Two Ca^{2+}-Requiring *p*-Nitrophenylphosphatase Activities of the Highly Purified Ca^{2+}-Pumping Adenosinetriphosphatase of Human Erythrocyte Membranes, One Requiring Calmodulin and the Other ATP†

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ABSTRACT: The highly purified Ca^{2+}-pumping ATPase from human erythrocyte membranes displays two *p*-nitrophenylphosphatase (NPPase) activities: one of these requires calmodulin and low concentrations of Ca^{2+}, while the other requires ATP and higher Ca^{2+} concentrations. The free Ca^{2+} concentrations required for the expression of the two NPPase activities differed very substantially. Both activities required high free Mg^{2+} concentrations and displayed simple hyperbolic kinetics toward *p*-nitrophenyl phosphate (NPP) with a K_m in the range of 5–20 mM. Study of the dependence of the calmodulin-stimulated NPPase on Mg^{2+} and NPP indicated that the Mg–NPP complex is not the substrate of the enzyme. Under conditions optimal for ATP-requiring NPPase (1 mM free Ca^{2+}), the Ca^{2+}-ATPase displayed simple hyperbolic kinetics with a low K_m for ATP. NPP competitively inhibited this activity, and the apparent K_i for NPP was less than 1 mM, much lower than the K_m for NPP as a substrate. If NPP were inhibiting the ATPase by binding at the same site at which NPP is hydrolyzed, the apparent K_i for NPP as inhibitor would be the same as the K_m for NPP as substrate. (Under these circumstances, the apparent K_i and the K_m can be directly compared, since NPP was being hydrolyzed under both circumstances.) Since K_i was much lower than K_m, NPP must have been inhibiting at another site; thus, these data show the existence of two types of NPP sites on the enzyme, one at which NPP is hydrolyzed and the other at which it inhibits ATP hydrolysis. ATP competitively inhibits the calmodulin-stimulated NPPase with an apparent K_i of 0.46 mM. This is comparable to the K_m reported for the "low-affinity" ATPase activity and suggests that NPP is hydrolyzed at the low-affinity ATP site. Vanadate and *N*-ethylmaleimide inhibit NPPase and ATPase concurrently, further emphasizing the similarities between these two activities.

A *p*-nitrophenylphosphatase (NPPase)[1] activity was first attributed to the Ca^{2+} pump of erythrocyte membranes by Rega et al. (1973); they observed an NPPase activity which required both Ca^{2+} and ATP. This same group has subsequently studied this ATP-requiring NPPase more extensively (Caride et al., 1982, 1983) and have proposed a relationship between the sites at which ATP is hydrolyzed and those at which NPP is hydrolyzed.

While the experiments reported in this paper were in progress, Lucas et al. (1981) reported the presence of a Ca^{2+}- and calmodulin-requiring NPPase activity in human erythrocyte membranes.

We present here a study on these two NPPase activities in the highly purified Ca^{2+}-pumping ATPase from human erythrocyte membranes. The availability of this enzyme has allowed us to show that both the ATP-requiring and the calmodulin-requiring NPPase activities are indeed properties of the Ca^{2+}-pumping ATPase. Our study confirms some of the conclusions reached by the Buenos Aires group (Rega et al., 1973; Caride et al., 1982, 1983) with regard to the ATP-requiring NPPase, but we differ from them in some respects. Our characterization of the calmodulin-requiring NPPase allows us to compare the properties of the two NPPase activities and to relate NPP hydrolysis to ATP hydrolysis in both cases.

Students of the Ca^{2+}-pumping ATPase in human erythrocyte membranes have assumed the existence of two types of sites for ATP hydrolysis to explain the nonlinear double-reciprocal plots frequently seen when Ca^{2+}-ATPase activity is observed as a function of ATP concentration (Richards et al., 1978; Muallem & Karlish, 1979). These sites have been referred to as having "high affinity" and "low affinity" for ATP, although the actual affinity of ATP for the enzyme has not been measured. To conform to past practices, these terms will be used here, but the reader should understand that the basis for these terms has heretofore been nonlinear double-reciprocal plots. As will be seen, the data presented here provide additional evidence for the existence of two types of sites.

