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Differential effects of compound 48/80 on the ATPase and phosphatase activities of the Ca^{2+} pump of red cells

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The calmodulin antagonist compound 48/80 inhibits the phosphatase activity of the Ca^{2+}-ATPase lowering its maximum velocity and leaving unaltered its apparent affinity for the substrate regardless on whether phosphatase activity is elicited by Ca^{2+} plus ATP or by calmodulin. Compound 48/80 has no effect on the K_i for ATP as inhibitor of the phosphatase. These results contrast sharply with the large increase that compound 48/80 induces in the apparent affinity of the regulatory site for the nucleotide of the Ca^{2+}-ATPase and suggest that the active site for phosphatase activity is different from the regulatory site for ATP of the Ca^{2+}-ATPase.

Introduction

The Ca^{2+}-ATPase from human red cells displays a phosphatase activity towards *p*-nitrophenyl phosphate [1,2]. This Ca^{2+}-phosphatase activity is expressed only in media with calmodulin and/or ATP, and the effects of calmodulin can be mimicked by partial proteolysis with trypsin [3]. Findings from this laboratory have shown that activation of the Ca^{2+}-ATPase by ATP at its low-affinity, regulatory site, is associated with a parallel inhibition of Ca^{2+}-phosphatase activity so that ATP inhibits Ca^{2+}-phosphatase activity with the same apparent affinity as it activates the Ca^{2+}-ATPase at its regulatory site [4]. On the other hand, *p*-nitrophenyl phosphate inhibits the fraction of the Ca^{2+}-ATPase activity that depends on the occupation of the low-affinity site by ATP with the same apparent affinity as that for *p*-nitrophenyl phosphate as the substrate of Ca^{2+}-phosphatase [4]. On this basis we have proposed that the catalytic site for *p*-nitrophenyl phosphate is the low-affinity regulatory site for ATP of the Ca^{2+}-ATPase [4]. After these findings we have demonstrated that the calmodulin antagonist compound 48/80, induces a large decrease in the apparent affinity of the regulatory site of the Ca^{2+}-ATPase for ATP [5]. Since this provided a valuable tool to obtain further information about the role of this site during Ca^{2+}-phosphatase activity, we have performed experiments designed to test the phosphatase activity under conditions in which the apparent affinity for ATP of the Ca^{2+}-ATPase is lowered by compound 48/80.

Results show that the sites for ATP that compound 48/80 modifies during Ca^{2+}-ATPase activity are kinetically different from the sites for ATP and *p*-nitrophenyl phosphate that are modified by compound 48/80 during phosphatase activity.

Abbreviation: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

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Materials and Methods

Fresh blood from hematologically normal adults collected on acid/citrate/dextrose solutions was always used. Calmodulin-depleted membranes were obtained by the procedure of Gietzen et al. [6].

Phosphatase activity was measured estimating the release of *p*-nitrophenol from *p*-nitrophenyl phosphate [1]. Except when otherwise indicated in Results, the incubation media contained: 120 mM KCl, 5.5 mM $MgCl_2$, 30 mM Tris-HCl (pH 7.40 at 37 °C), 10 mM *p*-nitrophenyl phosphate (di-Tris salt), 1.0 mM EGTA, 0 or 0.5 mM ATP, 0 or 120 nM calmodulin, 1 mM ouabain, 50 to 100 μg/ml of membrane protein and various concentrations of $CaCl_2$. When the incubation media contained less than 0.5 mM ATP, to maintain the concentration of ATP constant, 1 mM phosphocreatine and 10 U/ml of creatine phosphokinase plus 0.1% (w/v) of bovine serum albumin to protect this enzyme against inactivation was added. Control experiments (not shown), indicated that compound 48/80 does not interfere with this ATP regenerating system. ATPase activity was measured from the release of $[^{32}P]P_i$ from $[\gamma\text{-}^{32}P]ATP$ [7] in media of similar composition as those used to estimate phosphatase activity except that they contained various concentrations of ATP. Ca^{2+}-dependent activities were calculated from the difference between the activities in media with and without $CaCl_2$. All incubations were carried out at 37 °C.

The concentration of free Ca^{2+} in the incubation media was measured with an IS561 Ca^{2+}-selective electrode [8]. Protein was estimated by the method of Lowry et al. [9].

