The Activation of Lymphocyte Plasma Membrane (Na,K)-ATPase by EGTA Is Explained Better by Zinc Than Calcium Chelation*

(Received for publication, December 30, 1980, and in revised form, March 4, 1981)

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We have observed that maximal lymphocyte membrane (Na,K)-ATPase activity occurred only when a chelating agent for divalent cations such as ethyleneglycol bis(β-aminoethy ether)-N,N,N',N'-tetraacetic acid (EGTA) was present in the reaction mixture. In the absence of EGTA, the ionized calcium was 2 μM and the (Na,K)-ATPase was 25 to 40% of maximum. Twenty-five μM EGTA reduced the ionized Ca to 0.6 μM and caused maximal (Na,K)-ATPase activity. Three observations indicated that Ca chelation was not causal in the enzyme activation. First, an excess of additional Ca, 9 as compared to 2 μM, was required to inhibit the (Na,K)-ATPase to its base-line activity. Second, when 250 μM histidine, which does not chelate Ca, was substituted for EGTA, the (Na,K)-ATPase was activated comparably. Third, the addition of Ca to the ATPase assay fully activated by histidine caused no inhibition at ionized Ca concentrations as high as 12 μM. These data indicate that EGTA binds a cation other than Ca to activate the (Na,K)-ATPase.

Trace amounts of Zn (2 μM) were found by atomic absorption analysis of the assay system. Three observations supported the causal role of Zn as the inhibitor. First, when Zn was added to the (Na,K)-ATPase, partially activated by 50 μM histidine, inhibition of the ATPase was observed. Second, at 35 μM Zn, the calculated ionized Zn concentration was 2 μM, similar to the initial Zn value, and the inhibition of the ATPase was similar to that in the absence of chelator. Third, mixtures of histidine and Zn calculated to produce partial activation of the (Na,K)-ATPase produced results predicted by the free Zn concentration achieved. These data established that enhancement of the activity of the lymphocyte (Na,K)-ATPase by EGTA is not explained by chelation of Ca; rather, the chelation of Zn activates the (Na,K)-ATPase under the conditions of these studies.

(Na,K)-ATPase activity of human lymphocyte membrane vesicles does not reach maximal velocity unless ethyleneglycol bis(β-aminoethy ether)-N,N,N',N'-tetraacetic acid is present in the reaction mixture (1). Many assays of erythrocyte (Na,K)-ATPase activity have contained chelators of divalent cations (2-5) but others have omitted these agents without apparent adverse effect on enzyme activation (6-8). Ca is an inhibitor of the erythrocyte (Na,K)-ATPase only when added in concentrations of 50 to 100 μM (9). In the present studies we initially tested the hypothesis that Ca in the reaction mixture inhibited lymphocyte (Na,K)-ATPase activity in vitro and that Ca chelation explained the activating effect of EGTA.* The data, however, suggested that Zn rather than Ca inhibited the (Na,K)-ATPase under these conditions.

MATERIALS AND METHODS

RESULTS

Chelator Activation of (Na,K)-ATPase

EGTA—(Na,K)-ATPase activity was measured in membranes exposed to EGTA from 0 to 100 μM (Fig. 1). "Ca before ATP" indicates the ionized calcium during the 10-min preincubation before the addition of ATP to the reaction mixture. The preincubation is necessary for maximal activation of the ATPase. "Ca after ATP" indicates the ionized calcium after ATP was added to start the assay.

In the absence of EGTA at an ionized Ca before and after the addition of ATP of 9 and 2 μM, respectively, the mean (Na,K)-ATPase activity of three membrane preparations was 0.3 nmol of Pi/μg of protein/30 min. Maximal ATPase activity of 1.3 nmol of P/μg of protein/30 min occurred at an EGTA concentration of 25 μM and an ionized Ca before and after ATP of 3 and 0.6 μM, respectively.

Histidine—Histidine, a chelating agent with a high affinity for many divalent cations bound by EGTA, has a negligible affinity for Ca. (Na,K)-ATPase activity was measured at histidine concentrations from 0 to 1000 μM (Fig. 2). The ionized Ca of 9 before and 2 μM after the addition of ATP remained unchanged regardless of the histidine concentration. The mean ATPase activity in four membrane preparations in the absence of histidine was 0.8 nmol of P/μg of protein/30 min and maximal activation, 2.2 nmol of P/μg of protein/30 min, was achieved at a concentration of 250 μM histidine.