Materials and Methods

L-α-Phosphatidylcholine (type IX-E from egg yolk), *p*-nitrophenyl phosphate di-Tris salt, ATP, and cyanogen bromide activated Sepharose 4B were purchased from Sigma Chemical Co. [γ-^{32}P]ATP (2–10 Ci/nmol) was purchased from New England Nuclear. Bovine brain calmodulin was prepared according to the method of Watterson et al. (1976) and stored at −20 °C. All other chemicals were the best available.

Ca^{2+}-ATPase Purification. Ca^{2+}-ATPase from human erythrocyte ghosts was purified by calmodulin affinity chromatography as previously described (Graf et al., 1982). The enzyme was eluted in a medium containing 10 mM Tes–TEA, pH 7.4, 0.1 mM phenylmethanesulfonyl fluoride, 0.1 mM benzamidine hydrochloride, 2 mM dithiothreitol, 300 mM

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[1] Abbreviations: NPP, *p*-nitrophenyl phosphate; NPPase, *p*-nitrophenylphosphatase; Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; TEA, triethanolamine; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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KCl, 0.05% Triton X-100, 1 or 5 mM EDTA, and 0.1% phosphatidylcholine.

Nitrophenylphosphatase Assay. NPPase activity was quantitated by the release of *p*-nitrophenol from *p*-nitrophenyl phosphate. The standard reaction mixture was 25 mM Tes–TEA buffer, pH 7.4, 5 mM EGTA–TEA, pH 7.4, and 1 mM EDTA–TEA, pH 7.4 (where indicated this was changed to 0.8 or 2.5 mM). The concentrations of the other components are indicated in the description of each experiment. ATP and NPP were adjusted to pH 7.4 before they were added. The standard incubation time was 30 min at 37 °C. In some experiments, where activity was low, incubation time was increased to 60 min. Under these circumstances, the NPPase activity was linear with time for at least 60 min. The reaction was stopped by the addition of 1.0 mL of 1 M NaOH. The tubes were centrifuged at 5000 rpm for 15 min, and the clear supernatants were monitored for the optical density at 410 nm. Blanks obtained without protein were subtracted from experimental values. These blanks gave about the same reading as those obtained in the presence of magnesium alone. A standard curve of *p*-nitrophenol was prepared to convert optical density into micromoles of *p*-nitrophenol released. The final specific activity was expressed as micromoles of substrate split per minute per milligram of protein of the enzyme. In some experiments measuring ATP-stimulated NPPase where activities were low, optical densities were monitored at 430 nm. This resulted in an increased ratio of experimental to blank optical densities and gave more reliable data.

Ca^{2+}-ATPase Assay. ATPase activity was quantitated by measuring the release of inorganic phosphate from [γ-^{32}P]ATP at 37 °C (Jarrett & Penniston, 1977).

The standard reaction mixture for ATPase assays consisted of 25 mM Tes–TEA, pH 7.4, and 5 mM EGTA–TEA, pH 7.4. The concentration of EDTA–TEA (pH 7.4) was 1 mM unless indicated otherwise. The amounts of $CaCl_2$ and $MgCl_2$ were adjusted to obtain the desired free divalent metal ion concentrations. These were calculated by using computer programs (Perrin & Sayce, 1967) which took into account all complexes involving Mg^{2+}, Ca^{2+}, EGTA, EDTA, and ATP. These computations also took into account the effects of temperature and ionic strength on the formation of the above complexes.[2] Standard solutions of $CaCl_2$, $MgCl_2$, EDTA, and EGTA were employed. In the purified Ca^{2+}-ATPase, 0.05% Triton X-100 and 0.1% phosphatidylcholine were present. During the assays of enzyme activities, the concentration of Triton X-100 was 0.01% or less, and that of phosphatidylcholine was 0.02% or less. In some experiments, an ATP-regenerating system was employed. To achieve this, 5 mM creatine phosphate and 5 units/mL creatine phospholinase were included in the reaction mixture.

Protein Determination. Protein concentrations were determined according to the method of Lowry et al. (1951) as modified by Bensadoun & Weinstein (1976) with bovine serum albumin as standard.

Figures were plotted by using a Houston Instruments plotter, according to a program which plots error brackets only when errors are more than 3% of the total ordinate values.

Effect of Including NPP on the Free Magnesium and Calcium Concentrations. Caride et al. (1982) used an association constant for Mg^{2+} and NPP of 300 M^{-1}. Using a divalent metal ion sensitive electrode, we found that NPP did not significantly lower the free Mg^{2+} concentration in our system (results not shown). Therefore, we disregarded the effect of NPP in calculating the free magnesium and free calcium concentrations.

