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ATPase and Phosphatase Activities from Human Red Cell Membranes: II. The Effects of Phospholipases on Ca^{2+}-Dependent Enzymic Activities

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Summary. Treatment of human red cell membranes with pure phospholipase A_2 results in a progressive inactivation of both Ca^{2+}-dependent and (Ca^{2+} + K^+)-dependent ATPase and phosphatase activities. When phospholipase C replaces phospholipase A_2, Ca^{2+}-dependent ATPase activity and Ca^{2+}-dependent phosphorylation of red cell membranes are lost, while Ca^{2+}-dependent phosphatase activity is enhanced and its apparent affinity for Ca^{2+} is increased about 20-fold. Activation of Ca^{2+}-dependent phosphatase following phospholipase C treatment was not observed in sarcoplasmic reticulum preparation. Phospholipase C increases the sensitivity of the phosphatase to N-ethylmaleimide but has little effect on the kinetic parameters relating the phosphatase activity to substrate and cofactors, suggesting that no extensive structural disarrangement of the Ca^{2+}-ATPase system has occurred after incubation with phospholipase C.

Since active Ca^{2+} extrusion was first demonstrated in red cells (Schatzmann, 1966), much knowledge has been gained on the characterization of the properties of the system responsible for this phenomenon (Schatzmann, 1975).

Although there is a large body of experience concerning the effects of phospholipases on other active transport systems, little is known about the response to phospholipases of the Ca^{2+}-dependent ATPase system of red blood cells.

Coleman and Bramley (1975) have recently shown that treatment with a partially purified preparation of phospholipase C from *Clostridium welchii* results in a progressive loss in Ca^{2+}-dependent ATPase activity.

Results in this paper confirm this finding, and, with the aid of highly purified phospholipase C or phospholipase A_2 preparations, extend the

studies of the effect of these enzymes to other catalytic activities of the Ca^{2+}-dependent ATPase system of human red cell membranes.

Materials and Methods

Treatment with Phospholipases

Human red cell membranes, prepared according to the procedure already described (Garrahan, Pouchan & Rega, 1969), were treated following a procedure similar to that described by Roelofsen and van Deenen (1973).

The incubation was carried out at 30° C for different lengths of time and was terminated by cooling and simultaneous addition of enough concentrated ethylene glycol-bis(aminoethyl)-tetraacetic acid (EGTA) solution as to give a final concentration of 10 mM. The membrane suspension was centrifuged and the pellet was washed twice with 50 volumes of Tris-HCl 15 mM (pH 7.4 at 20 °C). After the second wash the membranes were suspended in Tris HCl 15 mM to give a concentration of about 10 mg membrane protein/ml and submitted to rapid freezing and thawing before use. This procedure disrupts any permeability barrier that may appear as a consequence of incubation (Rega, Richards & Garrahan, 1973) and thus avoids artifacts due to compartmentalization, such as those pointed out by Coleman and Bramley (1975).

Measurement of Enzymic Activities

ATPase and phosphatase activities were measured as described previously (Richards, Rega & Garrahan, 1977). In all experiments a control was run with membranes submitted to preincubation during the same length of time and at the same temperature in media having identical composition to that used for treatment of membranes with phospholipases.

Analysis of Phospholipids

Membrane lipids were extracted by the procedure of Reed, Swisher, Marinetti and Eden (1960). The different phospholipid classes were separated by thin layer chromatography following the procedure of Wheeler and Whittam (1970). The amount of lipid phosphorus in each of the phospholipid classes was estimated by the procedure of Bohner, Soto and Cohan (1965).

Phosphorylation of red cell membranes was carried out using (γ-^{32}P)ATP by the procedure already described (Rega & Garrahan, 1975). Protein was measured by the method of Lowry, Rosebrough, Farr and Randall (1951).

