The Modulation of Ca$^{2+}$ Binding to Sarcoplasmic Reticulum ATPase by ATP Analogues Is pH-dependent*

(Received for publication, August 7, 1995, and in revised form, September 11, 1995)

Elisabeth Mintz, Ana M. Matař, Vincent Forge, Maria Passafiume, and Florent Guillaud

From the Commissariat à l’Energie Atomique and Unité de Recherche 1290 Associée au CNRS, Section de Biophysique des Protéines et des Membranes, Département de Biologie Cellulaire et Moléculaire, Centre d’Etudes de Saclay, 91191 Gif-sur-Yvette Cedex, France

Excess ATP is known to enhance Ca$^{2+}$-ATPase activity and, among other effects, to accelerate the Ca$^{2+}$ binding reaction. In previous work, we studied the pH dependence of this reaction and proposed a 3H$^+$/2Ca$^{2+}$ exchange at the transport sites, in agreement with the H$^+$/Ca$^{2+}$ counter transport. Here we studied the effect of ADP and nonhydrolyzable ATP analogues on the Ca$^{2+}$ binding reaction at various pH values.

At pH 6, where Ca$^{2+}$ binding is monophasic and slow, ADP, adenosine 5’-(β,γ-methylene)diphosphate (AMPPCP), or adeny1-5’-yl imidodiphosphate (AMPPNP) increased the Ca$^{2+}$ binding rate constant 20-fold. At pH 7 and 8, where Ca$^{2+}$ binding is biphasic, the nucleotides induce fast and monophasic Ca$^{2+}$ binding. At pH 7, AMPPCP accelerated Ca$^{2+}$ binding with an apparent dissociation constant of 30 µM.

At acidic pH, ADP, AMP-PCP, or AMP-PNP increased the equilibrium affinity of Ca$^{2+}$ for ATPase, whereas at alkaline pH, these nucleotides had no effect. At pH 5.5, AMPPCP increased equilibrium Ca$^{2+}$ binding with an apparent dissociation constant of 1 µM.

Ca$^{2+}$-ATPase is a membranous enzyme which pumps Ca$^{2+}$ from the cytoplasm into the sarcoplasmic reticulum lumen, requiring ATP hydrolysis. A simple description of the cycle is given in Scheme 1. Cytoplasmic Ca$^{2+}$ binding at the transport sites is a crucial step in the ATPase cycle, as it induces a change in the chemical reactivity of the catalytic site which is either phosphorylatable by P$_i$ in the absence of Ca$^{2+}$ or phosphorylatable by ATP in the presence of Ca$^{2+}$. Once the phosphoenzyme has been formed from ATP, the Ca$^{2+}$ bound at the transport sites can be released into the lumen, and dephosphorylation occurs.

Ca$^{2+}$-ATPase activity requires micromolar ATP and is enhanced by submillimolar or millimolar ATP (1–5). As the site of micromolar affinity is the catalytic site, there has been a great deal of discussion as to whether the lower affinity site is the catalytic or a regulatory site, especially because studies of ATP binding at equilibrium have shown one or two different sites (2, 5–8). ATP is known to accelerate Ca$^{2+}$ binding from the cytoplasm (9–13), Ca$^{2+}$ release into the SR lumen (14, 15) and dephosphorylation (16, 17). In other words, ATP increases the rate of all steps which start from a nucleotide-deprived species (steps 2, 3, and 4 in Scheme 1), suggesting that it binds to the nucleotide site, even on the phosphoenzyme, so that ATP enters the cycle as soon as ADP has dissociated. According to McIntosh and Boyer (18), Bishop et al. (19), and Champell et al. (16), the regulation occurs on the phosphoenzyme and probably at the catalytic site.

Cytoplasmic Ca$^{2+}$ binding to the ATPase is particularly important in studying the interaction between the catalytic and transport sites, because it is the step at which ATPase changes its chemical specificity. Keeping in mind that there is millimolar ATP in the cytoplasm, understanding how ATP modulates the Ca$^{2+}$ binding step will lead to a description of the Ca$^{2+}$ binding mechanism closer to the in vivo phenomenon.

To analyze the effect of a nucleotide at the catalytic site on the Ca$^{2+}$ binding reaction, the use of nonhydrolyzable ATP analogues is more convenient than ATP, as it avoids perturbing the Ca$^{2+}$ binding reaction by the phosphorylation reaction. ADP, AMPPCP, and AMP-PNP have been used by different authors who reached different conclusions. According to Ogawa et al. (20), the Ca$^{2+}$ affinity is increased by AMPPCP; according to Fernandez-Belda et al. (21), AMP-PNP does not affect the affinity or the rate of Ca$^{2+}$-binding; according to Wakabayashi and Shigekawa (13), AMPPNP increases the rate of Ca$^{2+}$ binding.