[2] Printouts of this program can be obtained by writing Dr. Penniston. It is written in Fortran for use on a PDP-11 with the multiterminal RT-11 operating system.

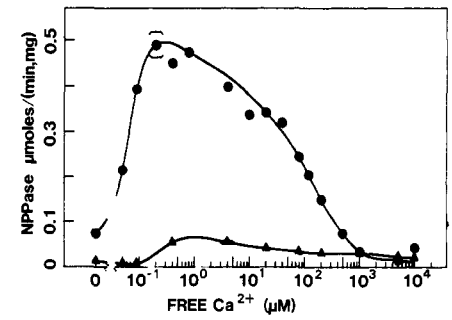

FIGURE 1: Stimulation of NPPase by calmodulin as a function of free calcium concentration. Incubation was for 30 min at 37 °C in the standard medium (0.5 mL) containing 0.8 mM EDTA, 50 mM KCl, and 6 μg/mL purified enzyme. Free magnesium concentration was kept to a constant level of 5 mM, and substrate (NPP) concentration was 6 mM. Points shown are mean ± $^1/_2$ range of duplicate values; the range is shown only where it exceeds 3% of the full range of the *y* axis. Triangles represent activity without calmodulin, circles with calmodulin. In all experiments where calmodulin was used, a saturating amount (10 μg/0.5 mL) was employed.

Table I: Mg^{2+}, Ca^{2+}, and Calmodulin Requirement of Calmodulin-Requiring NPPase[a]

additions			sp act. [nmol/(mg·min)] (mean ± $^1/_2$ range)	% max activity
[Mg^{2+}] (mM)	[Ca^{2+}] (μM)	calmodulin (μg)		
0	1.0	10	77 ± 1	13.4
4.95	0	10	37 ± 3	6.4
4.95	1.0	0	25 ± 2	4.3
4.95	1.0	10	575 ± 1	100

[a] Incubation was for 40 min at 37 °C in the standard medium (0.5 mL) containing 0.8 mM EDTA–TEA, pH 7.4, 50 mM KCl, 20 mM NPP, and 4.1 μg/mL purified enzyme. For Mg^{2+} and Ca^{2+}, the concentrations shown are free rather than total.

Results

Calmodulin-Requiring NPPase

Figure 1 shows the NPPase activity of the purified ATPase with and without calmodulin as a function of the free Ca^{2+} concentration. It is evident that calcium alone was not enough for the expression of the NPPase activity; calmodulin was also a requirement. The typical free calcium ion concentration for the optimal calmodulin-stimulated activity was in the range of 0.1–1 μM with $K_{1/2}$ around 0.05 μM.

Calmodulin-Requiring NPPase in Erythrocyte Membranes. A calmodulin-stimulated Ca^{2+}-NPPase activity was also present in erythrocyte membranes. At 10 mM NPP, 0.8 mM EDTA, 20 mM NaCl, 124 mM KCl, and 5 mM free Mg^{2+}, 1.05 mg/mL plasma membranes displayed a basal Mg^{2+}-NPPase activity of 5.43 ± 0.07 nmol/(mg·min) (n = 2). Here also, the addition of only Ca^{2+} (0.2 μM free Ca^{2+}) was not enough to cause significant increase over the basal level, giving a value of 5.85 ± 0.13 nmol/(mg·min). The addition of 20 μg/mL calmodulin increased the activity to 11.23 ± 0.21 nmol/(mg·min).

Requirement for Mg^{2+}. Table I shows that Mg^{2+} and also Ca^{2+} and calmodulin were all required for this NPPase activity of the highly purified Ca^{2+}-ATPase.