Calmodulin was purified from bovine brain as described by Kakiuchi et al. [10]. $[\gamma\text{-}^{32}P]ATP$ was prepared according to the procedure of Glynn and Chappell [11], except that no unlabelled orthophosphate was added to the incubation media. $[^{32}P]$Orthophosphate was provided by the Comisión Nacional de Energía Atómica (Argentina). Compound 48/80, ATP, *p*-nitrophenyl phosphate, enzymes and cofactors for the synthesis of $[\gamma\text{-}^{32}P]ATP$ were obtained from Sigma (U.S.A.). Salts and reagents were of analytical reagent grade.

Statistical treatment of the data. Theoretical equations were adjusted to the experimental results by least-squares non-linear regression using the algorithm of Gauss-Newton with optional damping. The concentration variables were assumed to have negligible error and the variance of velocities to be constant. The program was run on a microcomputer with 14-digit precision [12]. The following procedure was used when the velocity was measured as a function of the concentration of two ligands: A theoretical equation was individually adjusted to each set of data of velocity vs. the concentration of one of the ligands, keeping constant the concentration of the other. The best-fitting parameters generated in this way were plotted as a function of the second ligand in order to find the functions relating its effects on the parameters. These functions were introduced into the first equation and the function of two independent variables thus obtained was adjusted to all the experimental points by multiple non-linear regression.

Results

Effects of compound 48/80 on the velocity versus ATP-concentration curve of the Ca^{2+}-ATPase in the presence of p-nitrophenyl phosphate

Present experimental evidence indicates that the effects of compound 48/80 on Ca^{2+}-ATPase activity are exerted through the blockage of the action of calmodulin on the activation of the Ca^{2+}-ATPase by ATP [5,13]. Since some organic anions mimic the actions of calmodulin (for references, see Ref. 2), it cannot be discarded that *p*-nitrophenyl phosphate, acting as these organic anions modifies the effects of compound 48/80 on the Ca^{2+}-ATPase activity. This point was studied measuring Ca^{2+}-ATPase activity as a function of the concentration of ATP in a 150 to 4000 μM concentration range. It is known [7] that the curve representing the activations of the Ca^{2+}-ATPase by ATP can be described by the sum of two Michaelis-Menten equations one of high ($K_{m1} < 8$ μM) and the other of low ($K_{m2} > 200$ μM) apparent affinity so that even at the lowest concentration of ATP tested (150 μM), the term governed by K_{m1} has reached its maximum velocity (V_1). For this reason an equation containing the Michaelis-Menten equation of low-apparent

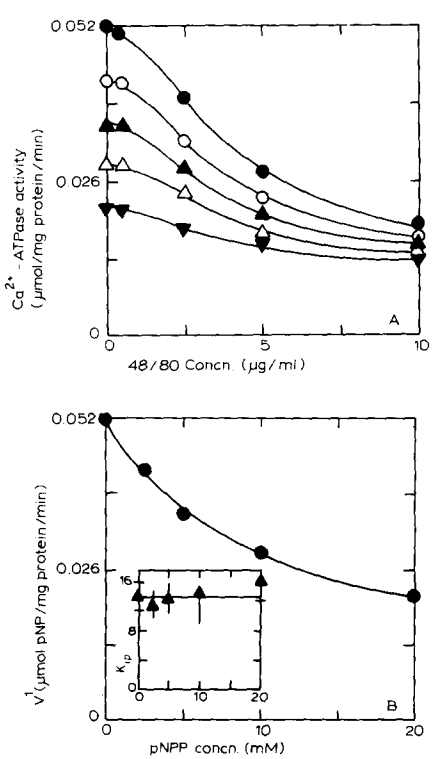
TABLE I

EFFECTS OF *p*-NITROPHENYLPHOSPHATE AND COMPOUND 48/80 ON KINETIC PARAMETERS OF THE Ca^{2+}-ATPase ACTIVITY

Ca^{2+}-ATPase activity was measured as a function of the concentration of ATP from 150 to 4000 μM. pNPP, *p*-nitrophenyl phosphate. The kinetic parameters were estimated adjusting the following equation (see Results) to the experimental data (mean of three independent experiments)