The (Na,K)-ATPase activity in the presence of EGTA and

* This work was supported by United States Public Health Service Research Grant CA-12790, by the University of Rochester Blood Research "Jimmy" Fund, by National Institute of Environmental Health Services Grant ES01247, and by the Ruth Estrin Goldberg Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviation used is: EGTA, ethyleneglycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid.

§ Portions of this paper (including "Methods and Materials" and "Mathematical Appendix") are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20014. Request Document No. 80M-3760, cite author(s), and include a check or money order for $1.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

6629
Activation of Lymphocyte (Na,K)-ATPase

**FIG. 1.** EGTA activation of (Na,K)-ATPase. The phosphohydrolysis of ATP by the (Na,K)-ATPase was measured in the presence of increasing concentrations of EGTA. The ionized Ca before (\(\text{Ca}^+\)-ATP) and after the addition of ATP (\(\beta\)-ATP) are shown below the corresponding concentrations of EGTA. The ionized Ca in this study was measured using a Radiometer Ca electrode. The data represent the mean ± S.E. of 3 lymphocyte plasma membrane populations, each measured in triplicate.

Histidine—In the absence of histidine and added Ca, the ATPase activity was 0.8 nmol of P_\text{i}/\mu g of protein/30 min. The addition of 10 mM histidine maximally increased the (Na,K)-ATPase to 1.8 (Fig. 4). No significant change in the histidine-activated ATPase was observed at a concentration as high as 100 \(\mu\)M added Ca, corresponding to an ionized Ca greater than 50 before and 12 \(\mu\)M after the addition of ATP, approximately 6 times the initial ionized Ca concentration.

The Effect of Zn on (Na,K)-ATPase

Atomic absorption analysis of the reaction mixture revealed no detectable Cd or Pb and trace amounts of Zn. We examined, therefore, the effect of added Zn in the assay system (Fig. 5). The (Na,K)-ATPase activity without added Zn or histidine was 0.5 nmol of P_\text{i}/\mu g of protein/30 min at an ionized Zn of 2 \(\mu\)M calculated from the atomic absorption measure-
A partially activating concentration of histidine, 50 μM, reduced the ionized Zn to 3 nM and increased the ATPase to 1.2. The addition of Zn caused an increase in the ionized Zn and inhibition of the (Na,K)-ATPase. When 35 μM Zn was added to the ATPase, activated by 50 μM histidine, the calculated free Zn concentration was near 2 μM, the starting value, and the inhibition of ATPase was similar to that in the absence of chelator, about 0.5 nmol of P_i/μg of protein/30 min (Fig. 5). Once established, the Zn inhibition of the lymphocyte ATPase was not reversed by the addition of 10 μM histidine. The pattern of ATPase activation and inhibition corresponded inversely to the Zn concentration in the ATPase assay.

Since added Zn inhibited the (Na,K)-ATPase, we tested whether additional histidine would prevent inhibition of the ATPase activity. From the logarithmic affinity constants K1 and K2 of histidine for Zn given under “Materials and Methods,” we calculated that 230 and 275 μM additional histidine (total histidine, 280 and 325 μM) would be necessary to reduce the ionized Zn to 3 nM when the total Zn was 35 and 45 μM, respectively. When the additional histidine was added followed by the addition of Zn to produce a total of 35 and 45 μM, the ATPase was restored quantitatively from an inhibited to a partially activated state.

The Effect of Preincubation on (Na,K)-ATPase Activity

If Zn in the assay system accounted for the suboptimal activity of the (Na,K)-ATPase, it should produce irreversible inactivation of the (Na,K)-ATPase in the absence of added chelator. To study this, the assay system was preincubated from 5 to 45 min at 37 °C without chelator prior to the addition of both ATP and EGTA (Fig. 6). Under these conditions, preincubation for 5 min led to a decrease in ATPase, and preincubation for 15 min led to maximal reduction of the (Na,K)-ATPase activity. When 50 μM EGTA was present throughout the preincubation period, the ATPase remained maximally activated at approximately 1.9 nmol of P_i/μg of protein/30 min when no EGTA was present during the preincubation or assay, the ATPase activity remained low (Fig. 6). These data indicate that chelator activation of the ATPase requires its presence as soon as the membranes are brought to 37 °C and that the enzyme inhibition is irreversible.