Is the Mg–NPP Complex a Substrate for the Calmodulin-Stimulated NPPase? A high concentration of free Mg^{2+} (about 5 mM) was required to achieve maximum hydrolysis of NPP. Kinetic experiments were done to determine whether

the Mg–NPP complex was the substrate for the NPPase activity observed; 25 data points were collected, covering NPP concentrations of 4–20 mM and free Mg^{2+} concentrations of 0.5–5.8 mM. Total Ca^{2+} concentration was varied so as to maintain the free Ca^{2+} concentration at 1 μM, KCl concentration was 50 mM, calmodulin concentration was 0.1 mg/mL, purified enzyme concentration was 4 μg/mL, and the assay volume was 200 μL. Incubation was for 60 min at 37 °C. In order to test whether Mg–NPP was the substrate, a computer program called ABSUB was written to fit the data to the following rate equation:

$$v = \frac{V[Mg^{2+}][NPP]}{K_m K_d + [Mg^{2+}][NPP]}$$

This is the equation expected if Mg–NPP were the substrate. The program was written by using the principles described by Cleland (1967). The fit to the data given by this program was compared with that given by the sequential rate equation

$$v = \frac{V[Mg^{2+}][NPP]}{K_{ia}K_b + K_a[NPP] + K_b[Mg^{2+}] + [Mg^{2+}][NPP]}$$

as given by the program SEQUEN (Cleland, 1979). The standard deviation obtained from the fit by SEQUEN was 0.024, while that from ABSUB was 0.052. ABSUB gave $V = 0.94 \pm 0.06$ unit and the product $K_m K_d = 23.1 \pm 0.1$ mM^2. The fit given by ABSUB was particularly bad for some of the most accurately measured points, those for which Mg^{2+} concentration was highest (5.8 mM).

SEQUEN gave $V_{max} = 1.6 \pm 0.2$ μmol/(mg·min), $K_a = 1.0 \pm 0.3$ mM (Mg^{2+}), $K_{ia} = 2.4 \pm 0.6$ mM (Mg^{2+}), and $K_b = 9 \pm 2$ mM (NPP). The poor fit by ABSUB indicated that Mg–NPP was not the substrate and that Mg^{2+} and NPP bind independently to the enzyme (although not necessarily at sites distant from one another).

Inhibition of the Calmodulin-Stimulated NPPase by ATP. ATP was found to inhibit the calmodulin-stimulated NPPase; 87 data points were collected covering ATP concentrations of 0–4 mM and NPP concentrations of 2–20 mM. Free Ca^{2+} and Mg^{2+} were maintained at 1 μM and 5 mM, respectively; EDTA concentration was 0.8 mM, KCl concentration was 50 mM, calmodulin concentration was 8 μg/mL, purified enzyme concentration was 5.3 μg/mL, and the assay volume was 500 μL. Incubation was for 60 min at 37 °C. The data were fit to the rate equations for competitive, noncompetitive, and uncompetitive inhibition, as described by Cleland (1979). The competitive model gave the best fit for those data. The standard deviation (σ) values were 0.0096 for competitive inhibition, 0.0365 for uncompetitive inhibition, and 0.0089 for noncompetitive inhibition. Here, the σ value for the noncompetitive model was less than that obtained for the competitive mechanism. However, the improvement in the fit obtained by introducing the extra constant (K_{ii}), which appears in the noncompetitive model, was negligible; the value obtained for K_{ii} was negative, indicating that the slightly improved fit was not meaningful. The competitive model gave $V_{max} = 0.57 \pm 0.01$ μmol/(mg·min), $K_m = 6.2 \pm 0.3$ mM (NPP), and $K_i = 0.46 \pm 0.02$ mM (ATP).

ATP-Requiring NPPase Activity

Figure 2 shows the NPPase activity of the highly purified ATPase as a function of the free calcium ion concentration with and without added ATP (1.5 mM). A respectable amount of ATP-stimulated NPPase activity was detected. The free calcium ion concentration necessary for the maximum expression of the NPPase was in the vicinity of 0.1–1.5 mM; 1 mM free calcium concentration was used in further experiments. A Lineweaver–Burk plot of these data gave a K_m for Ca^{2+} of 27 μM.

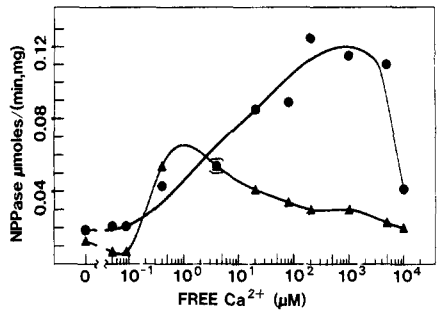

FIGURE 2: Stimulation of NPPase by ATP as a function of free calcium concentration. Incubation conditions were the same as those described for Figure 1, except the free Mg^{2+} concentration was maintained at 10 mM and no calmodulin was present. Triangles show specific activity in the absence of ATP and circles show it in the presence of 1.5 mM ATP. Points shown are mean ● $^1/_2$ range of duplicate values.

