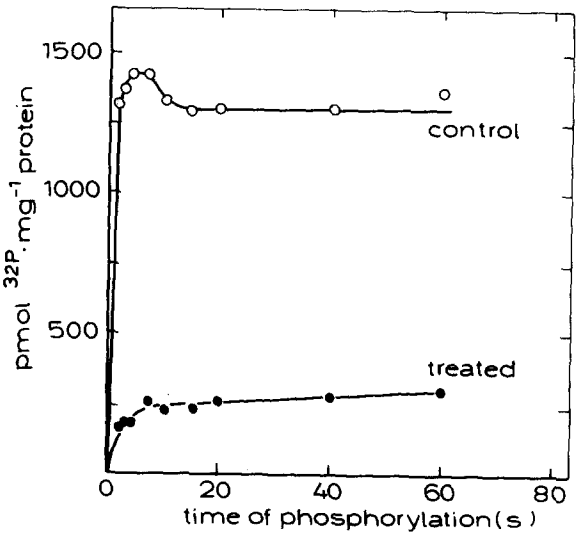

Fig. 6. Phosphorylation level of gastric membranes with (•) or without (o) sequential phospholipase C treatment. Phosphorylation procedure is described under Materials and Methods. A typical experiment out of two is shown.

affinity ATP site is in agreement with the value of 74 μM reported by Sachs et al. [8]. Recently, the same group [31] reported a high affinity K_m value of 3.5 μM without explaining the large difference with the earlier value. In our opinion, the difference may be due to the higher KCl concentration and temperature used by Sachs et al. [8] and by us: K^+ is known to decrease the affinity of $(K^+ + H^+)$-ATPase for ATP [8,14,22,23], while lowering the temperature decreases the low affinity K_m value of $(Na^+ + K^+)$-ATPase [32].

The activation energies and transition temperatures for $(K^+ + H^+)$-ATPase, obtained from Arrhenius plots, do not differ significantly in treated and control membranes (Fig. 5 and Table IV). These results suggest that most properties of the $(K^+ + H^+)$-ATPase preparation are not greatly affected by treatment with phospholipase C, except that the enzyme activity is greatly reduced, maximally by 70%.

We have also investigated the effect of phospholipase C treatment on some partial reaction steps. Substrate binding has been studied with the non-phosphorylating ATP analogue, adenylyl imidodiphosphate (Ado*PP*[NH]*P*). The K_d value for Ado*PP*[NH]*P* is not changed, but the maximal binding capacity of the treated membranes is reduced

(Table IV). In line with this observation, the phosphorylation level for ATP is reduced by more than 70% after sequential treatment (Fig. 6). From these observations it may be concluded that the inactivation of $(K^+ + H^+)$-ATPase activity by phospholipase C is due to a decreased number of available substrate binding sites.

Discussion

Lipid content and composition of gastric $(K^+ + H^+)$-ATPase preparation

The determination of the phospholipid contents does not seem to be vitiated by preferential extraction of any given phospholipid, since 98% of the total lipid phosphorus content of the membrane preparation is extracted in our procedure. The phospholipid composition can be reliably calculated without having recourse to the revovery (ranging from 60% to 90% in our experiments), as pointed out by Roelofsen et al. [33].

Our findings for the phospholipid content and composition of gastric membranes (105 mol per 100 000 g protein, consisting of 29% phosphatidylcholine, 29% sphingomyelin, 28% phosphatidylethanolamine, 10% phosphatidylserine and 4% phosphatidylinositol) resemble those of Saccomani et al. [12] but they differ greatly from those of Sen and Ray [9]. Our molar cholesterol/phospholipid ratio of 0.48 is much lower than the extremely high value of 1.95 reported by the latter authors, who apparently used a phospholipid molecular weight of 1000. Even if their molar ratio is recalculated on the basis of an average phospholipid molecular weight of 753 (calculated by us from their data for phospholipid and fatty acid composition), the still relatively high value of 1.4 is obtained. Their aberrant value is mainly due to the low phospholipid content, which amounts to only 23% and 30% of that found by us and Saccomani et al. [12], respectively.

The absence of cardiolipin indicates a very low mitochondrial contamination in our $(K^+ + H^+)$-ATPase preparation, which is in agreement with earlier conclusions based on the low cytochrome *c* oxidase activity [14]. The absence of lysophospholipids and phosphatidic acid indicates that the gastric membranes must posses a rather rigid structure.