Sources of Materials

Two phospholipase C preparations were used. Pure phospholipase C from *Bacillus cereus* (Zwaal & Roelofsen, 1974, specific activity about 1000 IU) was a kind gift from Dr. R. F. A. Zwaal (Department of Biochemistry, University of Utrecht) and phospholipase C from *Clostridium welchii* (specific activity 5 IU) was from Sigma Chemical Co. Phospholipase A_2 was purified in this laboratory from porcine pancreas following the procedure of Nieuwenhuizen, Kunze and de Haas (1974). ATP, *p*-nitrophenylphosphate, EGTA and N-ethylmaleimide were from Sigma Chemical Co. All other salts and reagents were of A.R. degree. Solutions were prepared in doubly glass-distilled water.

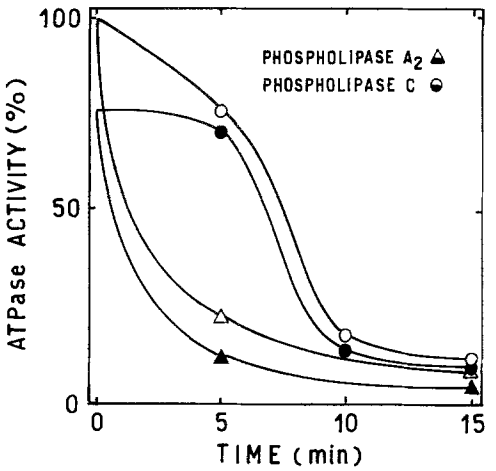

Fig. 1. Ca^{2+}-dependent (●, ▲) and ($Ca^{2+}+K^+$)-dependent (○, △) ATPase activities of red cell membranes after treatment for different lengths of time with either phospholipase C from *Bacillus cereus* (1 IU/mg membrane protein) or phospholipase A_2 (0.5 IU/mg membrane protein). Results are expressed taking as 100% the ($Ca^{2+}+K^+$)-dependent activity of untreated membranes

Results

Effects of Phospholipases on Ca^{2+}-Dependent ATPase and Phosphatase Activities

Results in Fig. 1 show the Ca^{2+} and ($Ca^{2+}+K^+$)-dependent ATPase activities of red cell membranes that had been preincubated for various lengths of time with either phospholipase C from *Bacillus cereus* or Phospholipase A_2 from porcine pancreas.

It is clear that both phospholipase C and phospholipase A_2 lead to rapid inactivation of Ca^{2+}- and ($Ca^{2+}+K^+$)-dependent ATPase activities. After a 15-min preincubation almost 90% of the activities are lost; the activities that remain after that time persist even if preincubation is prolonged to 120 min with a concentration of phospholipase C ten times higher than that used in the experiment in Fig. 1. Essentially similar results to those in Fig. 1 were obtained with a preparation of phospholipase C from *Clostridium welchii.*

Results in Fig. 2 show that in contrast to the rapid inactivation of Ca^{2+}-dependent ATPase activities, treatment of red cell membranes with phospholipase C has little effect in their ability to catalyze Ca^{2+}-dependent hydrolysis of *p*-nitrophenylphosphate, the main effect of the treat-

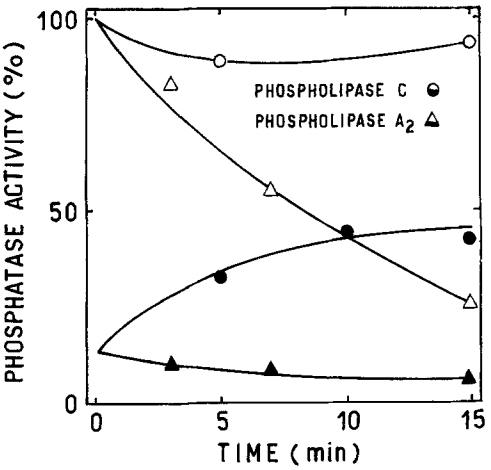

Fig. 2. Ca^{2+}-dependent (●, ▲) and ($Ca^{2+}+K^+$)-dependent (○, △) phosphatase activities of red cell membranes after treatment for different lengths of time with either phospholipase C from *Bacillus cereus* or phospholipase A_2 under identical conditions as those in Fig. 1

ment being to increase from 15 to 40% the fraction of the total ($Ca^{2+}+K^+$)-dependent phosphatase activity that can be elicited by Ca^{2+} alone.

