Two Simultaneous Binding Sites for Nucleotide Analogs Are Kinetically Distinguishable on the Sarcoplasmic Reticulum Ca 2+ −ATPase †

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Two Simultaneous Binding Sites for Nucleotide Analogs Are Kinetically Distinguishable on the Sarcoplasmic Reticulum Ca^{2+}-ATPase†

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ABSTRACT: Erythrosin B and eosin Y stimulate *p*-nitrophenyl phosphate hydrolysis by purified sarcoplasmic reticulum Ca^{2+}-ATPase by nearly 2−3-fold in the presence of Ca^{2+}. This stimulation is not due to a change on the apparent affinity for substrate but is indeed due to acceleration of the turnover rate of the enzyme. Stimulation reaches a maximum at approximately 5 μM erythrosin or 20 μM eosin and is strictly dependent on the presence of Ca^{2+} in reaction media, while higher concentrations of dye progressively inhibit phosphatase activity. Labeling with fluorescein isothiocyanate (FITC) largely shifts the K_m for *p*-nitrophenyl phosphate (pNPP) and completely abolishes the stimulation of phosphatase activity induced by erythrosin in the presence of Ca^{2+}, apparently by FITC impairing dye binding to an activator site and allowing only manifestation of an inhibitory binding site. In the absence of Ca^{2+}, both erythrosin and eosin inhibit pNPP hydrolysis with Ic_{50} values 3−4-fold higher than the maximally stimulatory concentrations for Ca^{2+}-phosphatase. This inhibitory effect is not modified by previous labeling of the enzyme with FITC, which by its turn does not affect pNPPase activity in absence of Ca^{2+}. It is suggested that stimulation and inhibition of phosphatase activity are related to two simultaneous and physically different nucleotide analog binding sites.

After the description of the so-called "muscle vesicular relaxing factor" three decades ago (Portzehl, 1957; Ebashi, 1961; Ebashi & Lipmann, 1962), an enormous effort was made to elucidate what today is known as the Sarcoplasmic reticulum Ca^{2+}-ATPase. These efforts led to the current knowledge of the whole primary structure of this enzyme (MacLennan *et al.*, 1985; Clarke *et al*., 1989) and the prediction of secondary and tertiary structures by computer modeling (Taylor & Green, 1989; Stokes *et al.*, 1994). Electron microscopy recently provided improved structure images for this enzyme (Stokes *et al.*, 1994), giving some insight on its topology, although resolution of the images remains far from ideal. Despite this progress, a doubt persists concerning the stoichiometry of nucleotide binding sites, as specific activity presents a biphasic dependence to ATP concentrations (Verjovski-Almeida & Inesi, 1979; Taylor & Hattan, 1979; Suzuki *et al*., 1990). Such behavior is attributed to the existence of a catalytic, phosphorylatable site and a regulatory site (Dupont *et al.*, 1984; Coll & Murphy, 1991) which further increases the hydrolysis rate by 2−3-fold without causing a concurrent increase in phosphoenzyme levels.

An interesting feature of the SR[1] Ca^{2+}-ATPase is the lack of specificity displayed for both substrate hydrolysis and enzyme phosphorylation. Compounds such as acetyl phosphate, carbamyl phosphate, umbelliferone phosphate, *p*-nitrophenyl phosphate, GTP, UTP, and ITP can be used as phosphate donors, although with a lower affinity, and are also able to support Ca^{2+} transport (Inesi, 1971; Pucell & Martonosi, 1971; Nakamura & Tonomura, 1978; Rossi *et al.*, 1979; Bodley & Jencks, 1987). Nevertheless, there seems to be a stringency for the occupation of the regulatory site since none of these compounds elicits the same biphasic response observed for ATP. Three different working hypotheses for the phenomenon of ATP-induced acceleration of turnover are favored. (1) Two physically different accessible sites coexist in the same 110 kDa Ca^{2+}-ATPase peptide. (2) Two sites exist on the same peptide and are physically different, although the regulatory site is occluded and becomes available for ATP binding only after phosphorylation. (3) The regulatory site is a kinetic manifestation of the catalytic site and is expressed only after enzyme phosphorylation and ADP release from the enzyme. Thus, evidence supporting one-site (Cable *et al.*, 1985; Bishop *et al.*, 1987; Champeil *et al.*, 1988) and two-site models (Dupont *et al*., 1985; Carvalho-Alves *et al.*, 1985; Suzuki *et al.*, 1990; Coll & Murphy, 1991) exists. In this paper we addressed this problem by studying the effects of halogenated fluorescein derivatives, erythrosin B and eosin Y, as non-hydrolyzable nucleotide analogs, on the hydrolysis of *p*-nitrophenyl phosphate as a pseudosubstrate.

