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Sensitivity and reversibility of Ca-dependent inhibition of the $(Na^+ + K^+)$-ATPase of human red blood cells

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The sensitivity of the $(Na^+ + K^+)$-ATPase to inhibition by Ca was increased 30-fold by a partially purified extract of human red cell hemolysate. The hemolysate fraction reduced the concentration of free Ca required for 50% inhibition from 30 μM to approx. 1 μM. Ca-dependent inhibition of the $(Na^+ + K^+)$-ATPase in the presence and absence of the hemolysate fraction was completely reversible. The hemolysate fraction also stimulated the Ca^{2+}-ATPase and increased its affinity for Ca. In the presence of the hemolysate fraction, the concentration of free Ca that inhibited the $(Na^+ + K^+)$-ATPase by 50% was similar to that which half-maximally stimulated the Ca^{2+}-ATPase. Boiling the fraction destoryed its effect on the $(Na^+ + K^+)$-ATPase, but did not impair its stimulation of the Ca^{2+}-ATPase.

Introduction

Although the inhibitory effect of Ca on the $(Na^+ + K^+)$-ATPase has been known for quite some time [1], the reversibility of such inhibition has never been established. Recent evidence suggests that the sensitivity of the $(Na^+ + K^+)$-ATPase to inhibition by Ca is increased by a factor from the hemolysate of human red blood cells which is sensitive to heat and trypsin and distinct from calmodulin [2,3]. This factor may also affect Ca inhibition of ion transport through the sodium pump of red cells [4]. Addition of hemolysate increases the otherwise low sensitivity of the $(Na^+ + K^+)$-ATPase, making it sensitive to μM concentrations of intracellular free Ca that have been shown to regulate other enzymes [5–8]. If the inhibition caused by Ca and the hemolysate factor are reversible, then intracellular Ca might regulate the $(Na^+ + K^+)$-ATPase via the proposed protein. On the other hand, the increased inhibition caused by the addition of hemolysate could be due to an agent, such as a Ca-dependent proteinase [9], whose actions would be irreversible.

The reversibility of Ca inhibition on the sodium pump in human red blood cells has been examined in at least two studies. In the first, the sodium pump regained its uninhibited rate as the cell pumped out Ca that had previously been introduced [10]. In the other, the sodium pump remained inhibited after intracellular Ca was removed by chelating the extracellular Ca in the presence of ionophore A23187 to make the membrane permeable to Ca [11].

In the present paper, we examine the reversibility of Ca-induced inhibition of the $(Na^+ + K^+)$-ATPase of human red blood cells in the presence and absence of a partially purified hemolysate fraction. The results indicate that intracellular Ca and the proposed cytoplasmic inhibitor could be reversible regulators of the $(Na^+ + K^+)$-ATPase.

Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Materials and Methods

Preparation of red cell membranes. The membranes used in this study were prepared by the Dodge procedure modified to deplete the membranes of calmodulin and to lower the sensitivity of the $(Na^+ + K^+)$-ATPase to inhibition by Ca [2].

Preparation of the hemolysate fraction. Human red blood cells not more than 3 days old were obtained from the Red Cross and washed three times in 10 vol. 172 mM Tris-HCl (pH 7.6), centrifuging at $12000 \times g$ for 1 min at 4°C, and removing the plasma and buffy coat by aspiration. The cells were then centrifuged at $12000 \times g$ for 5 min and the supernatant was removed, leaving packed cells with a hematocrit exceeding 95%. The packed cells were added to 20 vol. 20 mM Tris-HCl/0.1 mM EGTA (pH 6.8) and hemolyzed at 0°C for 5 min. The hemolysate was centrifuged at $49000 \times g$ at 4°C for 10 min. The supernatant was decanted into a flask held at 0°C. After all the supernatant had been collected, 1 vol. DEAE-Sephadex (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated in 20 mM Tris-HCl/0.1 mM EGTA (pH 6.8) was added to 20 vol. of the supernatant, and the mixture was gently swirled at 0°C for 2 h. The Sephadex was collected by gravity and repeatedly washed in 20 mM Tris-HCl/0.1 mM EGTA (pH 6.8). The Sephadex was then poured on a column and eluted with 4 bed vol. of a solution comprising 300 mM NaCl/20 mM Tris-HCl/0.1 mM EGTA (pH 6.8), and then by an equal number of bed vol. of 400 mM NaCl/20 mM Tris-HCl/0.1 mM EGTA (pH 6.8). This last fraction was washed in 1000 vol. 20 mM Tris-HCl/0.1 mM EGTA (pH 6.8) and concentrated under nitrogen at 4°C on a PM 30 membrane (Amicon Corporation, Danvers, MA). The final protein concentration of the fractions was approx. 1 mg/ml.

