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THE ROLE OF THE SITES FOR ATP OF THE Ca^{2+}-ATPase FROM HUMAN RED CELL MEMBRANES DURING Ca^{2+}-PHOSPHATASE ACTIVITY

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1. In the presence of ATP, the Ca^{2+} pump of human red cell membranes catalyzes the hydrolysis of *p*-nitrophenyl phosphate. The requirement for ATP of the Ca^{2+}-*p*-nitrophenylphosphatase activity was studied in relation to the two classes of site for ATP that are apparent during Ca^{2+}-ATPase activity. 2. (a) The $K_{0.5}$ for ATP as activator of the Ca^{2+}-*p*-nitrophenylphosphatase extrapolated at 0 mM PNPP is equal to the K_m of the Ca^{2+}-ATPase. (b) PNPP competes with ATP and its effectiveness is the same regardless the nucleotide acts as the substrate of the Ca^{2+}-ATPase or as activator of the Ca^{2+}-*p*-nitrophenylphosphatase. 3. PNPP at the high-affinity site does not substitute for ATP as activator of the Ca^{2+}-*p*-nitrophenylphosphatase. 4. At ATP concentrations that almost saturate the high-affinity site, Ca^{2+}-*p*-nitrophenylphosphatase activity increases as a function of PNPP along an S-shaped curve, while Ca^{2+}-ATPase activity is partially inhibited along a curve of the same shape and apparent affinity. The fraction of Ca^{2+}-ATPase activity which is inhibited by PNPP is that which results from occupation of the low-affinity site by ATP. 5. Activation of the Ca^{2+}-ATPase by ATP at the low-affinity site is associated with inhibition of the Ca^{2+}-*p*-nitrophenylphosphatase activity. Both phenomena take place with the same apparent affinity and along curves of the same shape. 6. Experimental results suggest that: (a) the Ca^{2+}-*p*-nitrophenylphosphatase activity depends on ATP at the high-affinity site; (b) PNPP is hydrolyzed at the low-affinity site; (c) Ca^{2+}-ATPase activity at the high-affinity size persists during Ca^{2+}-*p*-nitrophenylphosphatase activity.

Introduction

The substrate curve of the Ca^{2+} pump is biphasic and can be represented by the sum of two Michaelis-like equations, one with high apparent affinity and low maximum velocity and the other with low apparent affinity and high maximum velocity [1,2]. The high-affinity site is that at which ATP phosphorylates the enzyme [3]. Combination of ATP at the low-affinity site increases the rate of hydrolysis of the phosphoenzyme, suggesting a regulatory role for this site during ATPase activity [3]. Although in this paper we will refer to low-affinity and high-affinity sites, it is important to mention that from kinetic data it is diffcult to establish if these are two different sites for ATP or different states of a single site.

In the presence, but not the absence, of ATP, red cell membranes show a *p*-nitrophenylphosphatase activity which is activated by Ca^{2+} [4] from the inner surface of the cell membrane [5]. There is strong experimental evidence in favor of the idea that this activity belongs to the Ca^{2+} pump [6].

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; PNPP, *p*-nitrophenyl phosphate; Ca^{2+}-ATPase, Ca^{2+}-dependent ATPase.

Results in this paper suggest that Ca^{2+}-*p*-nitrophenylphosphatase activity depends on occupation of the high-affinity site by ATP and that the active site for PNPP is related to the low-affinity site of the Ca^{2+} pump.

Materials and Methods

Materials

Fragmented membranes from human red blood cells were prepared as described previously [7]. [γ-^{32}P]ATP was prepared according to the method of Glynn and Chapell [8] except that no orthophosphate was added to the medium. [^{32}P]Orthophosphate was provided by Comisión Nacional de Energía Atómica, Argentina. ATP, enzymes and cofactors for [γ-^{32}P]ATP synthesis; *p*-nitrophenyl phosphate (diTris salt); phosphocreatine (diTris salt) and creatine phosphokinase (from rabbit muscle, 100–150 units/mg protein) were from Sigma Chemical Co. (U.S.A.). All other reagents were of analytical reagent grade.