Table II: Mg^{2+}, Ca^{2+}, and ATP Requirement of ATP-Requiring NPPase[a]

additions			sp act. [nmol/(mg·min)] (mean ± $^1/_2$ range)	% max activity
[Mg^{2+}] (mM)	[Ca^{2+}] (mM)	[ATP] (mM)		
0	1	0.6	49 ± 0	20
4.95	0	0.6	20 ± 5	8.2
5.87	1	0	39 ± 1	15.9
4.95	1	0.6	245 ± 5	100

[a] Incubation conditions were the same as those described in Table I. For Mg^{2+} and Ca^{2+}, the concentrations shown are free rather than total.

Table III: ATP-Requiring NPPase Activity in Ghosts as a Function of Free Ca^{2+} Ion Concentration[a]

[free calcium] (μM)	ATP-requiring NPPase act. [nmol/(mg·min)]		
	no ATP	0.53 mM ATP	ATP stimulation
0	1.81	0.61	0
0.1	1.83	0.75	0
1	2.43	1.12	0
10	1.67	1.88	0.21
100	1.61	2.56	0.95
1000	1.28	2.66	1.38

[a] Incubation was for 30 min at 37 °C in the standard medium (0.5 mL) containing no EDTA, 20 mM NPP, 50 mM KCl, and 1 mg/mL ghost protein. Free Mg^{2+} concentration was maintained at 10 mM.

Requirement for Mg^{2+}, Ca^{2+}, and ATP. All three, Mg^{2+}, Ca^{2+}, and ATP, were necessary for the expression of the NPPase activity observed here (Table II).

ATP-Requiring NPPase in Erythrocyte Membranes. The NPPase activity was measured as a function of the free calcium concentration with and without 0.53 mM ATP (Table III). The ATP-stimulated portion of the NPPase activity showed a much lower affinity for calcium than the total NPPase activity (basal plus ATP stimulated).

ATP-Requiring NPPase as a Function of Free Mg^{2+} Concentration. Like the calmodulin-requiring NPPase, the ATP-requiring NPPase also required millimolar concentrations of free magnesium in the media. A 10 mM concentration of magnesium was optimal (data not shown) and was routinely used in the other experiments.

Effect of ATP and ADP on NPPase Activity. In the experiment shown in Figure 3, ATP was found to increase the basal NPPase activity by more than 5-fold. About 0.3 mM

Table IV: Effect of Vanadate on NPPase and Ca^{2+}-ATPase Activities under Similar Assay Conditions[a]

[vanadate] (μM)	% max act. (mean ± $^1/_2$ range) ATP-requiring NPPase	Ca^{2+}-ATPase	calmodulin-requiring NPPase	Ca^{2+}-ATPase
0	100 ± 0.45	100 ± 0.00	100 ± 0.75	100 ± 3.85
0.5	90.0 ± 3.18	85.4 ± 1.70	80.1 ± 1.49	80.6 ± 0.08
1.0	78.6 ± 0.45	77.1 ± 6.23	64.8 ± 0.37	67.5 ± 1.25
2.0	69.1 ± 3.64	70.1 ± 0.19	47.9 ± 2.99	47.0 ± 6.46
3.0	63.2 ± 0.45	64.6	35.6 ± 1.12	44.8 ± 1.64
5.0	50.4 ± 0.45	50.8 ± 0.24	28.8 ± 0.00	35.3 ± 3.57
conditions				
[free Ca^{2+}] (μM)	200	200	0.8	0.8
[free Mg^{2+}] (mM)	10	10	5	5
calmodulin (μg)	0	0	10	10
[ATP] (mM)	0.6	0.6	0.6	0.6
[NPP] (mM)	10	10	10	10

[a] Incubation was for 30 min at 37 °C in the standard medium (0.5 mL) containing 0.8 mM EDTA, 50 mM KCl, and 5.3 μg/mL purified enzyme. Specific activities (in micromoles per miligram per minute) in the absence of vanadate were 0.134 for ATP-requiring NPPase, 0.415 for Ca^{2+}-ATPase in the presence of 0.6 mM ATP and 10 mM NPP, 0.162 for calmodulin-requiring NPPase, and 0.403 for Ca^{2+}-ATPase in the presence of calmodulin.

