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ATPase and Phosphatase Activities from Human Red Cell Membranes III. Stimulation of K^+-Activated Phosphatase by Phospholipase C

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Summary. Treatment of red cell membranes with pure phospholipase C inactivates $(Na^+ + K^+)$-ATPase activity and Na^+-dependent phosphorylation but increases K^+-dependent phosphatase activity. When phospholipase A_2 replaces phospholipase C, all activities are lost. Activation of K^+-dependent phosphatase by treatment with phospholipase C is caused by an increase in the maximum rate of hydrolysis of *p*-nitrophenylphosphate and in the maximum activating effect of K^+, the apparent affinities for substrate and cofactors being little affected. After phospholipase C treatment K^+-dependent phosphatase is no longer sensitive to ouabain but becomes more sensitive to N-ethylmaleimide. In treated membranes Na^+ partially replaces K^+ as an activator of the phosphatase. Although ATP still inhibits phosphatase activity, neither ATP nor $ATP + Na^+$ are able to modify the apparent affinity for K^+ of K^+-dependent phosphatase in these membranes.

There is now a copious amount of literature showing that the $(Na^+ + K^+)$-ATPase activity of cell membranes and its partial reactions require the integrity of the membrane phospholipids (Schatzmann, 1962; Wheeler & Whittam, 1970; Goldman & Albers, 1973; Roelofsen & van Deenen, 1973).

In the previous paper of this series (Richards *et al.*, 1977*b*) we have analyzed the changes induced in the behavior of the Ca^{2+}-ATPase system of red cell membranes by treatment with purified phospholipases C and A_2. In the experiments reported in this paper the effects of an identical treatment on the $(Na^+ + K^+)$-ATPase system of red cells are analyzed.

The results show that the response of this system to phospholipase C treatment is remarkably similar to that of the Ca^{2+}-ATPase system.

Materials and Methods

Treatment with phospholipases and analysis of phospholipids were carried out following the procedure described in the previous paper (Richards *et al.*, 1977*b*). $(Na^+ + K^+)$-ATPase and K^+-dependent phosphatase activities were measured as already described (Richards, Rega & Garrahan, 1977).

Na^+-dependent phosphorylation was performed by the procedure already described (Rega, Richards & Garrahan, 1974) in media containing (mM): $MgCl_2$, 0.04; EGTA, 0.05; Na_2HPO_4, 0.03; (γ-^{32}P)ATP (0.5 μC/μmole), 0.03; Tris-HCl (pH 7.4 at 0 °C), 125; and NaCl, 25. Na^+-dependent phosphoenzyme was taken as the difference between the amount of ^{32}P-phosphoprotein formed in this medium and in a medium in which all NaCl was replaced by an equivalent amount of Tris-HCl. Phospholipase C and phospholipase A_2 preparations were those used in the previous paper (Richards *et al.*, 1977*b*). All salts and reagents were A.R. grade.

Results

Effect of Phospholipase C on $(Na^+ + K^+)$-ATPase

The effect of treatment of red cell membranes with phospholipase C from *Bacillus cereus* on $(Na^+ + K^+)$-ATPase activity is shown in Fig. 1. It is evident that after a 10 min long treatment $(Na^+ + K^+)$-ATPase activity is reduced to less than 20% the control value. The fraction of activity remaining could not be eliminated by increasing by a factor of 10 the concentration of phospholipase used in the experiment in Fig. 1 or by prolonging up to 120 min the incubation with enzyme.

Effect of Phospholipase C on Na^+-Dependent Phosphorylation

The effect of phospholipase C on the ability of cell membranes to form phosphoenzyme on incubation with (^{32}P) ATP was tested by measuring the amount of Na^+-dependent phosphoenzyme in intact membranes and in membranes that had been preincubated with phospholipase C during 40 min. After incubation with phospholipase C the steady-state level of phosphoenzyme dropped from 0.54 to 0.036 pmoles/mg protein.

The Effect of Phospholipases on K^+-Dependent Phosphatase Activity

In sharp contrast with the response of $(Na^+ + K^+)$-ATPase, treatment of red cell membranes with phospholipase C increases K^+-dependent phosphatase activity which, under our experimental conditions, reaches after treatment a value about twice that of control membranes (Fig. 2). The increase in phosphatase activity is restricted to its K^+-dependent