$$v = V_1 + \frac{V_2}{(1 + K_m/[ATP])} \quad (1)$$

Additions		V_1	V_2	K_{m2}
pNPP (mM)	48/80 (μg/ml)	(μmol/mg protein per min)	(μmol/mg protein per min)	(μM)
0	0	0.00805	0.03372	124
		±0.00071	±0.00062	±21
5	0	0.00949	0.03140	666
		±0.00310	±0.00210	±32
0	50	0.00566	0.03901	6892
		±0.00011	±0.0201	±504
5	50	0.00681	0.03563	7549
		±0.00081	0.01815	±714



affinity and a constant value representing V_1 (see legend to Table I) was adjusted to the data. The values of the parameters that gave best fit are shown in Table I. Results show that, as described before, both *p*-nitrophenyl phosphate [4] and compound 48/80 [5] increase K_{m2}. The large in-

Fig. 1. (A) Ca^{2+}-ATPase activity as a function of the concentration of compound 48/80 in media with 120 nM calmodulin, 30 μM Ca^{2+}, 1 mM ATP and either 0 (●), 2.5 (○), 5.0 (▲), 10.0 (△) or 20 (▼) mM *p*-nitrophenyl phosphate (pNPP). The following equation was fitted to the whole set of points

$$V' = \frac{V_0' K_i + V_r' [48/80]^2}{K_i + [48/80]^2} \quad (2)$$

where V_0' and V_r' are the activities at zero and at infinite concentration of compound 48/80, respectively. The concentration of compound 48/80 is squared to account for the sigmoidal shape of the initial part of the curve. Hence the concentration of compound 48/80 needed for half-maximal inhibition will be the square root of K_i. This constant was assumed to be independent of the concentration of *p*-nitrophenyl phosphate. Since from previous results [5], it is known that inhibition by *p*-nitrophenyl phosphate is partial and takes place along a rectangular hyperbola, V_0' and V_r' were assumed to vary with the concentration of *p*-nitrophenyl phosphate according to the following equations

$$V_0' = \frac{V_0 + V_{00}}{1 + [pNPP]/K_{ip}} + V_{00} \quad (3)$$

$$V_r' = \frac{V_r + V_{00}}{1 + [pNPP]/K_{ip}} + V_{00} \quad (4)$$

where V_0 and V_r are the value of V_0' and V_r' (Eqn. 2) in the absence of *p*-nitrophenyl phosphate, V_{00} is the Ca^{2+}-ATPase activity which is not affected by *p*-nitrophenyl phosphate and/or compound 48/80. The concentration of *p*-nitrophenyl phosphate for half-maximal inhibition (K_{ip}), was assumed to be the same for V_0' and V_r'. The best-fitting values of the parameters in Eqns. 2, 3 and 4 were: $V_0 = 0.042893$ μmol P_i/mg protein per min, $K_i = 12.40$ (μg/ml), $V_r = 0.005380$ μmol P_i/mg protein per min, $K_{ip} = 8.22$ mM, and $V_{00} = 0.00989$ μmol P_i/mg protein per min. These were used to draw the continuous lines that fit the experimental points of two independent experiments.

(B) The effects of *p*-nitrophenyl phosphate (pNPP) on V_0', V_r' and K_i. The continuous lines were calculated from Eqns. 2, 3 and 4 and the numerical values given in the legend to Fig. 1A. The values represented by the symbols (●, ▲) were calculated by fitting Eqn. 2 to the data for each concentration of *p*-nitrophenyl phosphate. Vertical bars are standard error. Where not shown, standard errors fall within the size of the symbols. pNP, *p*-nitrophenol.

crease in the value of K_{m2} by compound 48/80 persists in media containing *p*-nitrophenyl phosphate. Compound 48/80 is known to lower by about 30% V_1 leaving unaltered V_2 in the Ca^{2+}-ATPase [5]. Table I shows that this is also the case in the presence of *p*-nitrophenyl phosphate for V_1. Unfortunately, due to the very high value of K_{m2} the estimated value of V_2 bears an error large enough as to hinder any comparison. Nevertheless, from results in Table I, it seems reasonable to conclude that the effects described previously for 48/80 on the Ca^{2+}-ATPase [5] persist in media containing *p*-nitrophenyl phosphate.