It has been previously reported that some fluorescein-related dyes bind with high affinity to enzymes which possess nucleotide binding sites (Jacobsberg *et al.*, 1975; Yip & Rudolph, 1976; Skou & Esmann, 1981; Morris *et al.*, 1982;

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[1] Abbreviations: SR, sarcoplasmic reticulum; FITC, fluorescein isothiocyanate; EGTA, ethylene glycol bis(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; pNPP, *p*-nitrophenyl phosphate; 3-OMFP, 3-*o*-methylfluorescein phosphate.

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Murphy, 1988; Gatto & Milanick, 1993; De Michelis *et al*., 1993). The possibility that these dyes are indeed binding to nucleotide sites is raised since they usually cause inhibition of the nucleotide-related catalytic properties of the assayed enzymes, and in some cases, the stoichiometry of dye binding is nearly coincident with the number of nucleotides bound by those enzymes (Gatto & Milanick, 1993; Morris *et al*., 1982; Skou & Esmann, 1981). We present kinetic data showing that at least and probably no more than two molecules of nucleotide analogs can be bound simultaneously to the SR Ca^{2+}-ATPase molecule and can even induce a kinetic manifestation of the regulatory site of the enzyme.

MATERIALS AND METHODS

Enzyme Preparation. Sarcoplasmic reticulum vesicles (SRV) were prepared from rabbit hind leg skeletal muscle as described by Eletr and Inesi (1972). SR Ca^{2+}-ATPase was purified by method 2 of Meissner *et al.* (1973) and stored in liquid nitrogen. The specific activity of the preparations was typically between 4 and 6 μmol of P_i mg^{-1} min^{-1} when measured under standard ATPase reaction conditions. Protein concentrations were determined according to Lowry *et al.* (1951), using bovine serum albumin as standard.

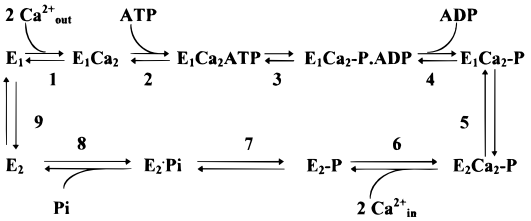
pNPP Hydrolysis. p-Nitrophenylphosphatase (pNPPase) activity was usually measured at 37 °C in media containing 20 mM Tris-HCl (pH 7.4) or Mes-NaOH (pH 6.0), 0.5 mM EGTA or 0.05 mM $CaCl_2$, 120 mM KCl, 10 mM $MgCl_2$, 10−20 μg/mL Ca^{2+}-ATPase, variable dye concentrations, and, except when specified, 3 mM pNPP. After 20−40 min, the reactions (0.5 mL) were quenched with 0.1 mL of 0.3 N NaOH, and 0.4 mL of deionized water was later added. The released *p*-nitrophenol was estimated by absorption at 425 nm, assuming an extinction coefficient of 1.1×10^4 M^{-1} cm^{-1}. V_{max} and K_m values were calculated by using the nonlinear regression program Enzfitter, assuming a simple Michaelian behavior.

3-OMFP Hydrolysis. 3-OMFPase activity was measured in conditions similar to those described for pNPPase activity, except that 50 μM substrate was added. After 2−5 min, hydrolysis was stopped by addition of 0.2 mL of activated charcoal in 0.1 N HCl. After centrifugation, the P_i remaining in the supernatant was measured with the use of acid molybdate and malachite green as described by Van Veldhoven and Mannaerts (1987).

Ca^{2+} Dependence of Phosphatase Activity. The assays were done in media similar to that described above for pNPPase activity measurements, except that 1 mM EGTA and various $CaCl_2$ concentrations were added. Free Ca^{2+} concentrations were calculated with the Ca-EGTA dissociation constants of Schwarzenbach *et al.* (1957), following an algorithm developed by Fabiato and Fabiato (1979).