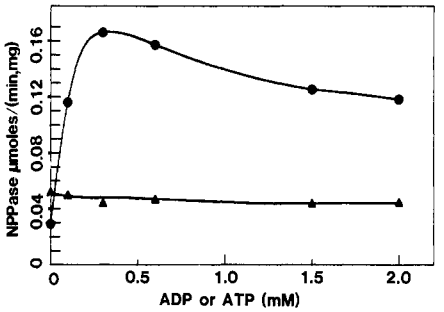

FIGURE 3: Specific activity of NPPase as a function of ATP and ADP concentrations. Incubation was for 30 min at 37 °C in the standard medium (0.5 mL) containing 0.8 mM EDTA, 50 mM KCl, 10 mM NPP, and 5.2 μg/mL purified enzyme. Free Mg^{2+} and Ca^{2+} concentrations were kept constant at 5 and 1 mM, respectively. Curves for ATP and ADP have been combined from two separate experiments. The upper curve is for ATP, and the curve showing no stimulation is for ADP. Points are mean ± $^1/_2$ range of duplicate values.

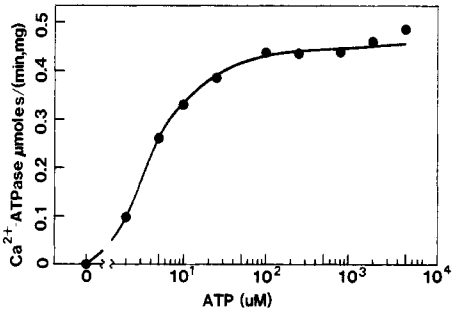

FIGURE 4: Specific activity of Ca^{2+}-ATPase as a function of ATP concentration. Incubation was for 5 min at 37 °C in the standard medium (0.2 mL) containing 1 mM EDTA, 50 mM KCl, and 3 μg/mL purified enzyme. Free magnesium and calcium concentrations were kept to a constant level of 10 and 1 mM, respectively. An ATP-regenerating system (as mentioned under Materials and Methods) was included in the medium. Points shown are mean ± $^1/_2$ range of duplicate values.

ATP was required for the maximum NPPase activity. When ADP was substituted for ATP, no increase in the activity was observed. The two curves shown in Figure 3 are from experiments done at different times; this accounts for the different values found with no added nucleotide.

Ca^{2+}-ATPase Activity at 1 mM Free Calcium. The Ca-ATPase activity was measured as a function of ATP concentration (Figure 4). This was done at 1 mM free calcium and 10 mM free magnesium. These conditions were similar to those used for measuring the ATP-requiring NPPase activity. Under such conditions, the Ca^{2+}-ATPase activity exhibited a linear double-reciprocal plot response with the K_m for ATP being 3 ± 0.5 μM. In Figure 4, the ATP concentration was plotted on a log scale, and that is why the curve appears to be sigmoid. In the above experiments, it was necessary to use a short incubation time and the ATP-regenerating system.

Inhibition of the Ca^{2+}-ATPase Activity by NPP. The conditions used were the same as those described in the legend to Figure 4, except 0.3 μg of enzyme was used; NPP concentrations from 0 to 10 mM and ATP concentrations from 2 to 20 mM were used. The 29 data points were analyzed by fitting with the competitive, uncompetitive, and noncompetitive computer programs (Cleland, 1979). The competitive mechanism was found to fit the data best. The σ value for the competitive mechanism was 4.73 vs. 18 for the uncompetitive model and 4.72 for the noncompetitive model. The slightly improved fit by the noncompetitive model was obtained at the cost of a negative K_{ii}, so this fit was rejected. The competitive model gave V_{max} = 182 ± 6 nmol/(mg·min), K_a = 3.5 ± 0.3 μM (for ATP as substrate), and K_i = 0.53 ± 0.04 mM (for NPP as inhibitor).

Inhibition of the Ca^{2+}-NPPase and Ca^{2+}-ATPase Activities by Vanadate and N-Ethylmaleimide. Vanadate has been shown to inhibit the activity of the highly purified Ca^{2+}-ATPase of human erythrocyte membranes (Niggli et al., 1981). Table IV shows the effect of vanadate on the calmodulin-requiring NPPase and the Ca^{2+}-ATPase activities assayed under the same conditions. The inhibition patterns of the two activities were strikingly similar. Table IV also shows vanadate inhibition of the ATP-requiring NPPase and Ca^{2+}-ATPase assayed under the same conditions. Here also, the inhibition patterns were quite similar.