Methods

Unless indicated, enzymatic assays were performed by incubating membranes at 37°C in media containing 0.5 mM EGTA, 0.6 mM $CaCl_2$, 100 mM KCl, 50 mM Tris-HCl (pH 7.8 at 37°C), 1 mM ouabain and various amounts of [γ-^{32}P]ATP, PNPP and fragmented membranes. When the incubation media contained less than 1 mM ATP, 1 mM phosphocreatine was also present. In the case of the Ca^{2+}-*p*-nitrophenylphosphatase assays the media also contained 5 units/ml of creatine phosphokinase. In all experiments ATP was added with an equimolar amount of $MgCl_2$, and PNPP and phosphocreatine with enough $MgCl_2$ to give a final concentration of 2 mM free Mg^{2+}, calculated assuming that for both compounds the association constant for complexing Mg^{2+} is 300 M^{-1}. Ca^{2+}-dependent activities were taken as the difference between the activities in the above-mentioned media and the activities measured in the same media except that $CaCl_2$ was omitted. Ca^{2+}-*p*-nitrophenylphosphatase was measured estimating the release of *p*-nitrophenol from PNPP following the procedure described previously [6]. Since in these experiments the concentrations of membranes was high, phosphocreatine and creatine phosphokinase were always present to keep the concentration of ATP constant by regenerating the hydrolyzed ATP. Ca^{2+}-ATPase was measured from the release of [^{32}P]P_i from [γ-^{32}P]ATP following the procedure described previously [1]. In these experiments the concentration of membranes was low and not more than 10% of the ATP was hydrolyzed. In spite of this, to keep the assay conditions for the Ca^{2+}-ATPase identical to those of the Ca^{2+}-*p*-nitrophenylphosphatase, phosphocreatine was always present. Creatine phosphokinase was omitted to avoid isotopic dilution of [γ-^{32}P]ATP. When Ca^{2+}-ATPase was measured in media containing PNPP, ^{32}P counts were corrected for quenching by PNPP using internal standards containing known amounts of PNPP. Protein was estimated by using the method of Lowry et al. [9].

Results

The requirement of ATP for Ca^{2+}-p-nitrophenylphosphatase activity

Fig. 1 shows the effects of ATP on the *p*-nitrophenylphosphatase activity of red cell membranes measured in the presence and absence of 100 μM Ca^{2+}. In the absence of Ca^{2+}, ATP is without effects on the phosphatase activity. When Ca^{2+} is present *p*-nitrophenylphosphatase activity increases with the concentration of ATP along a curve that from 5 to 500 μM ATP can be described by a single Michaelis equation with $K_{0.5} = 23$ μM. The *p*-nitrophenylphosphatase activity of the Ca^{2+} pump therefore does not show the biphasic response to ATP which is characteristic of the Ca^{2+}-ATPase activity.

Fig. 2a shows double-reciprocal plots of the results of an experiment in which Ca^{2+}-*p*-nitrophenylphosphatase activity was measured as a function of ATP concentration in media containing from 1.6 to 8 mM PNPP. It can be seen that both the $K_{0.5}$ and the maximum effect of ATP on Ca^{2+}-*p*-nitrophenylphosphatase activity increase with PNPP. Fig. 2b shows double-reciprocal plots of the substrate curves of the Ca^{2+}-ATPase at different fixed PNPP concentrations. The experiment was performed in media of composition similar to those used in Fig. 2a and at ATP concentrations (8 to 30 μM) low enough to occupy signifi-

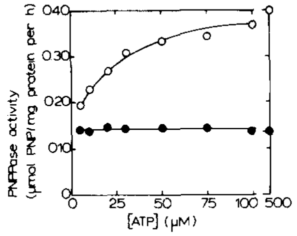

Fig. 1. (Left-hand figure.) *p*-Nitrophenylphosphatase (PNPPase) activity in the presence (○) and in the absence (●) of Ca^{2+} as a function of ATP concentration. The concentration of PNPP was 10 mM. The curve drawn for the activity in the presence of Ca^{2+} corresponds to a Michaelis equation with $V = 0.27$ μmol/mg protein per h and K_m 23 μM added to the activity in the absence of Ca^{2+}. The concentration of fragmented membranes was 1 mg protein/ml.