These contradictory conclusions are probably due to various experimental conditions, especially the pH. A previous study of the Ca$^{2+}$ binding step has led us to a detailed description of the 2Ca$^{2+}$/3H$^+$ exchange occurring at the transport sites (22, 23). Scheme 2 describes this exchange without Mg$^{2+}$. With Mg$^{2+}$, the species EH$_3$ and EC$_2$ were shown to be phosphorylated by P$_i$ and ATP, respectively, so that Scheme 2 can be taken as step 4 in Scheme 1. On the basis of this previous work, we have studied the effects of ATP on the cytoplasmic Ca$^{2+}$ binding step at various pH values, using nonhydrolyzable ATP analogues, and we show that ADP, AMP-PNP, and AMPPCP have a pH-dependent effect on both the rate and the affinity of Ca$^{2+}$ binding. (i) These nucleotides drastically increase the rate of Ca$^{2+}$ binding at pH 6 whereas they have little effect at pH 8. This is thought to occur via an increase in the rate of the deprotonation steps which are rate-limiting without nucleotides. (ii) These nucleotides also increase the Ca$^{2+}$ affinity at acidic pH; whereas they have no effect at neutral or alkaline pH. We suggest this could be due to a higher affinity of the Ca$^{2+}$-saturated ATPase for ATP analogues at acidic pH.

MATERIALS AND METHODS

SR vesicles were prepared and tested as described in Ref. 22 from rabbits subjected to a 48-h starvation diet (24). The experiments were carried out at 20 or 5 °C in thermostated rooms. Buffers were: 100 mM Tes-Tris (pH 8), 100 mM Mops-Tris (pH 7), or 100 mM Mes-Tris (pH 6.3–5.5) and were prepared with water filtered through a Milli-Q Water...
Purification System (Millipore). All salts were added as chlorides. FITC labeling was done as described in Ref. 16. All nucleotides were purchased from Boehringer, ADP was the purest available preparation.

Equilibrium and kinetic measurements involving \(^{45}\text{Ca}^2^+\) were performed as described in Ref. 23. The rapid filtration apparatus (25) (Biologic, Chaix, France) was used to perfuse \(^{45}\text{Ca}^2^+\) for times from 50 ms to 5 s. Once SR vesicles are adsorbed on the filter, the filter holder and the perfusion device come into contact for the chosen perfusion time. The perfusion of the buffer through the filter is performed by an electronically controlled stepping motor. At the end of the perfusion, the filter is removed by the manipulator.

The intrinsic fluorescence change induced by the nucleotides was measured at equilibrium as described in Ref. 5, and the \(\text{Ca}^2^+\) binding kinetics were measured using a stopped-flow apparatus (SFM3, Biologic, Chaix, France) as described in Ref. 23.

Free \(\text{Ca}^2^+\) concentrations were calculated as in Ref. 22 and using the following figures for the dissociation constants of the calcium complexes with nucleotides (in \(\text{M}\)): AMPPNP, \(4 \times 10^{-4}\) at \(\text{pH} 6\) and \(10^{-4}\) at \(\text{pH} 7, 7 \times 10^{-4}\) at \(\text{pH} 8\); AMPPCP, \(4 \times 10^{-4}\) at \(\text{pH} 8\). Keeping in mind that, without \(\text{Mg}^2^+\), equilibrium \(\text{Ca}^2^+\) binding affinities are \(9 \mu\text{M}\) at \(\text{pH} 6\) and \(0.1 \mu\text{M}\) at \(\text{pH} 8\) (22), excess concentrations of \(100 \mu\text{M}\) and \(10 \mu\text{M}\) were chosen to saturate ATPase at \(\text{pH} 6\) and 8, respectively. At \(\text{pH} 6\), \(\text{Ca}^2^+\) binding was slow and monophasic with a rate constant of \(0.7\) s\(^{-1}\); whereas at \(\text{pH} 8\), \(\text{Ca}^2^+\) binding was biphasic with a slow rate constant of \(3\) s\(^{-1}\) and a fast rate constant in the 25-45 s\(^{-1}\) range, in agreement with previous measurements (23). The presence of 0.1 mM ADP, AMPPCP, or AMPPNP increased the \(\text{Ca}^2^+\) binding rate constant at \(\text{pH} 6\) up to \(15\) s\(^{-1}\) and the rate of the slow phase at \(\text{pH} 8\), producing a unique fast phase with a rate constant of \(30\) s\(^{-1}\). Note the reduced amplitude of the signal with nucleotides present. This is due to nucleotide binding to the \(\text{Ca}^2^+\)-depleted ATPase which induced half of the total fluorescence enhancement due to \(\text{Ca}^2^+\) binding.