The effect of phospholipase C persisted even if preincubation time was prolonged to 120 min with a concentration of phospholipase C ten times higher than that used in the experiment of Fig. 2. Lack of inhibition of phosphatase activity was also observed using a phospholipase C preparation from *Clostridium welchii.* The absolute dependence on ATP of the phosphatase for activation by Ca^{2+} is still apparent in phospholipase C-treated membranes. Addition of Ca^{2+} in the absence of ATP results in inhibition of phosphatase activity from treated membranes. Fig. 2 also shows that the effect of phospholipase C on phosphatase activities, is not reproduced by phospholipase A_2. Preincubation of membranes with phospholipase A_2 leads to a progressive decline in both Ca^{2+}- and ($Ca^{2+}+K^+$)-dependent phosphatase activities.

Effects of Phospholipase C on Ca^{2+}-Dependent Phosphorylation

The lack of inhibition of phosphatase activity suggests that loss of Ca^{2+}-dependent ATPase activity after phospholipase C treatment results from the blockage of a reaction that preceeds dephosphorylation. To test this point the amount of Ca^{2+}-dependent phosphoenzyme formed

Table 1. The effect of treatment with phospholipase C on Ca^{2+}-dependent ATPase and phosphatase activities from sarcoplasmic reticulum of skeletal muscle

Preparation	Ca^{2+}-dependent ATPase activity	Ca^{2+}-dependent phosphatase activity
	μmole Pi/mg protein × hr	
Control	84.3	29.7
Phospholipase C-treated	13.1	6.4

Sarcoplasmic reticulum vesicles were prepared from rat skeletal muscle following the procedure described by Martonosi and Feretos (1964) for the preparation of the fraction called "grana 1". ATPase activity was measured estimating the Pi released from ATP after incubation of sarcoplasmic reticulum in media containing (mM): KCl, 100; Tris-HCl (pH 7.4 at 37 °C), 45; $MgCl_2$, 5; ATP (disodium salt), 4.5; $CaCl_2$, 0.05. Phosphatase activity was measured estimating the release of *p*-nitrophenol from *p*-nitrophenylphosphate after incubation in media having identical composition to that used for measuring ATPase activity, except that ATP was replaced by an equivalent amount of *p*-nitrophenylphosphate. The reaction was initiated by addition of 0.1 ml of suspension of sarcoplasmic reticulum vesicles (1 mg protein/ml) to 0.9 ml of incubation media. The reaction was stopped with trichloroacetic acid (5% w/v) after 5 min incubation at 37 °C. Sarcoplasmic reticulum vesicles were treated with phospholipase C from *Bacillus cereus* (10 IU/mg protein) during 1 hr at 30 °C following a procedure similar to that described in methods for red cell membranes.

after incubation of membranes with (γ-^{32}P)ATP was measured in intact membranes and in membranes treated for 30 min with phospholipase C from *Bacillus cereus*. Results showed that after treatment the steady state level of Ca^{2+}-dependent phosphoprotein dropped from 0.72 to 0.02 μmole/mg membrane protein.

Effects of Phospholipase C on Ca^{2+}-Dependent Activities of Sarcoplasmic Reticulum

Table 1 shows the effect of phospholipase C from *B. cereus* on Ca^{2+}-dependent ATPase and phosphatase activities of sarcoplasmic reticulum from skeletal muscle. After treatment with phospholipase C, the decrease in Ca^{2+}-dependent ATPase activity is parallelled by a similar decrease in phosphatase activity. It seems, therefore, that persistence of phosphatase activity in red blood cell treated with phospholipase C represents a property of this system rather than a general feature of Ca^{2+}-dependent membrane-bound enzymes.