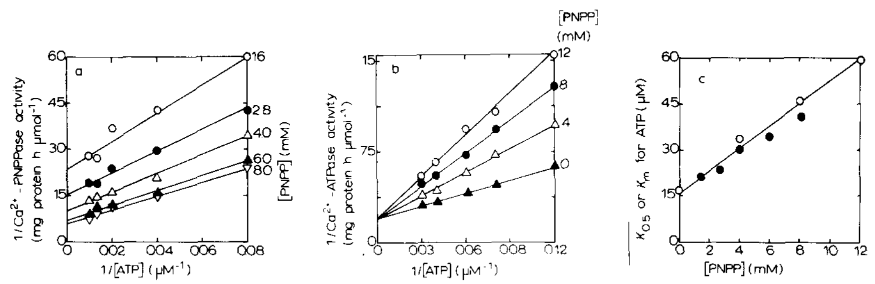

Fig. 2. (a) Lineweaver-Burk plots of Ca^{2+}-*p*-nitrophenylphosphatase (Ca^{2+}-PNPPase) activity as a function of ATP concentration in media with different concentrations of PNPP. The concentrations of fragmented membranes was 1 mg protein/ml. (b) Lineweaver-Burk plots of Ca^{2+}-ATPase activity as a function of ATP concentration in media with different concentrations of PNPP. The concentration of fragmented membranes was 0.1 mg protein/ml. (c) The effect of PNPP on the $K_{0.5}$ for ATP as activator of the Ca^{2+}-*p*-nitrophenylphosphatase (●) and on the K_m of the high-affinity site of the Ca^{2+}-ATPase (○). The values were calculated from the data in (a) and (b).

cantly the high-affinity site only. Results show that PNPP increases the $K_{0.5}$ for ATP but does not modify the maximum effect of the nucleotide at the high-affinity site.

The $K_{0.5}$ for ATP as activator of the Ca^{2+}-*p*-nitrophenylphosphatase and the K_m for ATP at the high affinity site of the Ca^{2+}-ATPase were calculated from Fig. 2a and b and plotted as a function of PNPP concentration in Fig. 2c. It can be seen that the experimental points are fitted by a straight line, as could be expected if PNPP were to compete with ATP. The straight line fits the values from both the ATPase and the phosphatase experiments. This indicates that PNPP is equally effective in competing with ATP for both enzymatic activities and that the $K_{0.5}$ for ATP as activator of the Ca^{2+}-*p*-nitrophenylphosphatase and the K_m of the ATPase at the high-affinity site have the same value. These results were confirmed in several independent experiments, the results of which are summarized in Table I. The similarities between the parameters that govern the interaction of ATP and PNPP with the Ca^{2+} pump during Ca^{2+}-ATPase and Ca^{2+}-*p*-nitrophenylphosphatase activity supports the idea that hydrolysis of PNPP requires combination of ATP at the high-affinity site of the Ca^{2+} pump.

Competition between ATP and PNPP suggests that PNPP, apart from combining at the hydrolysis site, also reacts with the high-affinity site of the Ca^{2+} pump. If this were so, it could be asked if PNPP at this site would be able to substitute for ATP as the cofactor of *p*-nitrophenylphosphatase activity. To answer this question, PNPP hydrolysis

TABLE I

KINETIC PARAMETERS FOR ATP AND *p*-NITROPHENYL PHOSPHATE AT THE HIGH-AFFINITY SITE

K_{mI} is the K_m of the Ca^{2+}-ATPase in the absence of PNPP and was obtained from plots such as those in Fig. 2b; K_A is the $K_{0.5}$ for ATP as activator of the Ca^{2+}-*p*-nitrophenylphosphatase in the absence of PNPP and was obtained by extrapolation of plots of $K_{0.5}$ for ATP as a function of PNPP concentration; K_{iA} is the K_i for PNPP as a competitive inhibitor of the Ca^{2+}-ATPase and K_{iP} is the K_i for PNPP as competitive inhibitor of the activation of the Ca^{2+}-*p*-nitrophenylphosphatase by ATP and were obtained from extrapolation to the abscissa of plots such as that in Fig. 2c. The numbers in parenthesis are the numbers of separate experiments.

Parameter	Mean value+S.E. (mM)
K_{mI}	0.0118±0.0013 (6)
K_A	0.0122±0.0013 (5)
K_{iA}	4.0 ±0.44 (6)
K_{iP}	4.4 ±0.89 (5)

by red cell membranes was measured in the absence of ATP at a wide range of PNPP concentrations in media with and without Ca^{2+}. Results in Fig. 3 show that, under these conditions, hydrolysis of PNPP by red cell membranes is not stimulated by Ca^{2+}. PNPP therefore does not replace ATP as the activator of the Ca^{2+}-*p*-nitrophenylphosphatase activity.