Labeling with FITC. Ca^{2+}-ATPase was labeled with FITC by a modification of previously described procedures (Pick & Bassilian, 1981). The enzyme was incubated for 20 min at room temperature in media containing 20 mM Tris-HCl (pH 8.5), 80 mM KCl, 0.05 mM $CaCl_2$, 5 mM $MgCl_2$, 0.01 mM FITC, and 1.0 mg of protein per milliliter. Reaction medium was neutralized by addition of an equal volume of 50 mM Tris-HCl (pH 7.0). This medium was further diluted to 20 μg of protein per milliliter during the assays of pNPPase activity. ATPase activity was always inhibited in excess of 95% of the control enzyme submitted to a similar process except that FITC was omitted, while phosphatase activity was completely retained by the labeled enzyme.

Scheme 1: Cycle of the Ca^{2+}-ATPase as Proposed by Carvalho et al. (1976)



Passive Ca^{2+} Efflux. Loading media were composed of 0.3 mM [^{45}Ca]$CaCl_2$, 80 mM KCl, 20 mM Tris-HCl (pH 7.4), 0.08 mg of SRV protein per milliliter, 10 mM $MgCl_2$, 2 mM ATP, and 20 mM P_i. After a 30 min loading incubation at 25 °C, media were centrifuged at 40000*g* for 15 min, and the supernatants were discarded. The SRV were then resuspended in deionized water to a protein concentration of 0.5 mg/mL. Efflux was started by dilution of SRV in efflux media, at 37 °C. Final composition of the media was 0.05 mg of SRV per milliliter, 20 mM Tris-HCl (pH 7.4), and 5 mM EGTA, with or without addition of 5 μM erythrosin and in presence or absence of 80 mM KCl and 10 mM $MgCl_2$. Aliquots were withdrawn at intervals and filtered through Millipore, followed by three washings with 5 mL of a solution with 5 mM $La(NO_3)_3$, 80 mM KCl, and 20 mM Tris-HCl (pH 7.4).

Reagents. pNPP (dicyclohexylammonium salt), 3-OMFP, 2-mercaptoethanol, Tris, MOPS, EGTA, ionophore A23187, and 5′-fluorescein isothiocyanate (FITC) were from Sigma. Fluorescein (free acid or sodium salt), erythrosin B, and eosin Y were from Riedel. [^{45}Ca]Ca^{2+} was from Dupont-New England Nuclear. All other reagents were of analytical grade.

RESULTS

The binding of Ca^{2+} to the high-affinity calcium sites of ATPase and the presence of a high-energy phosphate donor capable of phosphorylating the enzyme are requirements for making catalysis undergo the entire cycle proposed by Carvalho *et al.* (1975) (Scheme 1). The halogenated dyes erythrosin B and eosin Y bind noncovalently to the SR Ca^{2+}-ATPase (in the absence of light) and behave as activators of the hydrolysis of pseudosubtrates like pNPP and O-MFP, provided that there is enough Ca^{2+} in the reaction media. This effect is depicted in Figure 1 (A and B), where the activator effect is maximal at around 5 μM erythrosin or 10−20 μM eosin and is observed for both phosphatase substrates tested when assayed with eosin and for pNPP with erythrosin. Activation of hydrolysis of OMFP by erythrosin was not observed. One plausible explanation is the inhibitory effect of the dye compensates for the activation so almost a constant activity is observed up to 5 μM erythrosin.

In the absence of Ca^{2+}, the ATPase is known to develop a futile pNPPase activity which is not associated with Ca^{2+} binding and transport and is attributed to the E_2 conformation (Inesi, 1971). This activity, which is completely abolished by micromolar vanadate (not shown), is inhibited by erythrosin (Figure 2) and eosin (not shown), and approximately 20−50 μM erythrosin is needed to inhibit half of the activity.