To obtain additional information on the effects of compound 48/80 in media containing *p*-nitrophenyl phosphate, Ca^{2+}-ATPase activity was measured at a fixed concentration of ATP, as a function of the concentration of compound 48/80 in media with different concentrations of *p*-nitrophenyl phosphate. Results are shown in Figs. 1A and B. It can be seen that compound 48/80 inhibits Ca^{2+}-ATPase activity along a sigmoidal curve leaving a residual non-inhibitable activity. This curve can be described by Eqn. 2 in the legend to Fig. 1A. The second-order terms are only required to fit the inhibitory effect at low concentrations of compound 48/80. At higher concentrations, the function relating activity to the concentration of compound 48/80 tends to a rectangular hyperbola [5]. An excellent fit of Eqn. 2 to the experimental results is obtained if the activities at zero and at non-limiting concentrations of compound 48/80 are assumed to decrease with the concentration of *p*-nitrophenyl phosphate following the same hyperbolic function (Eqns. 3 and 4) and if K_i (the square of the concentration of compound 48/80 needed for half-maximal inhibition) is assumed to be independent of *p*-nitrophenyl phosphate (Fig. 1B). Apart from confirming previous observations that compound 48/80 [5] and *p*-nitrophenyl phosphate [4] are partial inhibitors of Ca^{2+}-ATPase activity, results in Figs. 1A and B show that the apparent affinities for *p*-nitrophenyl phosphate and compound 48/80 as inhibitors of the Ca^{2+}-ATPase are independent from each other. This provides further evidence that the effects of compound 48/80 on the Ca^{2+}-ATPase persist in media containing *p*-nitrophenyl phosphate.

Effects of compound 48/80 on Ca^{2+}-phosphatase at low ATP concentrations

We have shown previously [3,4] that ATP, acting at the high-affinity catalytic site of the Ca^{2+}-ATPase promotes Ca^{2+}-phosphatase activity in the absence of calmodulin and protects phosphatase activity against inhibition by excess Ca^{2+} in the presence of calmodulin. To see to what extent the effects of compound 48/80 on the high-affinity site for ATP of the Ca^{2+}-ATPase modify Ca^{2+}-phosphatase activity, the hydrolysis of *p*-nitrophenyl phosphate was measured as a function of low ATP concentrations in media with 15 mM *p*-nitrophenyl phosphate and different concentrations of compound 48/80. Results in Figs. 2A and B show that for all conditions tested, phosphatase activity increases with ATP along a Michaelis-Menten equation (Fig. 2A). Activation by ATP is half-maximal at about 8 μM ATP (Fig. 2B) and this value is independent of the concentrations of compound 48/80 tested. In contrast with this, the maximum effect of the nucleotide decreases with compound 48/80 along a hyperbola that tends to a value of about 40% of the control. These results indicate that, in keeping with the known dependence of the phosphatase activity on ATP at its high-affinity site and with the effects of compound 48/80 on this site, compound 48/80 does not change the shape of the activation curve of the phosphatase activity by ATP, its only effect being a partial reduction of the maximum velocity.

Effects of compound 48/80 on the velocity versus p-nitrophenyl phosphate-concentration curve of the Ca^{2+}-phosphatase

If, as proposed previously [4], the site from which ATP exerts its low affinity effect on the Ca^{2+}-ATPase were the catalytic site of the Ca^{2+}-phosphatase, it seems reasonable to think that compound 48/80 should also decrease the apparent affinity of the Ca^{2+}-ATPase for *p*-nitrophenyl phosphate. To test this prediction the effects of compound 48/80 on the kinetic parameters of the Ca^{2+}-phosphatase in media with and without ATP were studied.

The results of the experiment performed in media with ATP are shown in Figs. 3A and B. A Michaelis-Menten equation with its denominator