The results shown up to this point allow us to express the rate of hydrolysis of ATP at the high-affinity site (v_1) as

$$v_1 = \frac{V_1}{1 + \dfrac{K_{m1}}{[ATP]}\left(1 + \dfrac{[PNPP]}{K_{iA}}\right)} \tag{1}$$

where V_1 is the maximum velocity, K_{m1} the Michaelis constant and K_{iA} the apparent dissociation constant of PNPP at the high-affinity site.

When only the high-affinity site is occupied by ATP, the rate of hydrolysis of PNPP (v_P) can be expressed

$$v_P = \frac{V_P}{f[PNPP]\left[1 + \dfrac{K_A}{[ATP]}\left(1 + \dfrac{[PNPP]}{K_{iP}}\right)\right]} \tag{2}$$

where V_P is the maximum phosphatase activity, K_A the apparent dissociation constant of ATP as activator of the Ca^{2+}-*p*-nitrophenylphosphatase, K_{iP} the apparent dissociation constant of PNPP as competitive inhibitor of ATP as activator of the Ca^{2+}-*p*-nitrophenylphosphatase and f[PNPP] the function that relates v_P with PNPP concentration at non-limiting ATP.

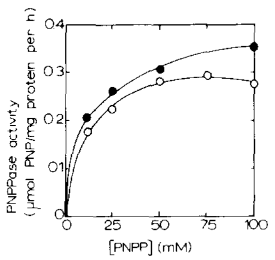

Fig. 3. *p*-Nitrophenylphosphatase (PNPPase) activity in the absence of ATP as a function of PNPP concentration in media with (○) and without (●) Ca^{2+}. The concentration of fragmented membranes was 1 mg protein/ml.

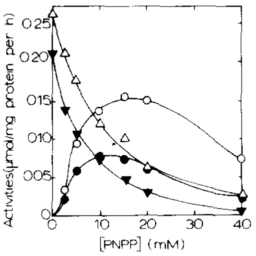

Fig. 4. Ca^{2+}-*p*-nitrophenylphosphatase (○, ●) and Ca^{2+}-ATPase activities (△, ▲) as a function of PNPP concentration. Activities were measured simultaneously in media containing 11 μM (●, ▲) or 30 μM (○, △) ATP. The concentration of fragmented membranes was 1 mg protein/ml in the Ca^{2+}-*p*-nitrophenylphosphatase experiments and 0.1 mg protein/ml in the Ca^{2+}-ATPase experiments.

Eqns. 1 and 2 predict that at constant ATP, Ca^{2+}-ATPase activity will decrease with PNPP and Ca^{2+}-*p*-nitrophenylphosphatase activity will first increase and then decrease tending to zero. The behavior was that observed when Ca^{2+}-*p*-nitrophenylphosphatase and Ca^{2+}-ATPase activities were measured simultaneously as a function of PNPP concentration (Fig. 4) at constant ATP concentration.

Since the data in Table I show that $K_{iP} = K_{iA}$ and $K_{m1} = K_A$, Eqns. 1 and 2 can be related to give:

$$\frac{v_P}{v_1} = \frac{V_P/V_1}{f[PNPP]} \tag{3}$$

which shows that if the only effect of PNPP on the ATPase is to displace ATP from the high-affinity site, the ratio between phosphatase and ATPase activity at the high affinity site will be a function independent of ATP and governed by f[PNPP]. Using the data from Fig. 4 the ratios v_P/v_1 were calculated and plotted against the concentration of PNPP. Results in Fig. 5 show that, in agreement with Eqn. 3, for the two concentrations of ATP used the points fall on the same curve. The curve is S-shaped and when the reciprocal of the square root of the velocity ratio in Fig. 5 is plotted against the reciprocal of PNPP concentration a straight line is obtained with positive intercept and slope (inset in Fig. 5). Eqn. 3 therefore can be written

$$\frac{v_P}{v_1} = \frac{V_P/V_1}{\left(1 + \dfrac{K}{[PNPP]}\right)^2} \tag{4}$$

where K is a constant, the value of which in the experiment shown was 14 mM.