Table 2. Phospholipids breakdown in red cell membranes treated with phospholipase C from *Bacillus cereus* and from *Clostridium welchii*

Condition of treatment	Phospholipid class (% of control)			
	Sphingo-myelin	Phos-phatidyl choline	Phos-phatidy-ethanolamine	Phos-phatidyl-serine
Phospholipase C (*B. cereus*)				
1.0 IU/mg membrane protein for 15 min	107	87	48	37
10.0 IU/mg membrane protein for 120 min	98	12	8	16
Phospholipase C (*C. welchii*)				
1.0 IU/mg membrane protein for 20 min	17	21	4	41

The figures are the results of a single typical experiment. Treated and control membranes were obtained from the same batch of red cells and run simultaneously.

Effects of Phospholipase C on Lipid Composition of Red Cell Membranes

Table 2 allows a comparison of the degree of breakdown of the main phospholipid classes of erythrocyte membranes after treatment with phospholipase C from both *B. cereus* and *C. welchii*. After a 15-min incubation with phospholipase C from *B. cereus*, the content of phosphatidylserine and phosphatidylethanolamine is less than half the control value, whereas little or no change is observed in the content of phosphatidylcholine and sphingomyelin. Prolonged incubation with a higher concentration of phospholipase C from *B. cereus* leads to a marked depletion of all phospholipid classes except sphingomyelin, whose content remains practically unchanged.

On the other hand when phospholipase C from *C. welchii* is used there is a marked drop in all phospholipid classes except that of phosphatidylserine, which decreases to a value similar to that obtained with phospholipase from *B. cereus*.

Comparison of Figs. 1 and 2 with Table 2 makes it clear that the effects of phospholipase C treatment on Ca^{2+}-dependent enzymic activities is completed considerably before full hydrolysis of phospholipids is achieved.

Table 3. The effect of treatment with phospholipase C of *Bacillus cereus* on the kinetic parameters of the Ca^{2+}-dependent phosphatase activities of red cell membranes

Kinetic parameter		Control	Treated
Substrate	K_m (mM)	29	29
	V_{max} (% control)	100	198
K^+ activation	$K_{0.5}$ (mM)	16	12
	V_{max} (% control)	100	70
Na^+ activation	$K_{0.5}$ (mM)	66	66
	V_{max} (% control)	100	72

For each parameter enzymatic activity was measured simultaneously on treated and control membranes from the same batch of membranes. The values were calculated from reciprocal plots of activity *vs.* concentration curves. Treated membranes were preincubated during 2 hr with 10 IU/mg membrane protein of phospholipase C.

Effects of Phospholipase C on the Kinetic Parameters of Ca-Dependent Phosphatase

Table 3 summarizes the effects of treatment of red cell membranes with phospholipase C on kinetic parameters of Ca^{2+}-dependent phosphatase. Results make it clear that after treatment the maximum rate of Ca^{2+}-dependent *p*-nitrophenylphosphate hydrolysis remains unchanged.

Table 3 also shows that the apparent affinity for Na^+ and K^+ are not significantly altered, the only effect of treatment on monovalent cation activation being a small decrease in the maximum effect of Na^+ and K^+. An additional and unexpected effect of phospholipase C is the change in the apparent affinity for Ca^{2+} of the Ca^{2+}-dependent phosphatase. Results in Fig.3 show that after incubation with phospholipase C, the concentration of Ca^{2+} for half maximal activation of the $(Ca^{2+}+K^+)$-dependent phosphatase drops from 9 to 0.45 μM Ca^{2+}.