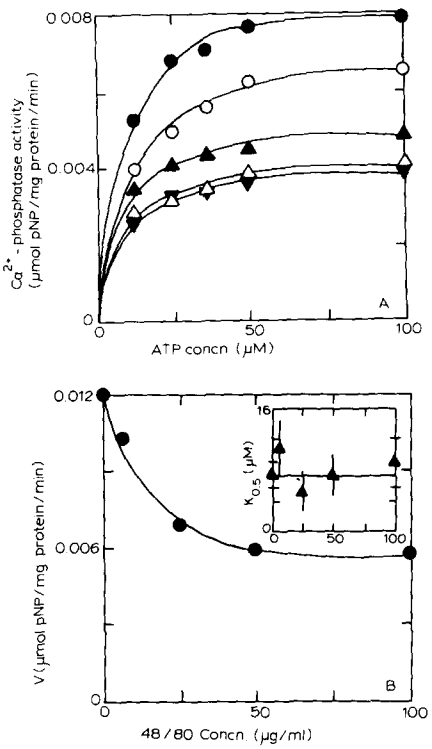


Fig. 2. (A) Ca^{2+}-phosphatase activity as a function of ATP concentration in media containing 120 nM calmodulin, 27 to 30 μM Ca^{2+}, 15 mM *p*-nitrophenyl phosphate, 10 U/ml creatine phosphokinase, 1 mM phosphocreatine, 0.1 g% (w/v) bovine serum albumin and either 0 (●), 6.125 (○), 25.0 (▲), 50.0 (△), or 100.0 (▼) μg/ml of compound 48/80. The following equation was fitted to the whole set of points.

$$v = \frac{V}{(1+[48/80]/K_i)(1+K_{0.5}/[ATP])} + \frac{V_r}{(1+K_{0.5}/[ATP])} \quad (5)$$

where $V+V_r$ is the Ca^{2+}-phosphatase activity in the absence of compound 48/80 and at non-limiting concentrations of ATP, $K_{0.5}$ is the concentration of ATP for half-maximal activation, K_i the concentration of compound 48/80 for half-maximal inhibition and V_r the Ca^{2+}-phosphatase activity not affected by compound 48/80. The best-fitting values of the parameters were: $V = 0.00680$ μmol *p*-nitrophenol/mg protein per min, $K_i = 11.17$ μg/ml $V_r = 0.00502$ μmol *p*-nitrophenol/mg protein per min and $K_{0.5} = 7.57$ μM. These values were used to draw the continuous lines that fit the experimental points of two independent experiments. pNP, *p*-nitrophenol.

(B) The effects of compound 48/80 on V and $K_{0.5}$. The continuous lines were calculated with Eqn. 5 and the numerical values given in the legend to Fig. 2A. The values represented by the symbols (●, ▲) were estimated by fitting individually for each level of compound 48/80 an equation similar to Eqn. 5 but omitting the function of the concentration of compound 48/80. Vertical bars are standard errors. Where not shown, standard errors fall within the size of the symbols.

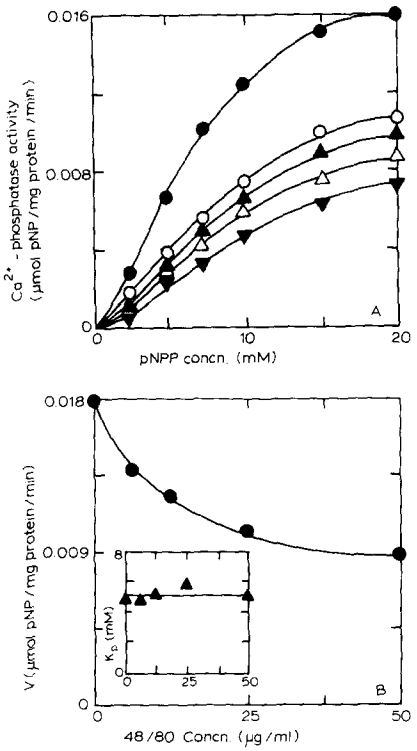


Fig. 3. (A) Ca^{2+}-phosphatase activity as a function of *p*-nitrophenyl phosphate (pNPP) concentration in media containing: 120 nM calmodulin, 27 μM Ca^{2+}, 0.5 mM ATP and either 0 (●), 6.25 (○), 12.50 (▲), 25.00 (△) or 50.00 (▼) μg/ml of compound 48/80. The following equation was fitted to the whole set of points.

$$v = \frac{V}{(1+[48/80]/K_i)(1+K_p/[pNPP])^2} + \frac{V_r}{(1+K_p/[pNPP])^2} \quad (6)$$

where $V+V_r$ is the activity at non-limiting concentrations of *p*-nitrophenyl phosphate and in the absence of compound 48/80, V_r is the activity that is insensitive to the inhibition by compound 48/80, K_i is the concentration of compound 48/80 for half-maximal inhibition and K_p is the concentration of *p*-nitrophenyl phosphate at which $v = (V/2)^{1/2}$. The best-fitting values of the parameters were: $V = 0.01071$ μmol *p*-nitrophenol/mg protein per min, $K_i = 7.45$ μg/ml, $V_r = 0.00651$ μmol *p*-nitrophenol/mg protein per min and $K_p = 5.10$ mM. These were used to draw the continuous lines that fit the experiment points representing the mean of three independent experiments. pNP, *p*-nitrophenol.

