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THE EFFECT OF CHOLERA TOXIN ON HUMAN RED CELL Ca-ATPase

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The effect of cholera toxin on transport Ca-ATPase was studied in membrane fragments from human red cells. A consistently moderate inhibition was found when fragments were previously incubated with toxin in the presence of β-NAD but not in its absence or after treatment with non-activated toxin. In calmodulin-free preparations, both Ca affinity and maximal rate of hydrolysis were affected whereas only affinity was altered in calmodulin-deficient membranes.

The human red cell Ca-ATPase is stimulated by CaM , increasing both Ca affinity and maximal rate of hydrolysis (4,17). Such an effect is thought to occur through Ca-mediated CaM binding to an enzyme domain, presumably affecting two different portions of the ATPase (1). This domain is located on a cytoplasmic segment, which bears the putative Ca binding sites (6).

The primary structure of the human plasma membrane Ca-ATPase was recently derived from complementary DNA sequencing (20). The CaM binding region was shown to be moderately enriched in arginine residues, locating about 11% of the total. Since cholera toxin A_1 subunit, catalyses ADPrybosilation of arginine and other guanidino related compounds (11), it was of interest to study the effect of this toxin on the Ca-ATPase from human red cells. It was found that the enzyme was moderately, but consistently inhibited by cholera toxin.

MATERIALS AND METHODS

All reagents were of analytical quality whenever possible, mainly obtained from SIGMA Chemical Co., USA and British Drug Houses, England.

Fresh O(+) blood from healthy human donors was used. The membranes were prepared by osmotic lysis (14), washed in the presence of 1 mM EDTA to be freed of CaM and fragmented by three cycles of freezing and thawing in ethanol-solid CO_2. Membrane fragments were stored at -80°C until use within not more than 30 days. In some experiments with CaM-containing preparations, EDTA was omitted from the washing medium.

Abbreviations: CaM, calmodulin; Pi, inorganic phosphate; Tris, tris-(hydroxymethyl)aminomethane; EDTA, ethylenediamine tetraacetic acid; EGTA, (ethylenebis(oxyethylenenitrilo))tetracetic acid.

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Activation of cholera toxin was done as described by Schleifer et al. (15), by incubating for 15 min at 37°C in a Pi-buffer (25 mM, pH 8.0) containing 20 mM dithiothreitol and 1 mg/ml bovine serum albumin.

ADPrybosilation was performed following Kahn & Gilman's method (7). Accordingly, membrane fragments were ADPrybosilated in either a 250 mM K_2HPO_4- or 100 mM Tris-buffer (pH 7.55 at room temperature), by preliminary incubation with activated cholera toxin (1:60; toxin to protein ratio) for 1 hr at 37°C, in the presence of (mM): GTP.Na_2, 0.1; creatine phosphate, 10; EGTA, 5; EDTA, 0.1; dithiothreitol, 1; thymidine, 10; β-NAD, 5; and 20 units creatine phosphokinase.

ATPase assays. Ca-ATPase activity of previously incubated membranes was determined after 2 hr incubation at 37°C, in a medium containing (mM): KCl, 130; $MgCl_2$, 2; EGTA, 1; ouabain, 0.1; Tris-HCl, 10 (pH 7.55 at room temperature) and different $CaCl_2$ concentrations set up to obtain free Ca levels varying between 1 μM and 1 mM. The required amounts of Ca and EGTA were calculated using a computer based program, as described by Fabiato & Fabiato (2). Ca-ATPase activity was obtained by substracting the activity found in the presence of Ca from that in its absence. In some experiments, Mg-ATPase was also measured after incubating in a similar medium but containing no Ca.

Pi determinations were done by a modification of the Fiske & Subbarow method (3), using $FeSO_4$ as reducing agent.

Proteins were determined by the Lowry method (9), using bovine serum albumin as standard.

Analyses of statistical significance were done using Student's t tests.