N-Ethylmaleimide has been shown to inhibit the Ca^{2+}-ATPase of human erythrocyte membranes (Hayashi & Penniston, 1973). *N*-Ethylmaleimide was equally effective in inhibition of the two NPPase activities of the highly purified Ca^{2+}-pumping ATPase (Table V). Both NPPase activities were inhibited to about equal degrees.

Discussion

These results demonstrate that the highly purified Ca^{2+}-pumping ATPase from the human erythrocyte plasma membranes displays a calmodulin-, Ca^{2+}-, and Mg^{2+}-requiring NPPase and an ATP-, Ca^{2+}-, and Mg^{2+}-requiring NPPase. Unlike the Ca^{2+}-ATPase, the calmodulin-requiring NPPase

Table V: Effect of *N*-Ethylmaleimide on Calmodulin- and ATP-Requiring NPPase Activities[a]

[*N*-ethylmaleimide] (mM)	% max act. (mean ± $^1/_2$ range)	
	calmodulin-requiring NPPase	ATP-requiring NPPase
0	100 ± 0.6	100 ± 0.7
0.1	44.7 ± 2.8	35.9 ± 0.7
0.2	34.1 ± 1.6	32.3 ± 1.1
0.5	24.5 ± 0.4	30.1 ± 3.6
2.0	13.9 ± 3.1	14.3 ± 0.5

[a] Incubation conditions were the same as those described for Table IV, except no ATP was present in the reaction mixture for measuring calmodulin-stimulated NPPase. Free calcium ion concentrations for calmodulin- and ATP-stimulated enzyme activities were 4 μM and 1 mM, respectively.

does not display any significant basal activities in the absence of calmodulin. The erythrocyte membranes show a higher basal NPPase activity in the absence of calmodulin or ATP, indicating the presence of other NPPases in the membrane.

The studies reported here demonstrate clearly the differing Ca^{2+} sensitivities of these two activities. The response to low levels of Ca^{2+} evident in the presence of calmodulin reflects the similar response displayed by the ATPase activity when calmodulin was added (Niggli et al., 1981). The response to higher levels of Ca^{2+} in the presence of ATP was similar to the response of the ATPase in the absence of calmodulin.

The analysis of the calmodulin-stimulated NPPase as a function of NPP and Mg^{2+} concentrations allowed a clear test of the hypothesis that Mg–NPP was the substrate for the calmodulin-stimulated NPPase. The model based on Mg–NPP as a substrate did not fit the data adequately, indicating that Mg^{2+} and NPP bind to the enzyme independently (although they probably interact directly once bound). Because of the complexity of the ATPase kinetics, it has been much more difficult to determine the nature of the substrate for ATP hydrolysis, but recent data suggest that the Mg–ATP complex is the substrate (Penniston, 1982; Enyedi et al., 1982). It is evident from the results reported here that, for a substrate which binds Mg^{2+} very weakly, the enzyme is capable of operating in a mode in which Mg^{2+} and the substrate are bound independently to the enzyme.

The K_m for NPP as substrate was 7.06 mM for the calmodulin-requiring NPPase and 4.59 mM for the ATP-requiring NPPase; in other experiments of the same type (not shown), the K_m for NPP as substrate ranged from 5 to 17 mM. These variations in K_m were due to variations in the properties of the ATPase preparation, since the error in K_m for a given experiment was relatively small. The apparent K_i for NPP as a competitive inhibitor of the ATPase activity was 0.53 mM, much lower than any values of K_m found for NPP as substrate.

Such a low apparent K_i is inconsistent with the idea that NPP inhibits by binding at the same site at which it is hydrolyzed. This can be seen by a consideration of the kinetic equations that would be obtained if NPP did inhibit by binding at the site of its hydrolysis. The equations which obtain to the situation in which two substrates compete for a single site have been developed by Laidler & Bunting (1973). If both of the substrates were being hydrolyzed by binding at the same site, each would inhibit the other with an apparent K_i which is equal to the K_m for that substrate when its own reaction is measured. This would be true regardless of whether only one enzyme–substrate complex is formed or whether two are formed (the situation considered by Laidler and Bunting). This result also does not depend on the reaction paths for the two substrates having any common intermediates; completely independent sets of reaction intermediates were assumed.