(B) The effects of compound 48/80 on V and K_p. The continuous lines were calculated using Eqn. 6 and the numerical values given in the legend to Fig. 3A. The values of the symbols (●, ▲) were calculated by fitting individually to the data for each concentration of compound 48/80 an equation similar to Eqn. 6 but omitting the function of the concentration of compound 48/80. Standard errors fall within the size of the symbols.

squared (Eqn. 6 in legend to Fig. 3A) was used to fit the data because previous studies [4] have shown that such an equation gives a good empirical description of the sigmoidal shape of the curve representing the velocity of the Ca^{2+}-phosphatase as a function of *p*-nitrophenyl phosphate. Inspection of Figs. 3A and B makes clear that an adequate fit of phosphatase activity as a function of both the concentration of *p*-nitrophenyl phosphate and compound 48/80 can be obtained assuming that this compound acts as a partial non-competitive inhibitor of Ca^{2+}-phosphatase that is, decreasing its maximum velocity along a hyperbolic function and leaving unaltered its apparent affinity for *p*-nitrophenyl phosphate.

When the incubation medium contains calmodulin and low (less than 30 μM) concentrations of Ca^{2+} there is Ca^{2+}-phosphatase activity in the absence of ATP [3,14]. The effects of compound 48/80 on the activation of the Ca^{2+}-phosphatase by *p*-nitrophenyl phosphate under these conditions, were explored in the experiment shown in Figs. 4A and B, in which Ca^{2+}-phosphatase activity in media containing calmodulin, 1.25 μM Ca^{2+} and different concentrations of compound 48/80 was measured as a function of *p*-nitrophenyl phosphate concentration. Results were adjusted to equations similar to those in Figs. 3A and B, except that the parameter V_r was omitted because when included, its best-fitting value proved to be not significantly different from zero. The results in Figs. 4A and B show that in the absence of ATP, compound 48/80 also acts as a non-competitive inhibitor of the Ca^{2+}-phosphatase activity. Hence under these conditions also the apparent affinity of the Ca^{2+}-phosphatase for *p*-nitrophenyl phosphate is unaltered by compound 48/80.