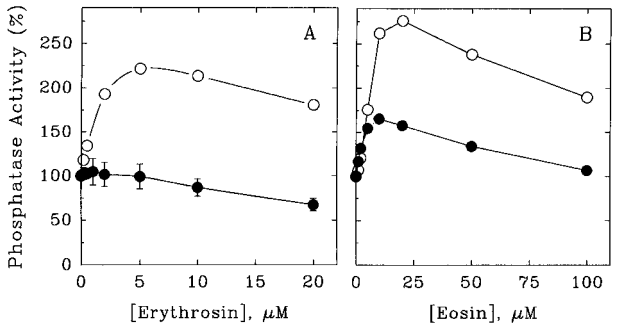


FIGURE 1: Modulation of the Ca^{2+}-stimulated phosphatase activity of the SR Ca^{2+}-ATPase by erythrosin B and eosin Y. Hydrolysis of pNPP (○) and 3-OMFP (●) were measured in the presence of increasing concentrations of erythrosin (A) or eosin (B). The media contained 50 μM $CaCl_2$, 20 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 120 mM KCl, 20 μg of purified Ca^{2+}-ATPase per milliliter, and either 3 mM pNPP or 50 μM 3-OMFP, at 37 °C. The phosphatase activity was assayed as described in Materials and Methods. Values presented for pNPPase and 3-OMFPase are means of two (in the case of activation of 3-OMFP by eosin) and four different independently normalized experiments from two different preparations. In these experiments, 100% phosphatase activity averaged 150−200 nmol of pNP mg^{-1} min^{-1} and 380−500 nmol of OMF mg^{-1} min^{-1}. Bars represent standard deviations.

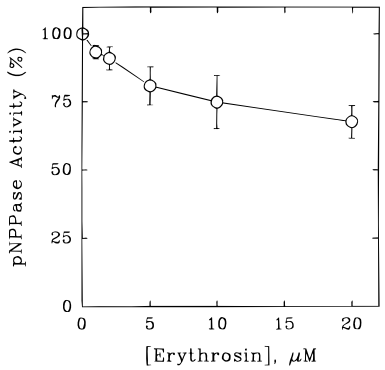


FIGURE 2: Effect of erythrosin on the phosphatase activity developed in the absence of Ca^{2+}. pNPP hydrolysis was measured as in Figure 1, but 0.5 mM EGTA was added instead of $CaCl_2$. Maximal velocity corresponds to 20−26 nmol of pNP mg^{-1} min^{-1}. Data represent means of four different independently normalized experiments ± the standard deviation, with different preparations.

As observed in Figure 1A, this dye concentration is high enough as to also induce inhibition of the "enhanced" phosphatase activity in presence of calcium. These data point to the existence of at least two different binding sites with two relatively similar affinities for the dyes. It must be observed that, although data are presented as percent values for the sake of simplicity, control Ca^{2+} dependent phosphatase is consistently 6−8-fold higher than the Ca^{2+} independent activity.

Nevertheless, it had to be determined whether the enhancement of phosphatase activity observed in the presence of Ca^{2+} was indeed related to a higher turnover rate of the enzyme and not to a change in affinity for the substrate. As shown in Figure 3, pNPPase activity is enhanced by erythrosin binding, without any change in the apparent affinity for substrate but almost doubling the extrapolated V_{max}. The values obtained by nonlinear regression to the points in Figure 3 are $V_{max} = 257.8 \pm 9.0$ nmol mg^{-1} min^{-1} and $K_m = 3.25 \pm 0.27$ mM in the absence and $V_{max} = 494.5 \pm 15.2$ nmol mg^{-1} min^{-1} and $K_m = 2.78 \pm 0.21$ mM in the presence of 5 μM erythrosin. This effect, in analogy to that proposed for ATP hydrolysis, could be attributed to acceleration of the interconversion of $E_1-P\cdot Ca_2$ to $E_2-P\cdot Ca_2$ or to an increased rate on the passage of the E_2 to the E_1 form of the enzyme (4 and 8, respectively, in Scheme 1). It is described elsewhere that these steps are accelerated by binding of ATP to the regulatory site of the enzyme (Scofano *et al.*, 1979; Dupont *et al.*, 1985; Champeil & Guillain, 1986), and thus, binding of erythrosin or eosin to this site could be responsible for some of the effects described above.

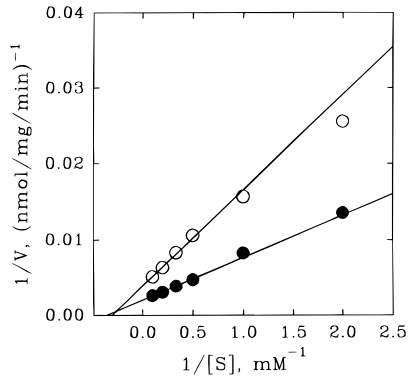


FIGURE 3: Affinity for pNPP of the SR Ca^{2+}-ATPase in the presence of erythrosin. Ca^{2+}-stimulated hydrolysis of pNPP was measured in the presence (●) or absence (○) of 5 μM erythrosin as in Figure 1, but in varying substrate concentrations. The data represent means of five experiments and were fitted as simple Michaelian kinetics with the nonlinear regression program Enzfitter. The values calculated for V_{max} and K_m are cited in the text.