The Effects of N-ethylmaleimide on Ca^{2+}-Dependent Phosphatase Activities of Red Cell Membranes Treated with Phospholipase C

In the preceding paper of this series (Richards *et al.*, 1977) we have shown that incubation of red cell membranes with N-ethylmaleimide leads to inhibition of Ca^{2+}-dependent phosphatase activity. The effect of N-ethylmaleimide is fully abolished by ATP. Calcium-ion, probably by combination at the sites at which it binds to activate ATPase and phosphatase,

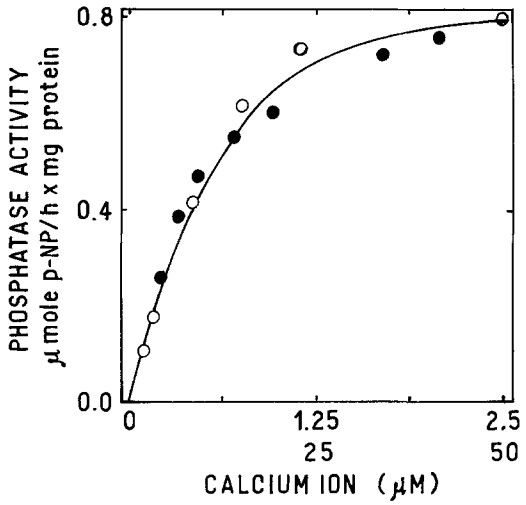

Fig. 3. The relation between the concentration of Ca^{2+} and $(Ca^{2+}+K^{+})$-dependent phosphatase activity in control (●) membranes and in membranes that had been pretreated for 2 hr with 10 IU/mg membrane protein of phospholipase C from *Bacillus cereus* (○). Ca^{2+} concentrations were adjusted using Ca-EGTA buffers following the procedure of Wolf (1973). For phospholipase-treated membranes the concentration of free Ca^{2+} was from 0 to 2.5 μM

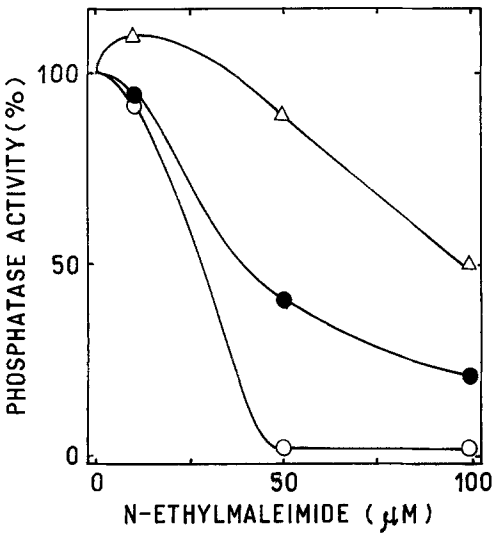

Fig. 4. The effects of different concentrations of N-ethylmaleimide during preincubation in control media (●); in media containing 100 μM Ca^{2+} (○); and in media containing 3 mM ATP (△) on $(Ca^{2+}+K^{+})$-dependent phosphatase activity of red cell membranes that had been pretreated for 2 hr with 10 IU/mg membrane protein of phospholipase C from *Bacillus cereus*. Treatment with N-ethylmaleimide was performed following the procedure already described (Richards *et al.*, 1977)

markedly increases the reactivity of the enzyme to N-ethylmaleimide, the concentration of N-ethylmaleimide for half-maximal effect dropping from 350 to 25 μM. Fig. 4 gives the result of an experiment in which the effects of increasing concentrations of N-ethylmaleimide were tested on Ca^{2+}-dependent phosphatase activity in membranes pretreated with phospholipase C.

Results show that after treatment with phospholipase C the concentration of N-ethylmaleimide for half-maximal inhibition of the phosphatase is 40 μM. It seems therefore that phospholipase C mimicks Ca^{2+} in increasing the sensitivity of the phosphatase to N-ethylmaleimide. This assertion gains support in the fact that Ca^{2+} during treatment with N-ethylmaleimide is with little effect if tested on phosphatase activity from membranes that have been preincubated with phospholipase C (Fig. 4). Results also show that when ATP is present during treatment with N-ethylmaleimide, the concentration of the sulfhydril blocking agent for half-maximal effect is increased to 100 μM, demonstrating that, although lowered (*cf.* Fig. 1*b* with Fig. 2*c* from Richards *et al.* 1977), protection by ATP against inhibition by N-ethylmaleimide is present in membranes treated with phospholipase C.