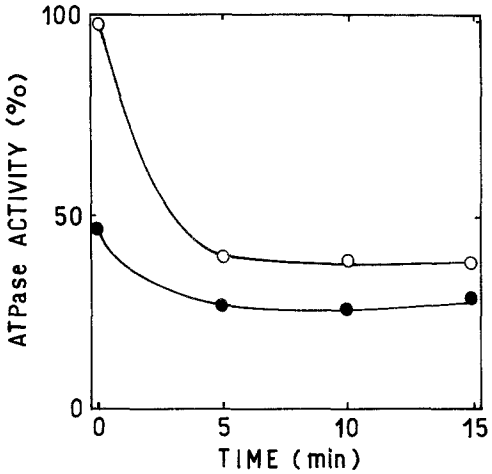

Fig. 1. Mg^{2+}-(●) and ($Mg^{2+} + Na^+ + K^+$)-ATPase (○) activities of red cell membranes after treatment for different lengths of time with 1 IU/mg membrane protein of phospholipase C from *Bacillus cereus*

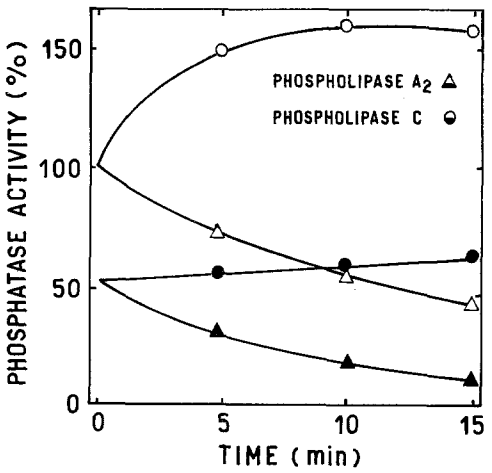

Fig. 2. Mg^{2+}-(●, ▲) and ($Mg^{2+} + K^+$)-phosphatase (○, △) activities of red cell membranes after treatment for different lengths of time with either 1 IU/mg membrane protein of phospholipase C from *Bacillus cereus* or 0.5 IU/mg membrane protein of phospholipase A_2 from porcine pancreas

fraction and does not seem to be a transient phenomenon since it persists even when preincubation is prolonged up to 120 min using ten times as much phospholipase C as in the experiment in Fig. 2. Results in Fig. 2 also show that when phospholipase A_2 is used instead of phospholipase C, K^+-dependent phosphatase activity is progressively inhibited.

Table 1. The effect of treatment with phospholipase C from *Bacillus cereus* on the kinetic parameters of the K^+-activated phosphatase activity of red cell membranes

Kinetic parameter	Control	Treated
Substrate		
Km (mM)	5	9
V_{max} (% control)	100	185
K^+ *activation*		
$K_{0.5}$ (mM)	8	16
$K_{0.5}$ in the presence of 20 mM Na^+ (mM)	–	27
Maximum effect (% control)	100	168
Na^+ *activation*		
$K_{0.5}$ (mM)	–	34
Maximum effect (% maximum effect of K^+)	none	31

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 for 120 min with 10 IU/mg membrane protein of phospholipase C

Other experiments (not shown) demonstrated that when intact red cells are incubated with phospholipase C from *Bacillus cereus* no change in K^+-dependent phosphatase activity can be detected.

Effects of Phospholipase C on Kinetic Parameters of K^+-Dependent Phosphatase

Table 1 compares some kinetic parameters of K^+-activated phosphatase before and after treatment with phospholipase C. Results make it clear that the increase in K^+-dependent phosphatase activity after treatment results from an increase in both the maximum velocity for *p*-nitrophenylphosphate hydrolysis and the maximum stimulatory effect of K^+. Table 1 also shows that competition between Na^+ and K^+ persists in treated membranes. However, Na^+, which cannot replace K^+ as an activator of the phosphatase in intact membranes, becomes able to stimulate this activity after treatment with phospholipase C. The stimulatory effect of Na^+ is exerted with an apparent affinity that is not much different from the apparent affinity for the competitive effect of Na^+ on K^+ activation. The maximum stimulatory effect of Na^+, however, is only 30% of that observed when K^+ is used as activator. Other experiments (not shown) demonstrated that Tl^+, Rb^+ and Cs^+ activated