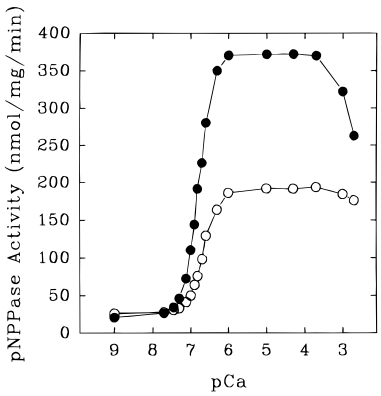


FIGURE 4: Ca^{2+} dependence of the erythrosin-stimulated pNPPase activity. The dependence of pNPP hydrolytic activity on Ca^{2+} concentration was assayed in media composed of 20 mM Tris-HCl (pH 7.4), 120 mM KCl, 10 mM $MgCl_2$, 20 μg of Ca^{2+}-ATPase. per milliliter, 3 mM pNPP, 1.0 mM EGTA, and varying Ca^{2+} concentrations, in the absence (○) or presence (●) of 5 μM erythrosin. The data presented are from a typical experiment.

As Ca^{2+} is a strict requirement for ATP hydrolysis and also for the observed enhancement of pNPPase activity, the dependence of phosphatase activity on Ca^{2+} concentration was studied, and an unusual profile was found in the presence of erythrosin. As shown above, when in the absence of Ca^{2+} or in Ca^{2+} concentrations low enough as not to saturate the enzyme, the dye acts as an inhibitor of the futile pNPPase activity developed in this condition. Accordingly, at the dye concentration used in this experiment (5 μM), a small inhibition is observed at pCa 9.0 (2 mM EGTA and 5−10 μM estimated contaminant Ca^{2+}). However, as the free calcium concentration is increased, the Ca^{2+}-ATPase begins to hydrolyze pNPP through the E_1-E_2 cycle, and thus, erythrosin acts as an activator, counteracting the initial inhibition. It is observed in Figure 4 that, although the apparent cooperativity on the high-affinity Ca^{2+} sites can

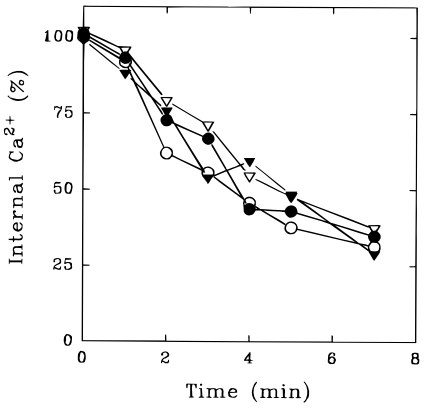


FIGURE 5: Passive Ca^{2+} efflux of SRV incubated with erythrosin. SRV were actively loaded with Ca^{2+}, centrifuged, and washed (see Materials and Methods). Efflux was started by dilution of the loaded SRV into media containing (final) 20 mM Tris-HCl (pH 7.4), 5 mM EGTA, 50 μg of SRV protein per milliliter, and no (○, ▽) or 5 μM (●, ▼) added erythrosin. The efflux media contained either no other addition (○,●) or 80 mM KCl and 5 mM $MgCl_2$ (▽,▼). The data presented are from a typical experiment.

not be easily interpreted, due to the overlapping of effects of the dye, back-inhibition by Ca^{2+} binding to the low-affinity sites is maintained and erythrosin does not appear to cause significant changes in Ca^{2+} binding.