**DISCUSSION**

Because of the biologic importance of Ca and the larger body of knowledge about the role of Ca as an activator or inhibitor of physiologic systems, the effects of EGTA are most likely to represent the result of Ca chelation, although the chelator has a high affinity for at least 12 divalent cations. In this regard, the addition of Ca to red cell (Na,K)-ATPase has been shown to inhibit its activity. This occurs as the Ca-dependent Mg-ATPase is activated, and inhibition of the (Na,K)-ATPase is maximal at 180 μM Ca (9). Some investigators have added chelators such as EGTA in relatively high concentrations, 0.5 mM, to eliminate the Ca-ATPase when studying the (Na,K)-ATPase (2). Also, Ca inhibition was suggested to explain the effect of chelators on (Na,K)-ATPase of rabbit brain (14). For these reasons, we suspected initially that Ca chelation was necessary to permit measurement of the (Na,K)-ATPase in lymphocyte plasma membranes.

Several lines of evidence indicated, however, that Ca chelation was not the explanation for the EGTA activation of the lymphocyte membrane (Na,K)-ATPase. First, histidine binds many of the divalent cations chelated by EGTA; however, histidine does not bind Ca even when present in concentrations as high as 10 mM, and histidine activated the (Na,K)-ATPase in a manner similar to EGTA. Furthermore, the addition of enough Ca to achieve 4 times the ionized Ca concentration present in the EGTA-free reaction mixture was required to reduce the EGTA-activated ATPase to the level observed in the absence of EGTA. Moreover, the addition of Ca to the ATPase maximally activated by histidine showed no inhibition until greater than 6 times the initial ionized Ca was present. Taken together, these data indicate that EGTA bound a cation other than Ca to activate the (Na,K)-ATPase.
Activation of Lymphocyte (Na,K)-ATPase

The addition of chelators such as EDTA or EGTA to prevent trace metals other than Ca from interfering with the (Na,K)-ATPase has been suggested because of erratic measurements noted early in the study of the erythrocyte enzyme (15). In addition, several investigators have reported enhanced (Na,K)-ATPase activity in beef brain, kidney cortex, and chick brain at EGTA concentrations less than 100 μM (16-18). However, the inhibiting ions present in the assay were not defined specifically. The inhibitors may have differed in the various studies since the addition of EGTA did not alter the enzyme activity under some conditions (8).

Zn at micromolar concentrations is a known irreversible inhibitor of the (Na,K)-ATPase in plasma membranes such as electrophorus (19), and 2 μM Zn was present in our reaction mixture. Several lines of evidence suggested that Zn chelation reduce the free Zn to nanomolar concentrations. The inhibition of the lymphocyte (Na,K)-ATPase in the absence of chelator was irreversible once it was established which is consistent with Zn inhibition. These data suggest that Zn is the principal inhibitor of the (Na,K)-ATPase assay under these conditions, and its chelation by EGTA or histidine accounts for the increase in enzyme activity observed.

Acknowledgments—We wish to thank Drs. Jacob Nusbacher and Joanna Heal, Rochester Regional Red Cross Blood Center, for supplying plateletpheresis residues and David Jackson for providing the measurements of total zinc. Geraldine Roberts gave expert technical assistance in these studies.

SUGGESTED MATERIAL TO

THE ACTIVATION OF LYMPHOCYTE PLASMA MEMBRANE Na,K-ATPASE
BY EDTA TO REFUTE ENZYMOLOGY BY ZINC THAN CALCIUM CHLORIDE

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MATERIALS AND METHODS

Preparation of Lymphocyte Plasma Membranes: Lymphocyte cell suspensions were isolated as previously described (16). The plasma membranes were prepared by step gradient centrifugation (17). These plasma membranes showed a greater than 35-fold enrichment of Na,K-ATPase and less than 5% contamination with succinate dehydrogenase when compared to the original homogenate.

Lymphocyte Membrane Enzyme Activity Assay: The enzyme activity was assayed in a reaction mixture containing 50 mM Tris-Cl, pH 7.4, 2 mM MgCl₂, 270 mM NaCl, and 0.1% bovine serum albumin. The assay was started by adding 0.1% bovine serum albumin. The inhibition of the lymphocyte (Na,K)-ATPase by Zn at micromolar concentrations is a known irreversible inhibitor of the enzyme activity under some conditions (19).

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