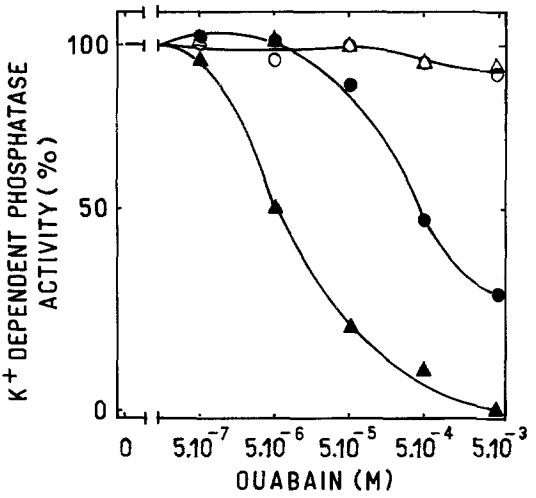

Fig. 3. The effect of ouabain on K^+-dependent phosphatase activity from control (●, ▲) and phospholipase C-treated (○, △) membranes, in the presence (▲, △) and in the absence (●, ○) of 0.5 mM ATP. Phospholipase C treatment was performed by incubating membranes with 10 IU/mg protein of phospholipase C from *Bacillus cereus* for 120 min

phosphatase of treated membranes with the same sequence of relative affinities as in untreated membranes.

Effects of Phospholipase C on Ouabain Sensitivity of K^+-Dependent Phosphatase

A salient feature of K^+-activated phosphatase activity is that the effect of K^+ is fully blocked by adequate concentrations of ouabain (Garrahan *et al.*, 1969). The effectiveness of ouabain is largely increased by ATP (Garrahan, Pouchan & Rega, 1970). Fig. 3 shows a comparison of the effects of different ouabain concentrations on K^+-dependent phosphatase activity before and after treatment of red cell membranes with phospholipase C. It is clear that this treatment results in an almost complete loss in the ouabain sensitivity of the K^+-activated phosphatase, both in the presence and absence of ATP.

Effects of Phospholipase C on the Effect of ATP on K^+-Dependent Phosphatase

In intact membranes, ATP lowers the apparent affinity and the maximum effect of K^+ on K^+-activated phosphatase. Addition of Na^+ to-

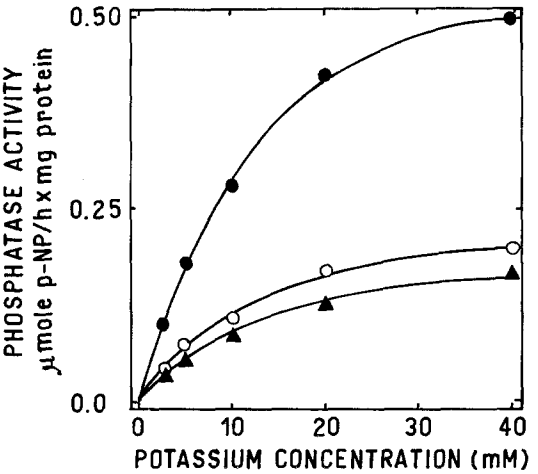

Fig. 4. K^+-dependent phosphatase activity of phospholipase C-treated membranes as a function of K^+ concentration in control media (●); in media with 0.25 mM ATP (▲); and in media with 0.25 mM ATP plus 20 mM Na^+ (○). Treatment with phospholipase C was performed as in Fig. 3. *p*-NP is *p*-nitrophenol

gether with ATP leaves unchanged the effects of ATP on the maximum velocity but increases about ten times the apparent affinity for K^+ (Garrahan *et al.*, 1970). In the experiment in Fig. 4 phosphatase activity of phospholipase C-treated red cell membranes was measured as a function of K^+ concentration in media with or without ATP or ATP+Na^+. The curves show that addition of ATP results in a marked decrease in the rate of K^+-dependent phosphatase activity, this effect being almost unchanged by the addition of Na^+ together with ATP. Double reciprocal plots of K^+-dependent phosphatase against K^+ concentration demonstrated that ATP reduced the maximum effect of K^+ from 0.28 to 0.12 μmole/mg protein and had no effect on the concentration of K^+ for half maximal activation which, both in the presence and absence of ATP, was 14 mM. It is clear therefore that as a consequence of phospholipase C treatment the effect of ATP and of ATP plus Na^+ on the apparent affinity for K^+ are lost.

Effects of Phospholipase C on the Reactivity to N-Ethylmaleimide of K^+-Dependent Phosphatase

Fig. 5 shows the effects of preincubation with increasing concentrations of N-ethylmaleimide on K^+-dependent phosphatase of membranes that had been pretreated with phospholipase C. The concentration of