Effects of phospholipase C on phospholipid content and composition

Phospholipase treatment can be used to study specific protein-lipid interactions in membranes. Phospholipase C has often been preferred, because the diacylglycerol produced by it do not seem to affect membrane bound enzyme activities per se, although they may affect the charge properties of the remaining phospholipids or cause their rearrangement through coalescence into droplets [34].

In our experiments incubation with phospholipase C results in a rapid decrease of lipid phosphorus content of the gastric membranes but the hydrolysis stops at a certain level. This residual phospholipid level shows little dependence on the type of phospholipase C used (51% for *B. cereus* and 50% for *Cl. welchii*), but is lower after treatment by both enzymes (35%). In our experiments the *B. cereus* enzyme hydrolyzes phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine but little or no sphingomyelin and phosphatidylinositol, while the *Cl. welchii* enzyme hydrolyzes phosphatidylcholine, phosphatidylethanolamine and sphingomyelin but little or no phosphatidylserine and phosphatidylinositol. These differences in substrate specificity partially explain the higher and approximately additive hydrolysis after sequential treatment.

The abrupt cessation of hydrolysis at a certain level requires an explanation. It is unlikely that it is due to accessibility of only the outer leaflet phospholipids, since sequential treatment leads to the rather high value of 65% hydrolysis and the membrane preparation does not seem to consist of closed vesicles (no stimulation of the activity by ionophore). One possibility is a different substrate preference for the fatty acid chains of the phospholipid, indicated by the finding of Roberts et al. [28] that the phospholipase C (*B. cereus*) prefers short fatty acid chains. This may also explain why the *Cl. welchii* enzyme causes less breakdown of phosphatidylcholine and sphingomyelin in the $(K^+ + H^+)$-ATPase preparation than in a highly purified $(Na^+ + K^+)$-ATPase preparation from rabbit kidney outer medulla [17]. Another possibility is that the limited hydrolysis is due to interactions of the lipids with membrane proteins, leading to their protection against phospholipase C action. This appears to be the case in myelin sheath, where after treatment with trypsin there is an

increased hydrolysis of phospholipids by phospholipase C [35]. If this applies to our membranes, it would imply that sphingomyelin and phosphatidylserine have a strong interaction with the gastric $(K^+ + H^+)$-ATPase.

Phospholipids from the immediate lipid environment of a lipoprotein are probably extracted less readily than other phospholipids. Thus it seems unlikely that the nearly complete inactivation of $(K^+ + H^+)$-ATPase during the second step of a sequential treatment with 15% ethanol for 60 s at 23°C and subsequently at 37°C can be due to the extraction of these very phospholipids [10,11]. More likely, it is an inactivation of the enzyme by ethanol, as has been reported for $(Na^+ + K^+)$-ATPase [36]. The sensitivity of the enzyme to ethanol would then be correlated with the conformation of the enzyme, which may be influenced by the presence of essential lipids and by temperature.

Effects of phospholipase C treatment on $(K^+ + H^+)$-ATPase

There is a remarkable parallelism between the lowering of the phospholipid content and of the $(K^+ + H^+)$-ATPase activity during treatment with phospholipase C, both in time and in final level. A similar relationship is found for the $(Na^+ + K^+)$-ATPase activity during phospholipase C treatment of crude preparations isolated from ox brain [37] or *Electrophorus electroplax* [38]. On the other hand, De Pont et al. [17] find only a 15% loss of enzyme activity upon 65% phospholipid hydrolysis during phospholipase C treatment of a highly purified $(Na^+ + K^+)$-ATPase preparation from rabbit kidney.

The *p*-nitrophenylphosphatase activity is lowered in parallel with the $(K^+ + H^+)$-ATPase activity during phospholipase C treatment. This contrasts with the effects of phospholipase A_2, where no loss of K^+ stimulated *p*-nitrophenylphosphatase activity was observed in the face of 75% loss of $(K^+ + H^+)$-ATPase activity [12]. Our observations also differ from the findings for crude $(Na^+ + K^+)$-ATPase preparations, where the *p*-nitrophenylphosphatase activity upon phospholipase C treatment falls either less [37] or more [38] than the $(Na^+ + K^+)$-ATPase activity.

The effects of phospholipase C treatment on various properties of the gastric $(K^+ + H^+)$-ATPase have been studied in an attempt to find out how phospholipids affect the reaction mechanism of the enzyme. No changes are found in the pH optimum and the high affinity K_m value for ATP (as is also the case for phospholipase C treated $(Na^+ + K^+)$-ATPase [39]). This finding and the constancy of the Arrhenius plot suggest that the loss of activity results from an allosteric change rather than from a direct involvement of the active site. In contrast, phospholipase C treatment of $(Na^+ + K^+)$-ATPase changes the Arrhenius plot, which is ascribed to a disruption of the contact between charged protein groups and lipids [40]. Discontinuities in Arrhenius plots are often considered to reflect lipid phase transitions [41] and consequently a lipid dependence of the enzyme, although a temperature dependent change in the protein moiety of the lipoprotein may also be responsible.