The substrate curve for PNPP of the Ca^{2+}-p-nitrophenylphosphatase

To see if Eqn. 4 represents the substrate curve of the Ca^{2+}-*p*-nitrophenylphosphatase at non-

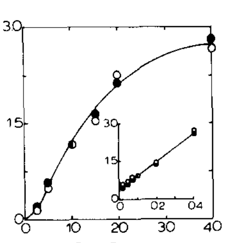

Fig. 5. The effect of PNPP concentration on the ratio between Ca^{2+}-*p*-nitrophenylphosphatase and Ca^{2+}-ATPase activities (v_P/v_1) calculated from the data in Fig. 4. Inset: a plot of the reciprocal of the square root of v_P/v_1 as a function of the reciprocal of the concentration of PNPP. 11 μM ATP (●); 30 μM ATP (○).

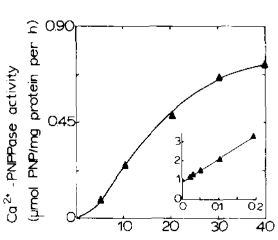

Fig. 6. Ca^{2+}-*p*-nitrophenylphosphatase (Ca^{2+}-PNPPase) activity as a function of PNPP concentration in media with 8 mM ATP. Inset: a plot of the reciprocal of the square root of Ca^{2+}-*p*-nitrophenylphosphatase activity as a function of the reciprocal of PNPP concentration. The concentration of fragmented membranes was 1 mg protein/ml.

limiting ATP concentrations, Ca^{2+}-*p*-nitrophenylphosphatase activity was measured as a function of PNPP concentration at an ATP concentration high enough (8 mM) as to occupy more than 95% of the high-affinity sites for ATP (see Eqn. 2). Results (Fig. 6) show that as a function of PNPP, phosphatase activity increases along an S-shaped curve which tends to saturation. An S-shaped curve is also obtained when total PNPP hydrolysis rate is plotted against PNPP concentration indicating that the shape of the curve in Fig. 6 is not an artifact caused by the substraction of Ca^{2+}-independent activity. The inset in Fig. 6 shows that when $1/v^{1/2}$ is plotted against 1/[PNPP] as in Fig. 5, a straight line is obtained. Therefore, when the high-affinity site is fully occupied by ATP the substrate curve of the *p*-nitrophenylphosphatase can be adequately represented by

$$v_P = \frac{V_P}{\left(1 + \frac{K}{[\mathrm{PNPP}]}\right)^2} \qquad (5)$$

which is formally identical to Eqn. 4. The simplest explanation of why Eqn. 5 describes the substrate curve of the phosphatase is to assume that in each Ca^{2+} pump unit there are two identical sites that have to be occupied by PNPP for Ca^{2+}-*p*-nitrophenylphosphatase activity with an apparent dissociation constant K. As will be shown later, the value of K depends on ATP.

Eqns. 3–5 were derived assuming that ATPase activity at the high-affinity site is independent of *p*-nitrophenylphosphatase activity. Therefore, the agreement of the experimental results with these equations suggests that during hydrolysis of PNPP the Ca^{2+} pump can also catalyze the hydrolysis of ATP at the high-affinity site.

The relation of the active site for PNPP with the low-affinity site

Ca^{2+}-*p*-nitrophenylphosphatase activity was measured in media containing 3, 6 or 9 mM PNPP as a function of ATP at concentrations ranging from 0.6 to 10 mM (Fig. 7a). Using the values in Table I and Eqn. 1 it can be estimated that under these conditions at least 95% of the high-affinity sites of the Ca^{2+}-ATPase will be occupied by the nucleotide. Results show that as ATP concentration increases Ca^{2+}-*p*-nitrophenylphosphatase activity declines, the inhibition by ATP being more evident at the lower PNPP concentration. Dixon plots of the data in Fig. 7a give straight lines, indicating that as ATP concentration rises, Ca^{2+}-*p*-nitrophenylphosphatase activity decreases along rectangular hyperbolae that tend to zero (Fig. 7b). The lines intercept at a point above the abscissa, indicating that ATP at high concentration acts as a competitive inhibitor of Ca^{2+}-*p*-nitrophenylphosphatase activity. The K_i for ATP calculated from the plots in Fig. 7b is 0.5 mM, which is close to the K_m of the low-affinity site for ATP measured in