Since the apparent K_i would be equal to K_m if NPP inhibited at the site at which it was hydrolyzed, the fact that the apparent K_i and K_m are quite different demonstrates that this is not the case. Therefore, NPP must inhibit the high-affinity Ca^{2+}-ATPase at a site with properties different from those of the site at which it is hydrolyzed. The simplest assumption is that NPP competitively inhibits the hydrolysis of ATP at the high-affinity ATP site without being hydrolyzed itself. A similar conclusion was reached by Caride et al. (1982) on the basis of less rigorous reasoning.

The Ca^{2+}-ATPase has been shown to exhibit a biphasic response toward ATP, one component having high apparent affinity and low maximum velocity and the other having lower affinity and higher maximum velocity (Richards et al., 1978; Muallem & Karlish, 1979, 1980, 1981, 1983). In an unpublished observation from this laboratory, we have also found that, at 5 μM free Ca^{2+}, the highly purified Ca^{2+}-pumping ATPase exhibits two apparent affinities toward ATP (K_m = 7.5 ± 0.6 and 269 ± 88 μM). The results presented in this paper show that the enzyme also has two different sites for NPP.

Our data show that NPP inhibits competitively an ATPase whose K_m corresponds to the high-affinity K_m seen in the biphasic response. This indicates that NPP inhibits ATPase by binding at the site which has a high affinity for ATP. On the other hand, ATP inhibits the calmodulin-stimulated NPPase with an apparent K_i comparable to the K_m for the low-affinity ATP hydrolysis site. In this case also, as discussed above, the apparent K_i can be compared with the K_m, since the apparent K_i incorporates the same microscopic rate constants as does the K_m when the inhibitor is itself being hydrolyzed. Therefore, approximate correspondence between the K_m for the low-affinity ATP site and the K_i for the competitive inhibition by ATP of the NPPase indicates that the site at which NPP is hydrolyzed corresponds to the low-affinity ATP hydrolysis site.

Caride et al. (1982) reported competitive inhibition of the ATP-requiring NPPase by ATP with an affinity (for ATP) appropriate to the low-affinity ATPase site, and we here report a similar competitive inhibition by ATP of the calmodulin-requiring NPPase. Thus, the properties of these two NPPases are rather similar, suggesting that both are due to hydrolysis of NPP at the same site.

Our data differ from previous results in several respects; it appears that these differences are due to our use of a highly purified enzyme, which avoided interferences encountered by others. We observed simple Michaelis–Menten kinetics for the NPPase activity regardless of how it was stimulated, whereas Caride et al. (1982) reported sigmoid kinetics. Similarly, we observed that NPP competitively inhibits ATPase with a K_i of 0.53 mM, while Caride et al. (1982) reported a K_i of 4.0 mM. Both of these differences would occur if erythrocyte membranes contain a relatively high concentration of NPP-binding sites which are absent from the highly purified enzyme. Binding of NPP by ghosts would cause the actual concentration of NPP to be lower than the amount added and thus would make the estimated values of K_i and K_m too high. Since a higher proportion of the NPP would be bound at lower NPP concentrations, sigmoidicity in the concentration curves would also be caused.

The $K_{1/2}$ for Ca^{2+} reported by Rega et al. (1973) is probably too low, as indicated by the data shown in Table III. The presence of a significant amount of NPPase that did not require ATP confused the situation in the ghosts which were the subject of the experiments shown in Table III. When the Ca^{2+}

dependence of the highly purified enzyme was observed, the K_m of the ATP-requiring enzyme was much higher than that for the calmodulin-requiring activity.

The similar vanadate and *N*-ethylmaleimide inhibition patterns of the ATPase and NPPase activities points to their close resemblance.

Further experiments would be required to show the mechanism of calmodulin or ATP activation of the NPPase. In particular, it was not possible, with the approach used here, to determine whether the high- and low-affinity sites are alternating forms of the same site (Muallem & Karlish, 1983) or physically separate sites.

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Registry No. ATPase, 9000-83-3; NPPase, 9073-68-1; NPP, 12778-12-0; ATP, 56-65-5; Ca, 7440-70-2; Mg, 7439-95-4; vanadate, 37353-31-4; *N*-ethylmaleimide, 128-53-0.

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