The only other change in enzyme parameters, apart from V, is a slight decrease in the K_a value for K^+. This would imply that phospholipase C action facilitates the K^+-induced conformation, previously reported by us [14]. This seems to differ from the $(Na^+ + K^+)$-ATPase, where detergent delipidation abolishes the K^+ induced decrease in ADP binding without changing the number of ADP binding sites [42].

Effects of phospholipase C treatment on partial steps of $(K^+ + H^+)$-ATPase

In order to determine whether the decreased enzyme activity is due to a decreased turnover number of all enzyme molecules or a complete inactivation of part of the enzyme molecules, we have studied the effects of phospholipase C treatment on the partial reactions of the overall enzyme activity. The binding capacity for the nonphosphorylating ATP-analogue, adenylyl imidodiphosphate (Ado*PP*[NH]*P*) and the maximal phosphorylation level are decreased to a similar extent. Rather than a general decrease in turnover number of all ATPase molecules, this suggests that part of the ATPase molecules are completely inactivated by losing their ability to bind substrate. This is in agreement with the lack of effect of phospholipase C treatment on most of the enzyme parameters.

The decreased substrate binding could be the result of a specific effect on the substrate binding centre due to hydrolysis of particular phospholipids.

Alternatively, it could be due to aggregation of protein molecules upon phospholipase C treatment. The striking parallelism between phospholipid content and $(K^+ + H^+)$-ATPase activity appears to favour the latter nonspecific phenomenon. Protein aggregation could be due to a decreased membrane surface, caused by the coalescence of diacylglycerols to droplets and extrusion of these droplets, as previously observed for the photoreceptor membrane [43]. However, the immediate decrease in $(K^+ + H^+)$-ATPase activity upon phospholipase C treatment (Figs. 1–3) indicates that this cannot be the full explanation, since diacylglycerol coalescence would most probably require a considerable degree of phospholipid hydrolysis.

Summarizing, the effects of phospholipase C treatment indicate that there is a general lipid dependence of $(K^+ + H^+)$-ATPase but no specific requirement for any particular phospholipid, and that the enzyme inactivation may occur through protein aggregation hindering substrate binding and subsequent phosphorylation.

Acknowledgements

The authors would like to thank Mr. P.A.A. Jansen for providing the purified phospholipase C preparation from *Bacillus cereus* and Dr. E.H.S. Drenthe for valuable discussion. This study has been supported in part by the Netherlands Organization for Basic Research (ZWO) through the Netherlands Biophysics Foundation and a U.S.P.H.S. grant (NS-14134) to A.O.