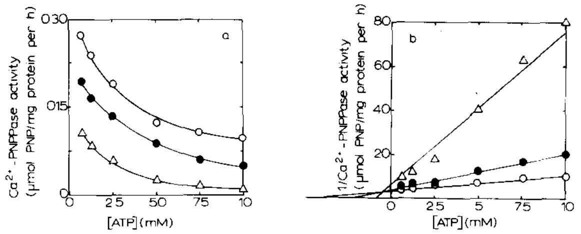

Fig. 7. (a) Ca^{2+}-*p*-nitrophenylphosphatase activity (Ca^{2+}-PNPPase) as a function of ATP concentration in media with 3 (△), 6 (●) or 9 (○) mM PNPP. The concentration of fragmented membranes was 1 mg protein/ml. (b) Dixon plots of the data in (a).

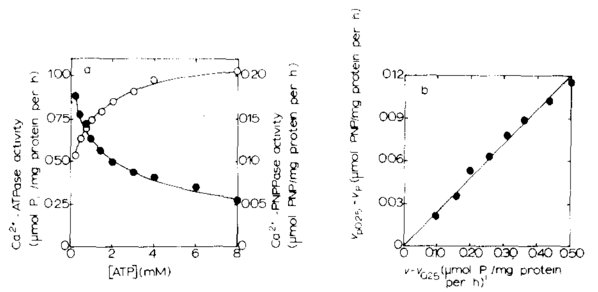

Fig. 8. (a) Ca^{2+}-ATPase (○) and Ca^{2+}-*p*-nitrophenylphosphatase (●) activities as a function of ATP concentration. Activities were measured simultaneously in media containing 4 mM PNPP and 1 mg protein/ml fragmented membranes. (b) A plot of $v_{P0.25} - v_P$ as a function of $v - v_{0.25}$ (see text) of the data in (a).

media of composition similar to those used for the experiment in Fig. 6 but without PNPP. This suggests a relationship of the low-affinity site for ATP with the site for PNPP. Further information on this was obtained from experiments in which Ca^{2+}-ATPase and Ca^{2+}-*p*-nitrophenylphosphatase activities were measured simultaneously on the same membrane preparation and under identical conditions. Experimental results in Fig. 8a indicate that, when measured as a function of ATP, activation of the Ca^{2+}-ATPase at the low-affinity site is accompanied by inhibition of Ca^{2+}-*p*-nitrophenylphosphatase activity. To allow a better comparison of both phenomena we proceeded as follows. In the case of the Ca^{2+}-ATPase, the activity at 0.25 mM ATP was substracted from the activity at the rest of the ATP concentrations tested. Since the ATP curve of the Ca^{2+}-ATPase can be represented by the sum of two Michaelis equations [1], and the high affinity component of the total activity will be near saturation at 0.25 mM ATP, the subtraction will cancel the contribution of the high-affinity component to the total activity and give a curve represented by the equation

$$v - v_{0.25} = \frac{\dfrac{V_2 K_{m2}}{0.25 + K_{m2}}}{1 + \dfrac{0.25 + K_{m2}}{[\mathrm{ATP}] - 0.25}} \qquad (6)$$

where V_2 represents the maximum effect of ATP at the low-affinity component, K_{m2} the apparent dissociation constant of the low-affinity component and $v_{0.25}$ the ATPase activity at 0.25 mM ATP. In the case of the *p*-nitrophenylphosphatase, the activity values at each of the ATP concentrations tested were substracted from the activity at 0.25 mM ATP ($v_{P0.25}$). Since the plots in Fig. 7b show that phosphatase activity decreases with the concentration of ATP following the equation

$$v_P = \frac{V_P}{1 + \dfrac{[\mathrm{ATP}]}{K_i}}$$

where K_i is the apparent inhibition constant for ATP, it is easy to show that

$$v_{P0.25} - v_P = \frac{\dfrac{V_P K_i}{0.25 + K_i}}{1 + \dfrac{0.25 + K_i}{[\mathrm{ATP}] - 0.25}} \qquad (7)$$

Fig. 8b shows a plot of $v_{P0.25} - v_P$ against $v - v_{0.25}$. The points fall on a straight line with zero intercept, indicating that inhibition of *p*-nitrophenylphosphatase activity by ATP is associated to activation of Ca^{2+}-ATPase activity at the low affinity site along curves of the same shape and apparent affinities.