Discussion

Results presented in this paper show that as a consequence of treatment with phospholipase C the Ca^{2+}-dependent ATPase system of human red cell membranes loses its ability to catalyze Ca^{2+}- and $(Ca^{2+}+K^{+})$-dependent hydrolysis of ATP, preserving its ability to catalyze Ca^{2+}- and $(Ca^{2+}+K^{+})$-dependent hydrolysis of *p*-nitrophenylphosphate. After treatment, Ca^{2+}-dependent phosphatase activity is increased and the activating effect of K^{+} on this activity is somewhat reduced in such a way that the overall rate of $(Ca^{2+}+K^{+})$-phosphatase activity remains almost unchanged after treatment. The response to phospholipase C of Ca^{2+}-dependent enzymatic activities of red cell membranes contrasts with that of the Ca^{2+}-dependent activities of the sarcoplasmic reticulum of skeletal muscle in which treatment with phospholipase C leads to a parallel decline of both ATPase and phosphatase activities.

Experiments in this paper strongly indicate that inhibition of Ca^{2+}-dependent ATPase of red cell membranes after treatment with phospholipase C has to be attributed to blockage of its phosphorylation step. In fact, treatment with phospholipase C drastically reduces the steady-state level of Ca^{2+}-dependent phosphorylated intermediate but has little

effect on Ca^{2+}-dependent phosphatase activity. Since phosphatase activity probably expresses the ability of the system to catalyze dephosphorylation, it is likely that the reduction in the level of phosphoenzyme is caused by the reduction in the rate of Ca^{2+}-dependent phosphorylation. The persistence of Ca^{2+}-dependent phosphatase activity after treatment with phospholipase C requires the conservation of the properties of the sites for ATP and Ca^{2+}. These sites seem to be the same as those of the Ca^{2+}-ATPase (Rega *et al.*, 1973). It seems therefore reasonable to postulate that blockage of phosphorylation after phospholipase C treatment is not due to lack of binding of ATP and Ca^{2+} but to the selective inhibition of the catalysis of phosphorylation. Furthermore, persistence of Ca^{2+}-dependent phosphatase in the absence of phosphorylation seems to indicate that the absolute dependence on ATP of this activity is due to the formation of an enzyme-ATP complex and not to the formation of phosphoenzyme.

The inability of phospholipase C treatment to inactivate phosphatase activity does not mean that dephosphorylation of the Ca^{2+} pump is completely insensitive to attack by phospholipases since treatment of red cell membranes with phospholipase A_2, under conditions which according to Roelofsen and van Deenen (1973) would eliminate the toxic products of the reaction, leads to progressive inhibition of Ca^{2+}-dependent phosphatase.

The experiments presented in this paper do not allow us to postulate a definite mechanism to account for the effect of treatment with phospholipases on Ca^{2+}-dependent enzymatic activities. Nevertheless, since the purity of the phospholipase preparations used precludes effects other than the enzymatic hydrolysis of phospholipids, it seems reasonable to think that the observed effects result from the perturbation of the lipid environment of the Ca^{2+}-ATPase system. If we take this for granted, our results may be taken as suggestive that: (i) removal by hydrolysis of a fraction of the polar head groups of membrane phosphodiglycerides does not impede the binding of ATP and markedly increases the affinity for the binding of Ca^{2+}, but blocks the catalysis of phosphorylation; (ii) catalysis of dephosphorylation seems to be independent of the integrity of the polar head groups but requires the persistence within the membrane structure of the diacylglycerol moeity of phosphoglycerides; and (iii) the integrity of sphyngomyelin does not seem to be required for the maintenance of the functional properties of the Ca^{2+}-ATPase system.