ATPase activity. The activity of the membrane ATPases was determined at a constant concentration of ATP by measuring the oxidation of NADH enzymatically coupled to the production of ADP [12]. The reaction was followed by monitoring the decrease in absorbance at 340 nm in the splitbeam mode of a DW-2a spectrophotometer (SLM-AMINCO, Urbana, IL). The solutions in the cuvettes were stirred and maintained at 37°C. After all additions, the assay mixture of 3 ml contained 50 mM NaCl, 50 mM KCl, 1 mM $MgCl_2$, 0.3 mM phospho*enyl*pyruvate, 10 U/ml pyruvate kinase, 50 U/ml lactate dehydrogenase, 1 mM ATP, 0.43 mM NADH, 0.1 mM EGTA, 20 mM Hepes, 0.2 mg/ml membrane protein, and where appropriate 0.1 mM ouabain, various concentrations of Ca and 0.02 mg/ml of the homolysate fraction. The reaction mixture minus the ATP, ouabain and Ca was preincubated for 15 min at 37°C. The reaction was begun by the addition of ATP. For measurements at 0 free Ca, ouabain was added 5 min later and the absorbance was monitored for an additional 5 min. When present, Ca was added just after the ATP, the mixture was equilibrated for 4 min, and the reaction measured for the next 5 min. Ouabain was then added, and the reaction followed for another 5 min.

The reversibility of Ca inhibition of the $(Na^+ + K^+)$-ATPase was determined at 1.5 μM free Ca in a series of three separated measurements. First, the activity of the $(Na^+ + K^+)$-ATPase was determined in the absence of added Ca as described above. Then, the activity was determined after the addition of 100 μM total Ca, giving a calculated concentration of 1.5 μM free Ca. In the third measurement, 0.56 mM EGTA was added to the assay mixture 9 min after the addition of Ca, the reaction was followed for the next 5 min, ouabain was added, and the absorbance was monitored for another 5 min.

Changes in the concentration of free Ca in the assay mixture were checked by means of a Ca electrode. Prior to adding either Ca or EGTA to the solution, the electrode was immersed in the assay mixture in order to verify that these additions caused the free Ca to change by an expected amount. Unfortunately, the electrode had to be inserted and removed from the solution multiple times during a given run, contributing to drift in its response. For this reason, the absolute values for free Ca as given in the figures and the text were calculated.

Calculation of free and bound ligands. The concentration of free Ca, free Mg, MgATP and CaATP were calculated from the total concentrations of Ca, Mg, ATP and EGTA by the method of successive approximation [13] considering all the major

equilibrium relationships and using the appropriate equilibrium constants [14]. The concentrations used in the calculations were 1 mM ATP, 1 mM Mg, 0.1 mM EGTA, and the following μM concentrations of total Ca to give the μM concentrations of free Ca indicated in parentheses: 81 (0.16), 91 (0.35), 101 (1.5), 111 (4.5), 141 (17) and 310 (100). All solutions contained a minimum concentration of 1 μM total Ca, as measured with the Ca-sensitive electrode, giving a calculated free Ca of 0.004 μM in the assay solution in the absence of added Ca. In the reversibility experiments, 0.56 mM EGTA was added to the assay solution when it contained 101 μM total Ca, reducing the free Ca to 0.009 μM. During the manipulation of the Ca concentration in the assays, the calculated free Mg was maintained in the range of 0.25–0.35 mM.

Calibration of the Ca electrode. The Ca electrode (WPI, New Haven, CT) was calibrated at 37°C by adding known concentrations of Ca to 50 mM NaCl/50 mM KCl/20 mM Hepes (pH 7.4) to achieve final concentrations of 10, 1, 0.1 and 0.01 mM added Ca. The value for zero free Ca was obtained by adding 5 mM EGTA to the above solution in the absence of any added Ca. These data were then graphed as relative millivolts vs. the concentration of free Ca, assuming that the free Ca equaled the added Ca. A least-squares line through the above points had a correlation coefficient close to 1 and a slope equal to that predicted by the Nernst equation. The value obtained in the presence of 5 mM EGTA intercepted the abscissa at 0.1 μM Ca. By means of this calibration, the concentration of Ca in the solution before the addition of either Ca or EGTA was approx. 1 μM. Since the electrode is also somewhat sensitive to Mg, the electrode was recalibrated in the presence of the concentration of free Mg expected in the assay mixture.