Fig. 9 shows that when measured as a function of PNPP in media containing 5 mM ATP, Ca^{2+}-*p*-nitrophenylphosphatase activity increases while Ca^{2+}-ATPase activity is inhibited. The activation curve of the Ca^{2+}-*p*-nitrophenylphosphatase is S-shaped and can be fitted by Eqn. 5 with $V_P =$ 1.54 μmol *p*-nitrophenol/mg protein per h and $K = 13$ mM. Inhibition of Ca^{2+}-ATPase activity takes place along an S-shaped curve also. When the reciprocal of the square root of the difference between ATPase activity at zero PNPP (v_0) and the activity at each PNPP concentration tested (v) is plotted against the reciprocal of PNPP concentration (Fig. 10), a straight line of positive intercept and slope is obtained. This indicates that at high ATP concentration inhibition of ATPase activity by PNPP can be expressed by the equation

$$v = v_0 - \frac{V_2}{\left(1 + \dfrac{K}{[\mathrm{PNPP}]}\right)^2} \qquad (8)$$

which is equal to the equation used to calculate the

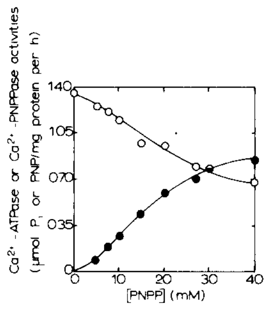

Fig. 9. Ca^{2+}-ATPase (○) and Ca^{2+}-*p*-nitrophenylphosphatase (Ca^{2+}-PNPPase) (●) activities as a function of PNPP concentration. The activities were measured simultaneously in media with 5 mM ATP and 1 mg protein/ml of fragmented membranes. The continuous line that fits the experimental points of Ca^{2+}-*p*-nitrophenylphosphatase activity represents Eqn. 5 with $V_P = 1.54$ μmol *p*-nitrophenyl/mg protein per h and $K = 13$ mM. The continuous line that fits the experimental points of Ca^{2+}-ATPase activity represents Eqn. 8 with $V_0 = 1.35$ μmol P_i/mg protein per h; $V_2 = 1.13$ μmol P_i/mg protein per h and $K = 13$ mM.

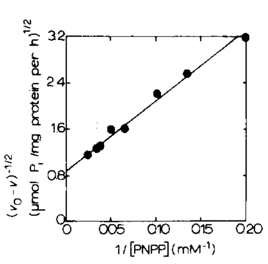

Fig. 10. A plot of the reciprocal of the square root of the difference between Ca^{2+}-ATPase activity in the absence of PNPP and the activity at each PNPP concentration as a function of the reciprocal of PNPP concentration. The experimental values were taken from Fig. 9.

theoretical curve that fits the experimental points of the ATPase activity in Fig. 9. In that experiment K was 13 mM, a value equal to that of the constant for Ca^{2+}-*p*-nitrophenylphosphatase activation measured under identical conditions. Therefore, inhibition of the Ca^{2+}-ATPase by PNPP takes place along a curve of the same shape and apparent affinity as that of the activation by PNPP of Ca^{2+}-*p*-nitrophenylphosphatase activity.

The calculated value of $V_2 = 1.13$ μmol P_i/mg per h (Fig. 10) is less than the experimental value of v_0 (1.35 μmol P_i/mg per h), suggesting that at high ATP only a fraction of the ATPase activity is inhibitable by PNPP. This result fits in with the suggesting we made above that during Ca^{2+}-*p*-nitrophenylphosphatase activity the Ca^{2+} pump catalyzes the hydrolysis of ATP at the high-affinity site which, under the conditions of the experiment in Fig. 9, will be occupied mostly by ATP. The inhibition of the Ca^{2+}-ATPase by PNPP shown in Fig. 9 therefore would represent the blockage of the activation of the Ca^{2+}-ATPase at the low-affinity site and V_2 in Eqn. 8 the ATPase activity that resulted from occupation of the low-affinity site at 4 mM ATP.