The demonstrations of these assertions, however, needs the experimental verification that lost or modified activities are restored back

to normal by addition of exogenous phospholipids. In this respect it is interesting to mention that Coleman and Branley (1975) have shown that after phospholipase C treatment Ca^{2+}-ATPase activity of red cell membranes can be restored by addition of mixtures of either diacylphosphoglycerides or monoacyl analogues (lysoderivatives).

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Note added in Proof

After this paper was submitted for publication B. Roelofsen and H.J. Schatzmann (*Biochim. Biophys. Acta* **464**:17 (1977)) published a description of the effects of pure phospholipases on the Ca^{2+}-dependent ATPase activity of red cell membranes. Their results agree with the effects of phospholipases on Ca^{2+}-dependent ATPase activity reported in this paper.

References

Bohner, L. S., Soto, E. F., Cohan, T. 1965. Quantitative analysis of phospholipids by thin-layer chromatography. *J. Chromatogr.* **17**:513

Coleman, R., Bramley, T. A. 1975. Hydrolysis of erythrocyte membrane phospholipids by a preparation of phospholipase C from *Clostridium whelchii*. Deactivation of Ca^{2+}, Mg^{2+}-ATPase and its reactivation by added lipids. *Biochim. Biophys. Acta* **382**:565

Garrahan, P. J., Pouchan, M. I., Rega, A. F. 1969. Potassium activated phosphatase from human red cells. The mechanism of potassium activation. *J.Physiol. (London)* **202**:777

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. 1951. Protein meassurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265

Martonosi, A., Feretos, R. 1964. Sarcoplasmic reticulum: I. The uptake of Ca^{++} by sarcoplasmic reticulum fragments. *J. Biol. Chem.* **239**:648

Nieuwenhuizen, W., Kunze, H., Hass, G. H. de 1974. Phospholipase A_2 (phosphatide acylhydrolase, EC3.1.1.4) from porcine pancreas. *In:* Methods in Enzymology. S. Fleischer and L. Paker, editors, vol. XXXII part *B*, p. 147. Academic Press, New York

Reed, C. F., Swisher, S. N., Marinetti, G. V., Eden, E. G. 1960. Studies of the lipids of the erythrocyte. I. Quantitative analysis of the lipids of normal human red blood cells, *J. Lab. Clin. Med.* **56**:281

Rega, A.F., Garrahan, P.J. 1975. Calcium ion-dependent phosphorylation of human erythrocyte membranes. *J. Membrane Biol.* **22**:313

Rega, A. F., Richards, E. E., Garrahan, P. J. 1973. Calcium ion-dependent *p*-nitrophenyl phosphate phosphatase activity and calcium ion-dependent adenosine triphosphatase activity from human erythrocyte membranes. *Biochem. J.* **136**:185

Richards, D. E., Rega, A. F., Garrahan, P. J. 1977. ATPase and phosphatase activities from human red cell membranes, I. The effects of N-ethylmaleimide. *J. Membrane Biol.* **35**:113

Roelofsen, B., Deenen, L. L. M. van 1973. Lipid requirement of membrane-bound ATPase. Studies on human erythrocyte ghosts. *Eur. J. Biochem.* **40**:245

Schatzmann, H. J. 1966. ATP-dependent Ca^{++} extrusion from human red cells. *Experientia* **22**:364

Schatzmann, H. J. 1975. Active calcium transport and Ca^{++}-activated ATPase in human red cells. *Curr. Top. Membr. Transp.* **6**:125

Wheeler, K. P., Whittam, R. 1970. The involvement of phosphatidylserine in adenosinetriphosphatase activity of the sodium pump. *J. Physiol. (London)* **207**:303

Wolf, H. U. 1973. Divalent metal ion buffers with low pH-sensitivity. *Experientia* **29**:241

Zwaal, R. F. A., Roelofsen, B. 1974. Phospholipase C (phosphatidylcholine choline phosphohydrolase, EC3.1.4.3) from *Bacillus cereus. In:* Methods in Enzymology. S. Fleischer and L. Paker, editors. Vol. XXXII part *B*, p. 154. Academic Press, New York