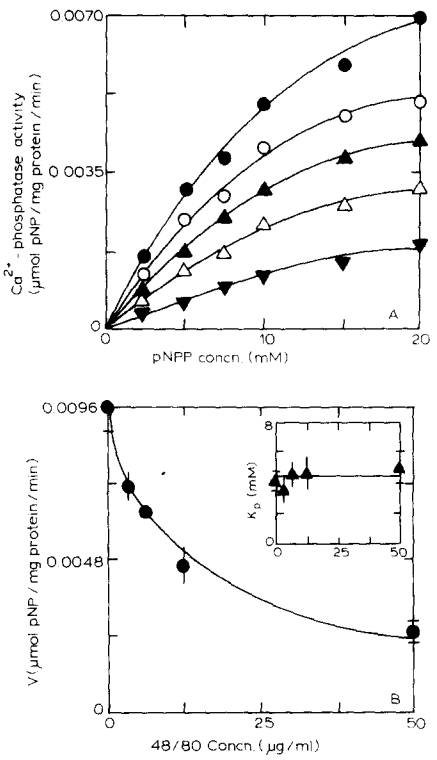


Fig. 4. (A) Ca^{2+}-phosphatase activity as a function of the concentration of *p*-nitrophenyl phosphate in media without ATP and containing 120 nM calmodulin, 1.25 μM Ca^{2+} and either 0 (●), 3.125 (○), 6.25 (▲), 12.50 (△) or 50.00 (▼) μg/ml of compound 48/80. An equation like Eqn. 6, but without the term V_r, was fitted to the whole set of points. The best-fitting values of the parameters were: $V = 0.00956$ μmol *p*-nitrophenol/mg protein per min, $K_i = 12.55$ μg/ml and $K_p = 4.21$ mM. These were used to draw the continuous lines that fit the experimental points representing the mean of two independent experiments. pNP, *p*-nitrophenol.
(B) The effects of compound 48/80 on V and K_p. The continuous lines were calculated with Eqn. 6 and the numerical values given in the legend to Fig. 4A. The values represented by the symbols (●, ▲) were calculated by fitting individually the data for each level of compound 48/80 (see legend to Fig. 3B). Vertical bars are standard errors. Where not shown, standard errors fall within the size of the symbols.

Effects of compound 48/80 on the inhibition of Ca^{2+}-phosphatase by high ATP concentrations

Although the lack of effect of compound 48/80 on the apparent affinity for *p*-nitrophenyl phosphate is in keeping with the idea that the active site of the Ca^{2+}-phosphatase is different from the low-affinity site for ATP, it could be argued that, as a consequence of the diverse chemical structures of *p*-nitrophenyl phosphate and of ATP, compound 48/80 affects the apparent affinity for ATP leaving unaltered that for *p*-nitrophenyl phosphate. A way to surmount this objection is to study the effects of compound 48/80 on the inhibitory action of ATP on the Ca^{2+}-phosphatase activity. In fact, if low-affinity stimulation of the Ca^{2+}-ATPase by ATP and catalysis of *p*-nitrophenyl phosphate hydrolysis took place at the same site, ATP would inhibit the phosphatase by displacing *p*-nitrophenyl phosphate with an ap-

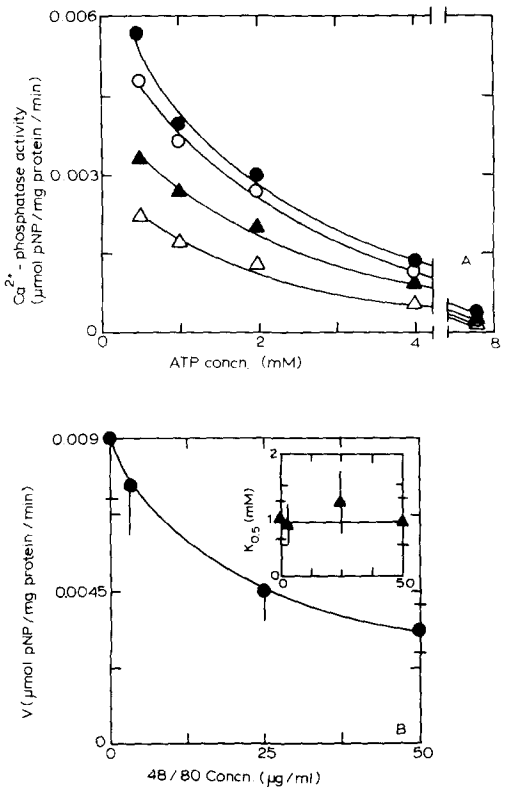


Fig. 5. (A) Ca^{2+}-phosphatase activity as a function of the concentration of ATP in media containing 120 nM calmodulin, 27–30 μM Ca^{2+}, 5 mM *p*-nitrophenyl phosphate (pNPP) and either 0 (●), 3.125 (○), 25.0 (▲) or 50.00 (△) μg/ml of compound 48/80. The following equation was fitted to the whole set of points.

$$v = \frac{V_0 - V_r}{(1+[48/80]/K_i)(1+[ATP]/K_{0.5})} + V_r \qquad (7)$$

where $V_0 - V_r$ is the Ca^{2+}-phosphatase activity that is sensitive to inhibition by compound 48/80, $K_{0.5}$ the concentration of ATP for half-maximal inhibition and V_r and K_i have the same meaning as in Figure 4. The best-fitting values of the parameters were: $V = 0.00902$ μmol *p*-nitrophenol/mg protein per min, $V_r = 0.00225$ μmol *p*-nitrophenol/mg of protein per min, $K_i = 11.25$ μg/ml and $K_{0.5} = 0.898$ mM. These values were used to draw the continuous lines that fit the experimental points representing the mean of two independent experiments. pNP, *p*-nitrophenol.