Discussion

Experiments in this paper give support to the idea that for the Ca^{2+} pump to exhibit phosphatase activity, ATP has to combine at the high-affinity site. This requirement explains the strict dependence of the Ca^{2+}-*p*-nitrophenylphosphatase activity on ATP that we have described before [4,6]. The conclusion that ATP is needed at the high-affinity site is based in two observations: (i) When extrapolated to zero PNPP, the value for the $K_{0.5}$ for ATP as activator of the Ca^{2+}-*p*-nitrophenylphosphatase is not significantly different from the K_m for ATP at the high-affinity site; and (ii) apart from being the substrate of the Ca^{2+}-phosphatase, PNPP competes with ATP at the high-affinity site during Ca^{2+}-ATPase activity and also competes with the nucleotide during activation of the Ca^{2+}-*p*-nitrophenylphosphatase with the same apparent affinity.

Two possible mechanisms to account for the activation of the phosphatase by ATP seem at hand. One is that ATP at the high-affinity site induces a conformational change leading to hydrolysis of PNPP by the Ca^{2+} pump. Another possibility is that only the phosphoenzyme of the Ca^{2+} pump is able to hydrolyze PNPP. Experimental results in this paper suggest that under conditions of maximum Ca^{2+}-*p*-nitrophenylphosphatase activity there is hydrolysis of ATP at the high-affinity site. This fact can be interpreted as evidence that net turnover of the Ca^{2+}-ATPase at the high-affinity site is required for Ca^{2+}-*p*-nitrophenylphosphatase activity. This view, however, is difficult to reconcile with our previous findings [10] showing the persistance of the ATP-dependent Ca^{2+}-*p*-nitrophenylphosphatase activity after most of the Ca^{2+}-ATPase and the Ca^{2+}-dependent phosphorylation have been abolished by phospholipase C. Therefore, to elucidate the mechanisms of activation of the Ca^{2+}-*p*-nitrophenylphosphatase activity by ATP, further experimental evidence will be necessary.

The *p*-nitrophenylphosphatase activity of the

Na^+ pump of red cells does not require ATP. However, ATP acting at the high-affinity site of the Na^+ pump markedly modifies the behavior of the phosphatase [11], indicating that also in this system there is strong interaction between the high-affinity site for ATP and the site for PNPP.

Results in this paper show that, in contrast with the persistence of Ca^{2+}-ATPase activity at the high-affinity site, activation of the Ca^{2+}-ATPase at the low-affinity site and Ca^{2+}-*p*-nitrophenylphosphatase activity are mutually exclusive functions of the Ca^{2+} pump. This means that those pump units that catalyze PNPP hydrolysis will not exhibit activation of the Ca^{2+}-ATPase by ATP at the low-affinity site and, conversely, those pump units in which ATP stimulates Ca^{2+}-ATPase activity will not catalyze PNPP hydrolysis. The simplest explanation for this is to assume that the low-affinity site for ATP is the active site for PNPP, being PNPP unable to substitute for ATP as activator of the ATPase. That the substrate site for the Ca^{2+}-*p*-nitrophenylphosphatase is the low-affinity site is also suggested by the finding that ATP competes with PNPP and inhibits Ca^{2+}-*p*-nitrophenylphosphatase activity with a K_i value close to its $K_{0.5}$ at the low-affinity site. It seems worthwhile to mention here that based in experiments of competition between PNPP and ATP at the low-affinity site of the $(Na^+ + K^+)$-ATPase, Robinson [12] has proposed that during K^+ dependent phosphatase activity PNPP is hydrolyzed by the Na^+ pump at the low-affinity site for ATP.

The shape of the substrate curve of the Ca^{2+}-*p*-nitrophenylphosphatase suggests that two identical sites for PNPP are present in each pump unit, both of which have to be occupied for *p*-nitrophenylphosphatase activity. The identification of these sites with the low-affinity site for ATP implies that each pump unit should have two sites with low-affinity for ATP. If this view is taken for granted, to explain why the effects of ATP follow Michaelis-like kinetics and those of PNPP follow sigmoid kinetics it seems necessary to assume that full activation of the ATPase is reached when only one of the low-affinity sites is occupied by ATP. Using this assumption it is easy to show that the different kinetic response of the Ca^{2+}-ATPase to PNPP and ATP cannot be used as an argument against simple competition between the two ligands. Our present knowledge of the Ca^{2+}-ATPase is too incomplete for a decision on whether or not a scheme like this represents the actual behavior of this system.

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