References

1 Forte, J.G., Ganser, A.L. and Tanisawa, A.S. (1974) Ann. N.Y. Acad. Sci. 242, 255–267
2 Sachs, G., Spenney, J.G. and Lewin, M. (1978) Physiol. Rev. 58, 106–173
3 De Pont, J.J.H.H.M. and Bonting, S.L. (1981) in New Comprehensive Biochemistry, vol. 2 (Bonting, S.L., De Pont, J.J.H.H.M., eds.), pp. 209–234, Elsevier/North Holland Biomedical Press, Amsterdam
4 Saccomani, G., Stewart, H.B., Shaw, D., Lewin, M. and Sachs, G. (1977) Biochim. Biophys. Acta 465, 311–330
5 Forte, J.G., Forte, G.M. and Saltman, P. (1967) J. Cell. Physiol. 69, 293–304
6 Limlomwongse, L. and Forte, J.G. (1970) Am. J. Physiol. 219, 1717–1722
7 Chang, H., Saccomani, G., Rabon, E., Schackmann, R. and Sachs, G. (1977) Biochim. Biophys. Acta 464, 313–327
8 Sachs, G., Berglindh, T., Rabon, E., Stewart, H.B., Barcellona, M.L., Wallmark, B. and Saccomani, G. (1980) Ann. N.Y. Acad. Sci. 341, 312–334
9 Sen, P.C. and Ray, T.K. (1979) Arch. Biochem. Biophys. 198, 548–555
10 Sen, P.C. and Ray, T.K. (1979) Biochem. J. 182, 637–640
11 Sen, P.C. and Ray, T.K. (1980) Arch. Biochem. Biophys. 202, 8–17
12 Saccomani, G., Chang, H.H., Spisni, A., Helander, H.F., Spitzer, H.L. and Sachs, G. (1979) J. Supramol. Struct. 11, 429–444
13 Sachs, G., Rabon, E. and Saccomani, G. (1979) in Cation Flux across Biomembranes (Mukohata, Y. Packer, L., eds.), pp. 53–66, Academic Press, New York
14 Schrijen, J.J., Luyben, W.A.H.M., De Pont, J.J.H.H.M. and Bonting, S.L. (1980) Biochim. Biophys. Acta 597, 331–344
15 Otnaess, A.B., Prydz, H., Bjørklid, E. and Berre, A. (1972) Eur. J. Biochem. 27, 238–243
16 Stahl, W.L. (1973) Arch. Biochem. Biophys. 154, 47–55
17 De Pont, J.J.H.H.M., Van Prooyen-Van Eeden, A. and Bonting, S.L. (1978) Biochim. Biophys. Acta 508, 464–477
18 Folch, J., Lees, M. and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497–509
19 Palmer, F.B.S.C. (1971) Biochim. Biophys. Acta 231, 134–144
20 Broekhuyse, R.M. (1968) Biochim. Biophys. Acta 152, 307–315
21 Neufeld, A.H. and Levy, H.M. (1969) J. Biol. Chem. 244, 6493–6497
22 Schrijen, J.J., Van Groningen-Luyben, W.A.H.M., De Pont, J.J.H.H.M. and Bonting, S.L. (1981) Biochim. Biophys. Acta 640, 473–486
23 Van de Ven, F.J.M., Schrijen, J.J., De Pont, J.J.H.H.M. and Bonting, S.L. (1981) Biochim. Biophys. Acta 640, 487–499
24 Pangborn, M.C. (1951) J. Biol. Chem. 188, 471–476
25 Van Amelsvoort, J.M.M., De Pont, J.J.H.H.M. and Bonting, S.L. (1977) Biochim. Biophys. Acta 466, 283–301
26 Chap, H.J., Zwaal, R.F.A. and Van Deenen, L.L.M. (1977) Biochim. Biophys. Acta 467, 146–164
27 Drenthe, E.H.S., Bonting, S.L. and Daemen, F.J.M. (1980) Biochim. Biophys. Acta 603, 117–129
28 Roberts, M.F., Otnaess, A.B., Kensil, C.A. and Dennis, E.A. (1978) J. Biol. Chem. 253, 1252–1257
29 Lee, H.C., Breitbart, H., Berman, M. and Forte, J.G. (1979) Biochim. Biophys. Acta 553, 107–131
30 Davies, O.L. and Pearson, E.S. (1934) J. R. Stat. Soc., Suppl. 1., 76–93
31 Wallmark, B., Stewart, H.B., Rabon, E., Saccomani, G. and Sachs, G. (1980) J. Biol. Chem. 255, 5315–5319

32 Robinson, J.D. (1976) Biochim. Biophys. Acta 429, 1006–1019
33 Roelofsen, B., Zwaal, R.F.A. and Woodward, C.B. (1974) in Methods in Enzymology, vol. 32, Biomembranes, part B (Fleischer, S.E., Packer, L., eds.), p. 135, Academic Press, New York
34 Gazotti, P. and Peterson, S.W. (1977) J. Bioerg. Biomembr. 9, 373–386
35 Gwarsha, K., Rumsby, M.G. and Little, C. (1980) Biochem. Soc. Trans. 8, 600–601
36 Lin, D.C. (1980) Biochem. Pharmacol. 29, 771–775
37 Taniguchi, K. and Tonomura, Y. (1971) J. Biochem. 69, 543–557
38 Goldman, S.S. and Albers, R.W. (1973) J. Biol. Chem. 248, 867–874
39 Sun, A.Y., Sun, G.Y. and Samorjski, T. (1971) J. Neurochem. 18, 1711–1718
40 Che Chung K'uo and Boldyrev, A.A. (1978) Biokhimiya 43, 2100–2104
41 Frenkel, E.J., Roelofsen, B., Brodbeck, U., Van Deenen, L.L.M. and Ott, P. (1980) Eur. J. Biochem. 109, 377–382
42 Jensen, J. and Ottolenghi, P. (1976) Biochem. J. 159, 815–817
43 Olive, J., Benedetti, E.L., Van Breugel, P.J.G.M., Daemen, F.J.M. and Bonting, S.L. (1978) Biochim. Biophys. Acta 309, 129–135