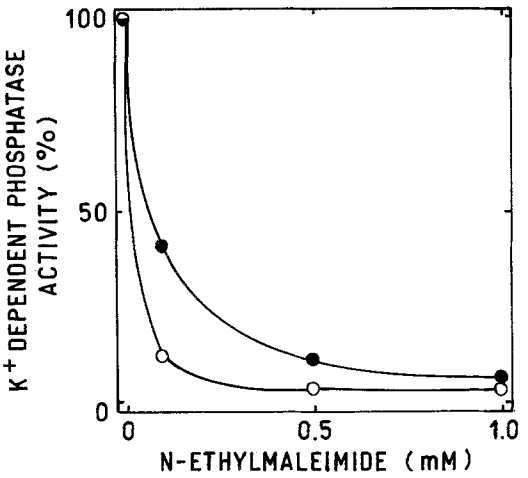

Fig. 5. The effects of preincubation with increasing concentrations of N-ethylmaleimide in the presence (●) and in the absence (○) of 3.0 mM ATP on K^+-dependent phosphatase activity of phospholipase C-treated membranes. Treatment with phospholipase C was performed as in Fig. 3

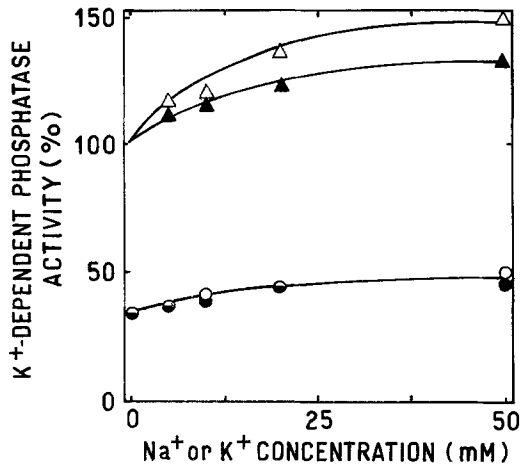

Fig. 6. The effects of increasing concentrations of either Na^+ (●, ▲) or K^+ (○, △) during preincubation with 0.1 mM N-ethylmaleimide in media with (▲, △) and without (●, ○) 3 mM ATP on K^+-dependent phosphatase activity of phospholipase C-treated membranes. Treatment with phospholipase C was performed as in Fig. 3

N-ethylmaleimide for half maximal effect is about 0.02 mM, a value about 100 times less than that for half maximal effect in intact membranes (Richards *et al.*, 1977*a*). Treatment with phospholipase C also increases the sensitivity to N-ethylmaleimide when preincubation with the reagent

is performed in the presence of ATP. Under these conditions, however, half maximal effect of N-ethylmaleimide is reached at about 0.07 mM showing that, although reduced, partial protection by ATP of the effect of N-ethylmaleimide on K^+-dependent phosphatase persists in phospholipase C-treated membranes. We have shown previously that the protective effect of ATP against inhibition by N-ethylmaleimide of K^+-dependent phosphatase is partially prevented if K^+ is present during preincubation with N-ethylmaleimide, Na^+ being ineffective in this respect (Richards *et al.*, 1977*a*). Fig. 6 shows that this effect of K^+ is lost after phospholipase C treatment. In fact, in treated membranes either Na^+ or K^+ during preincubation slightly reduce inhibition by N-ethylmaleimide regardless the presence or absence of ATP during preincubation.

Discussion

The main conclusion to be derived from the results presented in this paper is that treatment with phospholipase C drives the $(Na^+ + K^+)$-ATPase system of red cell membranes to a state characterized by a drastic reduction in catalysis of Na^+-dependent phosphorylation and $(Na^+ + K^+)$-dependent ATP hydrolysis and a significant increase in catalysis of K^+-dependent hydrolysis of *p*-nitrophenylphosphate. This state is reached after a period of time which allows phospholipase C to hydrolyze about 50% of membrane phosphoglycerides (Richards *et al.*, 1977*b*). Phospholipase A_2 cannot reproduce the effects of phospholipase C, and phospholipase C is ineffective when tested in intact cells, conditions under which it does not hydrolyze membrane lipids. (Verkleij *et al.*, 1973). This, together with the fact that a pure enzyme was used during this study, suggests that the effects of phospholipase C result from the enzymatic action of this enzyme on the membrane lipids related to the $(Na^+ + K^+)$-ATPase system. The demonstration of this needs the experimental verification that activities are restored by addition of exogenous phospholipids. This has been done for the $(Na^+ + K^+)$-ATPase activity (Roelofsen & van Deenen, 1973), but not for the other activities.

Our results are in partial agreement with those of Goldman and Albers (1973) who suggested that K^+-dependent phosphatase is not inactivated by phospholipase C, and with the findings of Wheeler and Walker (1975) who showed that K^+-dependent phosphatase is more resistant than $(Na^+ + K^+)$-ATPase activity to lipid extraction with Lubrol. On the other hand, concurrent inhibition of $(Na^+ + K^+)$-ATPase and activation of K^+-phosphatase activities has also been observed when either

glycerol or dimethyl sulfoxide is added to the reaction medium (Albers & Koval, 1972).