RESULTS

The Mg-ATPase activity of membranes preincubated in Pi-buffer with and without toxin, did not differ significantly from each other, being about 0.348±0.085 and 0.340±0.096 (mean value ± 1 S.D. of fourteen experiments), respectively. By contrast, Ca-ATPase activity, though variable among different membrane batches, showed a tendency to decrease upon previous toxin treatment. Thus, the average activity from fifteen experiments at 1 μM Ca and without added CaM, was 0.51±0.178 and 0.38±0.097 (mean ± 1 S.D.) after incubation in the absence or presence of toxin, respectively. Nonetheless, when each experiment was related to its respective control, by measuring the activity ratio between treated and non treated membranes (A/C ratio), a mean value less than unity and statistically different from 1 was found ($t=2.73$; $P<0.01$; see Table I). No effect on the ATPase activity ratio was obtained in the absence of NAD or if membranes were previously incubated with both NAD and non-activated toxin. On the other hand, when experiments were done using the same batch of membranes, a highly significant reduction of ATPase activity was obtained at above Ca concentration after toxin treatment ($P<0.005$; see Fig. 1).

The activity dependence on free Ca showed the usual low affinity-low Vmax pattern (Fig. 1), shifting to a high affinity-high Vmax state upon addition of 0.3 μM CaM (not shown). Such a behaviour was also observed with toxin-treated membranes. In this case, however, a slight but statistically significant reduction of both Ca affinity and Vmax was found (Fig. 1). This effect was consistently obtained with different membrane batches over a wide range of Ca concentrations, irrespective of CaM being present during incubation for ATPase assays. Thus between 0.1μM-1mM Ca, the A/C ratio was inferior to unity and statistically different from 1 under most conditions ($t>1.8$; $P<0.05$; Table I). The reduction of Ca-ATPase activity was more pronounced at the lowest Ca concentration tested. By contrast with these findings, no toxin action was essentially observed in membranes which had been preincubated in Tris-buffer ($t<1.4$; $P>0.1$; Table I).

TABLE I. Effect of ADPrybosilation on Ca-ATPase

Preincubation buffer	Incubated with:	Relative activity (toxin treated/control) Ionic Ca Concentration (μM) 0.1	1	10	50	100	500	1000
250 mM Pi	No CaM	0.60 ±0.132	0.65 ±0.124	0.63 ±0.086	0.74 ±0.075	0.71 ±0.091	0.80 ±0.063	0.60 ±0.091
	CaM (5 μg/ml)	0.58 ±0.108	0.68 ±0.113	0.78 ±0.092	0.80 ±0.087	0.83 ±0.091	0.78 ±0.123	0.70 ±0.118
100 mM Tris	No CaM	0.88 ±0.078	0.92 ±0.078	0.93 ±0.087	0.88 ±0.078	0.95 ±0.048	0.88 ±0.063	0.85 ±0.061
	CaM (5 μg/ml)	0.92 ±0.078	0.90 ±0.015	0.92 ±0.048	0.96 ±0.020	0.95 ±0.032	0,94 ±0.068	0.91 ±0.048

Membrane fragments were ADPrybosilated in either a Pi- or Tris-buffer (pH 7.55 at room temperature), by incubating with cholera toxin (1:60; toxin to protein ratio) for 1 hr at 37°C, in the presence of (mM): $GTP.Na_2$, 0.1; creatine phosphate, 10; EGTA, 5; EDTA, 0.1; dithiothreitol, 1; thymidine, 10;β-NAD, 5; and 20 units creatine phosphokinase. Control fragments were incubated as above but in the absence of both NAD and toxin. Thereafter, the fragments were washed thrice with isotonic K-medium, containing 50 mM Pi and once with Pi-free medium. Ca-ATPase was then assayed at the various free Ca concentration indicated above, in the presence and absence of 5 μg/ml calmodulin (CaM). Relative activity (toxin treated/control membranes) from 15 (Pi-incubated) and 5 experiments (Tris-incubated) is shown as the mean value ± 1 S.D.