Results

The addition of a partially purified fraction of red cell hemolysate increased Ca-dependent inhibition of the $(Na^+ + K^+)$-ATPase (Fig. 1) and stimulated the Ca^{2+}-ATPase (Fig. 2) in human red cell membranes. The addition of hemolysate decreased the concentration of free Ca required for

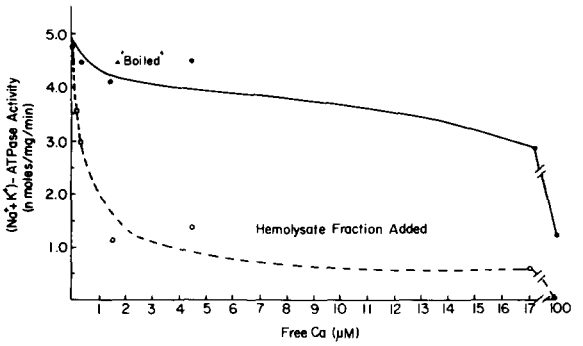

Fig. 1. The effect of the hemolysate fraction on increasing Ca inhibition of the $(Na^+ + K^+)$-ATPase. The activity of the $(Na^+ + K^+)$-ATPase is equal to the difference in the rate of ATPase activity in the presence and absence of ouabain. The final concentration of the hemolysate fraction was 0.02 mg/ml. The symbol marked 'Boiled' shows the effect of adding a hemolysate fraction that had been previously heated for 4 min in a bath of boiling water. Each point is the mean of two separate measurements done on two different days. All values are within 15% of the mean.

50% inhibition of the $(Na^+ + K^+)$-ATPase from approx. 30 μM to less than 1 μM (Figs. 1 and 3). The addition of the hemolysate fraction stimulated the Ca^{2+}-ATPase over 6-fold and reduced the concentration of free Ca required for 50% activation to approx. 1 μM (Fig. 2). Boiling the fraction destroyed the effect on the $(Na^+ + K^+)$-ATPase, but did not diminish its stimulation of the Ca^{2+}-

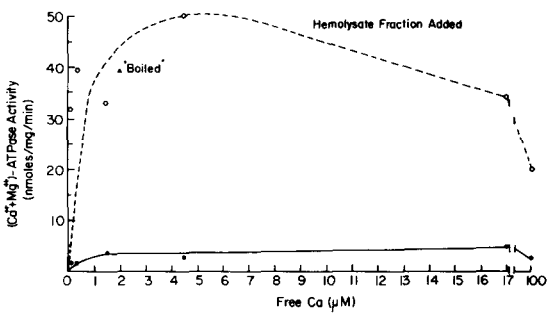

Fig. 2. The effect of the hemolysate factor on increasing Ca activation of the Ca^{2+}-ATPase. These measurements were carried out at the same time as those described in the legend to Fig. 1. The activity of the Ca^{2+}-ATPase is equal to the difference in the ouabain-insensitive rate in the presence and absence of Ca. The special symbol marked 'Boiled' is the rate measured in the presence of a hemolysate fraction that had been previously heated for 4 min in a boiling water-bath. Each point is the mean of two separate measurements. All values are within 15% of the mean.

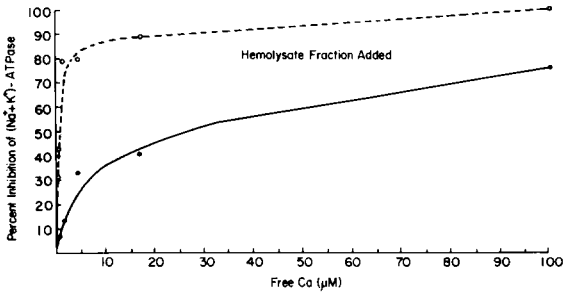

Fig. 3. Percent inhibition of the $(Na^+ + K^+)$-ATPase as a function of free Ca in the presence and absence of the hemolysate fraction. These values were calculated from the data in Fig. 1.

ATPase (see special symbols in Figs. 1 and 2).

As free Ca was increased in the experiments shown in Figs. 1 and 2, there were corresponding increases in CaATP and free Mg, and a decrease in MgATP (calculations not shown). These changes, however, do not explain the increased inhibition seen with the addition of the hemolysate, because the hemolysate did not change the concentration of free Ca as measured by the Ca electrode (data not shown).

The inhibitory effects of Ca on the $(Na^+ + K^+)$-ATPase both in the presence and absence of the hemolysate fraction are completely reversible. Increasing the free Ca from the nominally Ca-free value of 0.004 μM to 1.5 μM inhibited the $(Na^+ + K^+)$-ATPase $25 \pm 4.8\%$ in the absence of the hemolysate fraction and $70.5 \pm 10.5\%$ in the presence of 0.02 mg/ml of the hemolysate fraction. Upon exposure of the enzyme to 1.5 μM free Ca for the same length of time, followed by subsequent reduction of the free Ca to 0.009 μM, the rate of the $(Na^+ + K^+)$-ATPase in the absence of hemolysate returned to within $4.6 \pm 7.9\%$ of the value measured originally at 0.004 μM free Ca. In the presence of the hemolysate fraction, the rate of the $(Na^+ + K^+)$-ATPase returned to within $1.9 \pm 12.4\%$ of the original rate. These values are the mean and standard deviation of three separate measurements made on three different preparations of membranes on three different days. The absolute rates in the absence of added Ca on each of these days ranged from a high of 6.7 to a low of 2.1 nmol/mg per min.