Kinetic parameters of phosphatase activity show that stimulation of K^+-dependent phosphatase by phospholipase C treatment is caused by an increase in both the maximum rate of *p*-nitrophenylphosphate hydrolysis and in the maximum effect of K^+. Results do not allow us to determine whether the increase in the maximum rate of K^+-dependent phosphatase is due to a true increase in turnover or to an increase in the fraction of $(Na^+ + K^+)$-ATPase units that catalyze *p*-nitrophenylphosphate hydrolysis at nonlimiting concentration of substrate and activators. However, if the latter were true it cannot be attributed to the elimination by phospholipase C of permeability barriers (Coleman & Bramley, 1975) since the membrane preparation used is surely permeable to substrate and cofactors (Rega, Richards & Garrahan, 1973). Another consequence of treatment with phospholipase C is that Na^+ becomes able to partially replace K^+ as an activator of the phosphatase. Since the sequence of relative affinities to monovalent cations of treated membranes is not very different from that of control membranes, activation by Na^+ probably indicates that the treatment confers to the enzyme-Na^+ complex the ability to catalyze the hydrolysis of *p*-nitrophenylphosphate. After treatment with phospholipase C, the K^+-dependent phosphatase is no longer inhibited by ouabain. The sensitivity to ouabain cannot be restored by ATP which in untreated membranes increases markedly the effectiveness of the glycoside (Garrahan *et al.*, 1970). The reason for this effect remains obscure since Goldman and Albers (1973) have shown that phospholipase C treatment does not alter the binding of ouabain to $(Na^+ + K^+)$-ATPase from *electrophorus* electroplax. In phospholipase C-treated membranes $ATP + Na^+$ is no longer able to increase the apparent affinity for K^+ of the phosphatase. Since there is strong evidence that the increase of the affinity for K^+ induced by $ATP + Na^+$ is caused by the phosphorylation of the $(Na^+ + K^+)$-ATPase system (Garrahan *et al.*, 1970; Robinson, 1974) is not surprising that it should disappear after treatment with phospholipase C, which almost completely blocks phosphorylation. As judged by the inhibitory effect of ATP on the rate of *p*-nitrophenylphosphate hydrolysis and the protective effect of ATP on inhibition by N-ethylmaleimide, this nucleotide is still able to bind to the $(Na^+ + K^+)$-ATPase in phospholipase C-treated membranes. In view of this the inability of ATP to decrease the affinity for K^+ after treatment with phospholipase C may be taken as an indication that this treatment abolishes the interactions between the sites for

K^+ and the sites for ATP. Indirect evidence in favor of this is provided by the lack of effectiveness of K^+ in reversing the protective effect of ATP on inhibition by N-ethylmaleimide in phospholipase-treated membranes.

Comparison of the results presented here with those in the previous paper (Richards *et al.*, 1977*b*) indicates that the changes in the behavior of the $(Na^+ + K^+)$-ATPase system induced by treatment with phospholipase C have a significant number of features in common with the changes that the same treatment induces on the Ca^{2+}-ATPase system of red cell membranes, i.e., (i) catalysis of $(Na^+ + K^+)$- and of Ca^{2+}-dependent ATP hydrolysis is blocked; (ii) both Na^+-dependent phosphorylation and Ca^{2+}-dependent phosphorylation are largely inhibited; (iii) the K^+-dependent phosphatase activity of the $(Na^+ + K^+)$-ATPase system and the Ca^{2+}-dependent phosphatase activity of the Ca^{2+}-ATPase system are enhanced, and in both cases activation is due to an increase in the maximum rate of *p*-nitrophenylphosphate hydrolysis; and (iv) in both enzymic systems the effects are exerted by the same concentration of phospholipase C, reach completion with approximately the same time course, and cannot be reproduced by phospholipase A_2.

It is tempting to interpret the similarity in the response to phospholipases as an indication that the $(Na^+ + K^+)$-ATPase system has the same kind of dependence on membrane lipids as that proposed in the previous paper (Richards *et al.*, 1977*b*) for the Ca^{2+}-dependent ATPase system, which would suggest that there are structural similarities between both systems. Moreover, it seems interesting to note that after treatment, K^+-activated phosphatase becomes almost indistinguishable from Ca^{2+}-dependent phosphatase in regard to activation by Na^+, lack of sensitivity to ouabain, and high sensitivity towards N-ethylmaleimide. This may indicate that at least part of the functional differences between the phosphatase activities of the $(Na^+ + K^+)$-ATPase and the Ca^{2+}-dependent ATPase systems do not reside in their protein moeity.

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