On the other hand, CaM-deficient membranes showed a relative high-affinity and high Vmax ATPase activity, which was stimulated by CaM slightly (about 1.2-fold). Preliminary incubation of these membranes with toxin, also lead to a highly significant reduction in affinity. Thus, the A/C ratio (as mean

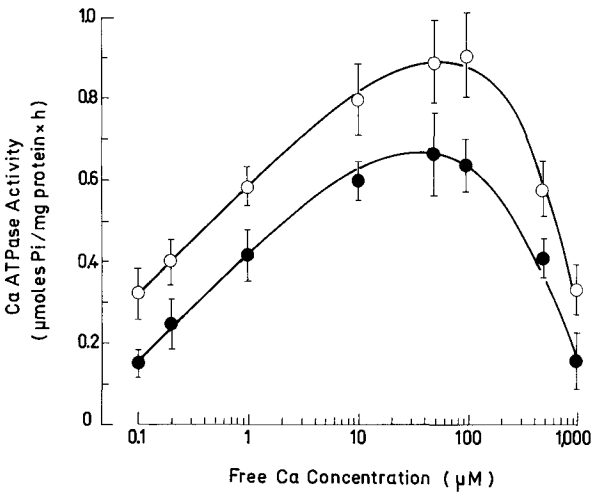

FIGURE 1. Decreased Ca-ATPase activity upon treatment with cholera toxin.
Membrane fragments were previously treated in a Pi-buffer with (filled symbols) and without (open symbols) cholera toxin and then assayed for Ca-ATPase activity, as described in legend to Table I. Each point is the mean value of seven experiments performed with the same batch of membranes. Vertical bars denote ± 1 S.D. of mean.

value ± 1 S.D. of seven experiments) being 0.75±0.05 at 0.5 μM Ca (t=4.68; P<0.005). By contrast, the same treatment practically had no effect on Vmax (A/C ratio= 1.02±0.08 at 20μM Ca; mean value ± 1 S.D. of five experiments; t=0.23).

DISCUSSION

The present work has shown that the human red cell Ca-ATPase is affected by cholera toxin. A moderate but consistent reduction in Ca affinity and Vmax was obtained when membrane fragments were previously incubated with activated toxin in Pi-buffer. Such an effect was absent from membranes treated with toxin in the absence of β-NAD. Similarly, the effect was not observed if membranes were incubated in a Tris-buffer or treated with non-activated toxin.

As cholera toxin A_1 subunit catalyzes ADP-rybosilation of arginine in the presence of β-NAD and its activity is markedly stimulated by Pi (10), the above results suggest that ADP-rybosilation of arginine residues of red cell membrane proteins results in a decreased Ca-ATPase activity.

The toxin effect appeared more marked on affinity than on Vmax, as may be inferred from the lowest A/C values found at very low Ca concentrations. When ATPase is assayed in the presence of added CaM, the characteristic stimulation of Ca affinity and Vmax is elicited but to a lower extent. By contrast, if CaM-deficient preparations are treated with toxin, only a reduction in affinity is observed. These findings suggest that CaM binding to the ATPase protects the enzyme against a reduction in Vmax but not in affinity. These results are consistent with the idea that the CaM-binding domain interacts with two different sites on the Ca-ATPase, as proposed by Enyedi et al. (1).

Previous work has demonstrated that cholera toxin catalyzes ADP-rybosilation of G proteins from human erythrocyte membranes (8). Our results do not allow us to distinguish whether the toxin is acting in a direct or indirect way. However, the fact that no effect on Vmax was found in CaM-deficient membranes, seem to suggest that the enzyme may be ADP-rybosilated by cholera toxin directly. The effect on Ca-ATPase seemed specific as preliminary incubation with toxin failed to affect the Mg-ATPase.

The human erythrocyte Ca-ATPase is modulated by a number of physiologically and non-physiologically occurring effectors. Except for some monovalent cations under particular conditions (13), all modulators described so far are activators of the enzyme (18). Moreover, covalent modifications mediated by protein kinases are also stimulatory (12,16). The reduction of the red cell Ca-ATPase activity by ADP-rybosilation is an interesting finding. This is not the first report in the literature of an effect of ADP-rybosil transferases on a transport Ca-ATPase. In rabbit skeletal muscle, ADP-rybosilation by endogenous enzymes ensued a marked decrease in sarcoplasmic reticulum Ca-ATPase activity (5). The presence of endogenous ADP-rybosil transferases within human erythrocytes (19) may be of physiological relevance in the control of Ca pump activity.

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