Discussion

This paper shows that the increased Ca inhibition of the $(Na^+ + K^+)$-ATPase observed in the presence of a protein fraction isolated from the hemolysate of human red blood cells is reversible, as is the inhibitory effects of Ca alone on the $(Na^+ + K^+)$-ATPase. The addition of the hemolysate fraction increased the sensitivity of the $(Na^+ + K^+)$-ATPase to inhibition by Ca by decreasing the concentration of free Ca causing 50% inhibition from approx. 30 to less than 1 μM. If this increased inhibition is caused by a cytoplasmic protein as formerly proposed [2], then this factor could be a physiological regulator of the $(Na^+ + K^+)$-ATPase, because its effects are reversible and it is activated in the same range as Ca-binding proteins such as calmodulin which are proposed regulators of cellular enzymes [5].

Previous reports have shown that the effects of the increased Ca inhibition caused by the hemolysate are probably due to an acidic protein distinct from calmodulin. The effects of the hemolysate are destroyed by trypsin and boiling [2] and are not inhibited by the drugs 48/80, R24571 and trifluroperazine which inhibit the effects of calmodulin [15]. This protein can also be extracted from human red cell membranes in the presence of EDTA and binds to DEAE-Sephadex more tightly than calmodulin [15,16]. In addition to increasing Ca inhibition of the $(Na^+ + K^+)$-ATPase, it also increases Ca inhibition of ouabain-sensitive sodium efflux in resealed ghosts [3,4]. The first report that the sodium pump was inhibited by intracellular Ca came from measurements made in resealed human red cell ghosts [17]. The relatively high sensitivity of the sodium pump to inhibition by Ca was later observed in resealed ghost containing arsenazo III to monitor intracellular free Ca [18,19].

In the absence of the inhibitory factor, the $(Na^+ + K^+)$-ATPase was inhibited 50% by approx. 30 μM free Ca (Fig. 2) which is in the same range as we [2] and others have recently reported [11]. This is the same concentration of free Ca that half-maximally stimulates the Ca^{2+}-ATPase, the enzymatic activity underlying the calcium pump [20], in the absence of added calmodulin [21]. In the presence of the hemolysate fraction, the $(Na^+ + K^+)$-ATPase was inhibited 50% by 1 μM free

Ca (Fig. 1). This is the same concentration which half-maximally stimulated the Ca^{2+}-ATPase (Fig. 2), in agreement with previous studies [21]. Stimulation of the Ca^{2+}-ATPase was almost certainly due to calmodulin that is known to be in this fraction [15]. The increased sensitivity of the $(Na^+ + K^+)$-ATPase to inhibition by Ca is not due to calmodulin, because boiling the fraction destroyed the effect on the $(Na^+ + K^+)$-ATPase, but did not diminish its stimulation of the Ca^{2+}-ATPase (see special symbols in Figs. 1 and 2). We interpret these data to mean that the sensitivity of both these enzymes to Ca can be regulated over approximately the same range of free Ca concentrations. If the Ca-sensitivity of both the calcium and the sodium pump are regulated by separate cytoplasmic proteins, then their exact sensitivities to Ca are not fixed and should not necessarily be the same as their corresponding enzymatic activity measured under a given set of conditions in vitro. A comparison of the effects of Ca on the sodium pump in intact human red cells indicated that the $(Na^+ + K^+)$-ATPase measured in the absence of exogenously added inhibitor was more sensitive to inhibition by Ca than was the sodium pump [11].

Our observation that the sensitivities of the Ca^{2+}-ATPase and $(Na^+ + K^+)$-ATPase can be varied over the same range of free Ca concentrations may have physiological implications. It was earlier thought that the calcium pump might keep the intracellular Ca low so as to prevent subsequent inhibition of the sodium pump [22]. With the demonstration of reversibility and relatively high Ca-sensitivity of the $(Na^+ + K^+)$-ATPase in the presence of the proposed inhibitor, it now seems possible that the sodium pump could become transiently inhibited by an increase in intracellular Ca. In cells with an electrogenic sodium pump, a transient increase in intracellular Ca could alter the membrane potential. In cells where the intracellular Ca is regulated by the Na-Ca exchange system or a combination of the calcium pump and Na-Ca exchange, regulation of the Ca-sensitivity of the sodium pump could play a fundamental role in controlling intracellular Ca.

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