In this paper, we are using purified leaky vesicles obtained according to Meissner *et al.* (1973); however, it is plausible that a certain percentage of sealed vesicles may remain. If this were the case, one possibility would be that erythrosin activated pNPP hydrolysis by leaking these vesicles to Ca^{2+}. Furthermore, it has been described that Ca^{2+}-ATPase can be led to an uncoupled state by fast binding of phenothiazines and β-adrenergic blocking agents (de Meis, 1991), and some anesthetics like procaine and dibucaine (Wolosker *et al.*, 1992), favoring a fast release of Ca^{2+} on loaded SRV. If the dyes were leading to enzyme uncoupling and/or vesicle leakage, an acceleration in Ca^{2+} efflux would be produced. As this was not observed in our Ca^{2+} release experiments (Figure 5), we believe that the acceleration of pNPP hydrolysis is due to a direct effect on the catalytic cycle of the enzyme. This is further supported by the fact that both K^+ and Mg^{2+} are present in the reaction medium, and these ions are shown to antagonize the efflux induced by uncouplers (de Meis, 1991; Wolosker *et al.*, 1992). Controls of phosphatase activity measured in the presence of the calcium ionophore A23187 did not show any enhancement of pNPP hydrolysis by addition of up to 10 μM ionophore. Moreover, the presence of A23187 in the media did not affect the pNPPase enhancement or the further inhibition induced by the dyes (data not shown).

Labeling with fluorescein isothiocyanate has been used as a tool to study events related to the nucleotide binding sites of Ca^{2+}-ATPase. FITC covalently binds to this enzyme on Lys-515 (Maruyama *et al.*, 1989), and this binding blocks ATP binding and hydrolysis (Pick, 1981; Pick & Bassilian, 1981). However, it was reported that the labeled enzyme is still able to hydrolyze small substrates like acetyl phosphate (Pick, 1981; Teruel & Inesi, 1988) or pNPP (Pick, 1981), to support Ca^{2+} transport (Teruel & Inesi, 1988), and to be phosphorylated by phosphate (Pick, 1981; Andersen *et al.*, 1982). We studied the substrate concentration dependence of the pNPPase activity of FITC-labeled Ca^{2+}-ATPase, measured both in the absence of calcium and in the presence

Table 1: Kinetic Parameters for the pNPPase Activity of the Sarcoplasmic Reticulum Ca^{2+}-ATPase Labeled with FITC[a]

	Ca^{2+} dependent		Ca^{2+} independent	
	K_m (mM)	V_{max} (nmol mg^{-1} min^{-1})	K_m (mM)	V_{max} (nmol mg^{-1} min^{-1})
control	3.41	263.5	5.44	33.8
FITC-labeled	9.68	358.0	6.02	35.0

[a] Ca^{2+}-ATPase was incubated with or without FITC for 20 min at pH 8.5 and then diluted 50-fold in media at pH 7.4. The residual ATPase activity of the labeled enzyme was less than 5% of the control. The values presented are means of two experiments.

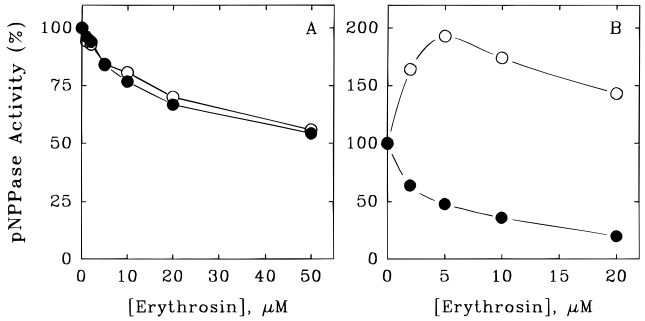


FIGURE 6: Effect of erythrosin on the pNPPase activity of FITC-labeled Ca^{2+}-ATPase. The enzyme activity was tested in media containing 20 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 120 mM KCl, 3 mM pNPP, and 20 μg of Ca^{2+}-ATPase per milliliter. Control (○) and FITC-labeled enzyme (●) phosphatase activities were assayed in the presence of increasing concentrations of erythrosin and with either 0.5 mM EGTA (A) or 50 μM $CaCl_2$ (B) added to the media. Data represent the means of three (A) and two (B) independently normalized experiments with different preparations.

of saturating calcium, and confirmed that both Ca^{2+} independent and Ca^{2+} dependent phosphatase activities were retained by the labeled enzyme. The maximum rate of pNPPase activity obtained by extrapolation of the Ca^{2+} dependent activity was higher in the labeled enzyme. This activation, though, was not observed for the Ca^{2+} independent activity.