(B) The effects of compound 48/80 on V and $K_{0.5}$. The continuous lines were calculated with Eqn. 7 and the numerical values given in the legend to Fig. 5A. The values represented by the symbols (●, ▲) were estimated by fitting individually for each level of compound 48/80 an equation similar to Eqn. 7 but omitting the function of the concentration of compound 48/80. Vertical bars are standard errors. Where not shown standard errors fall within the size of the symbols.

parent affinity which should necessarily change with compound 48/80 in the same way as the apparent affinity for the activation by ATP of the Ca^{2+}-ATPase.

In the experiment shown in Figs. 5A and B, Ca^{2+}-phosphatase activity was measured as a function of high (0.25–8.0 mM) ATP concentrations in media containing 5 mM *p*-nitrophenyl phosphate and different concentrations of compound 48/80. The best fit to the experimental data was obtained by an equation containing the product of two hyperbolic inhibitory components, one depending on the concentration of compound 48/80 and the other on the concentration of ATP (Eqn. 7 in the legend to Fig. 5A). Equations of this shape imply that the apparent affinities of the ligands involved are independent of each other. Hence results in Figs. 5A and B, show that the apparent affinity for ATP as inhibitory of Ca^{2+}-phosphatase is unaffected by compound 48/80.

Discussion

In a previous communication from this laboratory [4], it was proposed that the low-affinity site for ATP of the Ca^{2+}-ATPase acted as the active site for *p*-nitrophenyl phosphate during Ca^{2+}-phosphatase activity. The rationale of the experiments reported here is that if ATP and *p*-nitrophenyl phosphate shared the same site on the same conformer of the Ca^{2+}-ATPase, modification of the apparent affinity for ATP should be reflected on the apparent affinity for *p*-nitrophenyl phosphate.

Results in this paper are not in agreement with this prediction, as they show that compound 48/80, that induces a large reduction in the apparent affinity for ATP as activator of the ATPase at its low-affinity site, has no effect on the apparent affinities for *p*-nitrophenyl phosphate as the substrate of the Ca^{2+}-phosphatase. Furthermore inhibition of the Ca^{2+}-phosphatase by ATP, which according to our previous view was caused by displacement of *p*-nitrophenyl phosphate from the low-affinity site for ATP, takes place with an apparent affinity for ATP that is independent of compound 48/80.

These results indicate that the sites for ATP that compound 48/80 modifies during Ca^{2+}-

ATPase activity, which have been identified with the low-affinity sites for the nucleotide, are kinetically different from the sites for ATP and for *p*-nitrophenyl phosphate that are expressed during phosphatase activity.

Hence our previous view about the identity of the active site for *p*-nitrophenyl phosphate and the low-affinity site for ATP of the Ca^{2+}-ATPase [4] has to be modified. This modification may imply either that *p*-nitrophenyl phosphate is hydrolyzed at a site which is physically distinct from the low-affinity site for ATP of the Ca^{2+}-ATPase or that the same site is involved but that *p*-nitrophenyl phosphate hydrolysis and low-affinity activation of the ATPase take place in different steps of the reaction. The different steps may belong either to a reaction cycle which is in part shared by ATPase and phosphatase activities or pertain to reactions which are different depending on whether phosphatase or ATPase activity is taking place. Concerning the possibility that hydrolysis of *p*-nitrophenyl phosphate and low-affinity activation by ATP take place at different states of the same site, it is interesting to point out that Muallem and Karlish [15] have reported that low-affinity activation of the dephosphorylation of the Ca^{2+}-ATPase by ATP is independent of calmodulin, a property that contrasts with the calmodulin-dependence of the low-affinity activation of Ca^{2+}-ATPase by the nucleotide, which is usually considered to express in the overall reaction, the effect of ATP on dephosphorylation [16]. This finding may indicate that when the Ca^{2+}-ATPase is phosphorylated the apparent affinity of the regulatory site for ATP has a different dependence on calmodulin that when the enzyme is dephosphorylated. This may perhaps provide a clue to account for the dissociation between the effects of compound 48/80 on the apparent affinities of the sites for *p*-nitrophenyl phosphate and for the low-affinity activation of the Ca^{2+}-ATPase by ATP reported in this paper. Further experimental evidence is, however, needed to reach a more definite conclusion.

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