Likewise, there was a marked decrease in the apparent affinity of the FITC-labeled enzyme for pNPP when the assay was done under saturating Ca^{2+}, which was not observed for the Ca^{2+} independent phosphatase (Table 1). This finding perhaps reflects a conformational difference in the binding site for substrate when the enzyme is on the E_1 E_2 form, which has already been described by monitoring fluorescence changes of bound FITC (Highsmith, 1986; Markus *et al.*, 1989).

The effect of labeling Ca^{2+}-ATPase with FITC on the activation or inhibition of pNPPase activity was tested. Since erythrosin and eosin are halogenated fluorescein derivatives, there would be a high probability that both dyes would share with FITC a common binding site. Indeed, it was already reported (Murphy, 1988) that erythrosin and eosin protect against FITC labeling, and our own results showed that erythrosin competes with FITC for FITC labeling by delaying the loss of phosphorylation by ATP due to FITC covalent binding to the ATPase (data not shown). When pNPPase activity was tested on FITC-labeled Ca^{2+}-ATPase in the absence of calcium and with erythrosin added, the effect of the dye was inhibitory as described above and the dye concentration dependence was exactly the same for the control and labeled enzyme (Figure 6A). Nevertheless, when the response of phosphatase activity to erythrosin was

tested with saturating calcium, the activating effect previously described was completely abolished, and erythrosin behaved just as an inhibitor of phosphatase activity, much like the behavior of nonlabeled Ca^{2+}-ATPase in the absence of Ca^{2+} (Figure 6B), but with a higher apparent affinity for inhibition by the dye (compare panels A and B of Figure 6). These last observations suggest that, regardless of whether FITC is bound to the Ca^{2+}-ATPase or not, there is at least one site competent to bind erythrosin or eosin. One possibility is that binding of erythrosin to that site inhibits phosphatase activity and is dependent on the presence of Ca^{2+} in the assay media. A second site may exist which is responsible for the activator effect that both erythrosin and eosin induce when in the presence of Ca^{2+}. The covalent binding of FITC to the enzyme totally suppresses the activation elicited by erythrosin or eosin, possibly by impairing binding of the halogenated dyes to this same site. Moreover, the site that is occupied with FITC and that possibly binds the activating dye molecule seems to be silent or occluded when Ca^{2+} is omitted from the media.

DISCUSSION

Like other P-type ATPases, SR Ca^{2+}-ATPase exhibits biphasic kinetics when the hydrolytic rate is measured as a function of ATP (Verjovski-Almeida & Inesi, 1979; Taylor & Hattan, 1979). With a rise in substrate concentration in the micromolar range, hydrolytic activity increases and phosphoenzyme builds up until a maximum of 4−5 nmol mg^{-1} is reached. A further increase in ATP concentration leads to higher hydrolytic rates that are not accompanied by higher phosphoenzyme levels. Barrabin *et al.* (1984) reported that these phosphoenzyme values are obtained when almost all of the catalytically active ATPase monomers are phosphorylated, in agreement with estimates of the amount of Ca^{2+}-ATPase present in SR membranes (Coll & Murphy, 1984). These data basically rule out possible models based on ATPase oligomers with alternating sites and are an indication that the activator effect of millimolar ATP must be due to the existence of both a catalytic and a regulatory function on the same ATPase peptide.

As cited earlier, the basic proposals for the ATP regulatory effect can thus be restated in three possibilities for each monomer. (1) Two physically different accessible sites exist. (2) A regulatory site becomes accessible after phosphorylation of the catalytic site. (3) A single catalytic site behaves also as a regulatory site when phosphorylated. Champeil *et al*. (1988) demonstrated that phosphoenzyme formed with P_i has its rate of hydrolysis stimulated by addition of millimolar ATP but that previous labeling with FITC suppresses the effect of ATP in the submillimolar range. Such results were interpreted as suggesting that a single site with both catalytic and regulatory function exists per each Ca^{2+}-ATPase, as also proposed by Pick (1981). The single site model has further support since TNP−AMP, a fluorescent nucleotide analog (Dupont *et al*., 1982), binds to Ca^{2+}-ATPase in stoichiometric amounts, competes with ATP for phosphorylation and with ADP for dephosphorylation, and also inhibits the secondary activation by ATP. However, recent results show that the stoichiometry of binding of TNP−nucleotides can vary from one site per monomer, using TNP−AMP, to two sites per monomer, using TNP−ATP (Suzuki *et al*., 1990), in agreement with two classes of nucleotide sites described previously by competition with TNP−ATP and ATP (Dupont *et al*., 1985). Coll and Murphy (1991) reported that ATP and ADP exhibited noncompetitive behavior for ATP hydrolysis and synthesis, and addition of ATP even increased the rate of ATP synthesis during the reversal of the Ca^{2+} pump (reaction of $[^{32}P]E-P$ with ADP). These results were taken by the authors as evidence of the existence of two different nucleotide sites per Ca^{2+}-ATPase monomer.

Substrates other than ATP do not induce a secondary activation of hydrolysis. This behavior could be due to the absence of a nucleotide binding to the catalytic site of the enzyme after phosphoryl transfer from the substrate or to the lack of an appropriate binding to a different, low-affinity putative regulatory site. In this work, we found that erythrosin B and eosin Y, halogenated dyes which are considered to be good morphological analogs of adenosine phosphonucleotides [see Neslund *et al.* (1984)], induce a stimulation of the phosphatase activity developed by the SR Ca^{2+}-ATPase in presence of Ca^{2+}. This effect is observed without a concurrent change in the apparent affinity for pNPP as substrate, thus probably being due to the acceleration of a step which is rate-limiting for the hydrolysis of both substrates tested. A good guess would be that the dyes may be acting in the same step of the cycle accelerated by ATP. As there is evidence that ATP accelerates the steps between $E_1-P{\cdot}Ca_2$ and phosphoenzyme hydrolysis (Gould *et al.*, 1986; Champeil *et al.*, 1988; Michelangeli *et al.*, 1990) and/or between E_2 and phosphoenzyme formation (Gould *et al.*, 1986; Stahl & Jencks, 1984, 1987; Petithory & Jencks, 1986) (see Scheme 1), it is not surprising that the activator effect is observed only when the phosphatase activity is developed through the entire cycle of the enzyme and with Ca^{2+} as effector. The fact that labeling with FITC suppresses the stimulatory effect of erythrosin is a good indicator that binding of these dyes occurs in the same region that ATP binding does. Moreover, it is suggested by our results that, whatever should be the step accelerated by erythrosin or eosin, there is a stringency for the positioning and the interactions of a molecule within the site responsible for activation. As an example, free fluorescein does not induce any activation of phosphatase (not shown), but the extrapolated maximal velocity of the pNPPase activity appears to be higher for the FITC-labeled enzyme (Table 1). It is noteworthy that the degree of activation is dependent on both the activator and the substrate used and may indicate that the two binding sites are situated either in close proximity or in strict interdependence.

A second question concerns the inhibitory effect observed at scarcely higher dye concentrations in the presence of Ca^{2+}. This inhibition counteracts the initial activation and is indicative that high-affinity binding of a second dye molecule to Ca^{2+}-ATPase occurs. Labeling the enzyme with FITC eliminates the pNPPase enhancement, and only the inhibitory component for dye binding is observed, consistent with the fact that erythrosin is still binding to the enzyme with a relatively high affinity, however, in a site different from the activator one. This site could be related to the inhibitory site for the dyes observed in the absence of Ca^{2+}, the slight difference in affinities being probably attributed to a Ca^{2+}-induced conformational change of the binding site.

In this last case, the activator effect is not manifested since in the absence of Ca^{2+} the enzyme does not go through the E_1-E_2 cycle for hydrolysis. Enzyme labeling with FITC

does not induce any change in the concentration dependence of the inhibition by erythrosin in the absence of Ca^{2+}, thus once again suggesting a different site from the activator one observed with Ca^{2+}. It is noteworthy that, as FITC covalently labels the enzyme, this experiment implies that both nucleotide analogs (FITC and the inhibitory erythrosin molecule) are simultaneously and independently bound to the enzyme. However, a question remains unanswered which is whether activation and inhibition are due to effects at different portions (subsites) of the same site or to effects at two physically different sites with relatively near affinities. Alternatively, it should be considered that a single site exists, separated from the FITC site, and that it mediates the two effects. Whichever of the possibilities is preferred, the fact is that at least two dye molecules which are considered nucleotide analogs bind simultaneously to Ca^{2+}-ATPase, and at least one of them induces an effect which could be related to the well-documented ATP regulatory effect.

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