Similar experiments were performed by measuring radiolabeled \(\text{Ca}^2^+\) binding by the rapid filtration technique, which allows evaluation of the \(\text{Ca}^2^+\) specifically bound to ATPase. At \(\text{pH} 6\) where \(\text{Ca}^2^+\) binding was monophasic and slow, AMPPCP as well as AMPPNP increased the \(\text{Ca}^2^+\) binding rate (Fig. 2), thus confirming the results obtained from intrinsic fluorescence. Increasing the nucleotide concentration from 0.1 to 0.3 mM did not change the kinetics. The kinetics were identical, whether or not ATPase was incubated with the nucleotide, indicating that the binding of the nucleotide is faster than \(\text{Ca}^2^+\) binding. At \(\text{pH} 8\), it was very difficult to measure a clear acceleration of \(\text{Ca}^2^+\) binding in the presence of AMPPCP or AMPPNP, due to the small amplitude of the slow phase. Therefore, another series of experiments was performed at \(\text{pH} 7\) and \(5^\circ\text{C}\), varying the concentration of AMPPCP (Fig. 3). Under these conditions and without AMPPCP, \(\text{Ca}^2^+\) binding was biphasic. The fast phase, which represented half of the stoichiometry, was too fast to be measured by the filtration technique, even in absence of AMPPCP. The slow phase was accelerated with an apparent dissociation constant of 10 \(\mu\text{M}\) (see inset in Fig. 3).

Similar experiments were performed using FITC-labeled SR. In FITC-labeled SR, ATPase has covalently bound one molecule of FITC at Lys-515 which impairs nucleotide binding at the catalytic site (28–31). The results obtained at \(\text{pH} 6\) and with 3 mM \(\text{Mg}^2^+\) are shown in Fig. 4. With native SR, 0.1 mM AMPPCP increased the binding rate constant for free \(\text{Ca}^2^+\) at 30 \(\mu\text{M}\) from 1.5 to 7.4 s\(^{-1}\), whereas with FITC-labeled SR, AMPPCP did not affect \(\text{Ca}^2^+\) binding kinetics which had a rate constant of 2.5 s\(^{-1}\). Similar results were obtained when the nucleotide was AMPPNP without added \(\text{Mg}^2^+\). Note that with FITC present at the catalytic site, the \(\text{Ca}^2^+\) binding kinetics were faster than without FITC. This, together with the absence of effect of the nucleotide with FITC-labeled ATPase shows that...
acceleration of the Ca\(^{2+}\) binding kinetics is mediated by the nucleotide at the catalytic site.

Nucleotides Increase the Transport Sites Affinity for Ca\(^{2+}\) at Acidic pH—Ca\(^{2+}\) binding was studied at equilibrium, at various pH values and in the presence of nucleotides with or without added Mg\(^{2+}\) to determine whether the affinity for Ca\(^{2+}\) displayed the same sensitivity to the nucleotides as the Ca\(^{2+}\) binding kinetics. Fig. 5 shows equilibrium Ca\(^{2+}\) binding at various pH values, without (open symbols) and with (filled symbols) various nucleotides at 0.3 mM. The control curves showed a pH-sensitive affinity for Ca\(^{2+}\), i.e., Ca\(_{50}\), the Ca\(^{2+}\) concentration yielding half-saturation of the Ca\(^{2+}\) sites, varied from 0.3 \(\mu\)M at pH 8, to 10 \(\mu\)M at pH 6, as previously measured (22). At neutral and alkaline pH, AMPPCP did not affect the Ca\(^{2+}\) binding curve, whereas at acidic pH, the Ca\(^{2+}\) binding curve was shifted to the lower Ca\(^{2+}\) concentrations and Ca\(_{50}\) decreased, from 10 to 4 \(\mu\)M at pH 6 and from 4 to 2 \(\mu\)M at pH 6.3.

The experiments done with Mg\(^{2+}\) and ADP or AMPPNP displayed an additional Ca\(^{2+}\) binding stoichiometry which proved to be due to slow Ca\(^{2+}\) transport (data not shown). This led us to check the purity of the nucleotides by HPLC, using an ion exchange column (TSK DEAE-2SW). AMPPCP and ADP showed a single peak, whereas AMPPNP was not pure. The additional stoichiometry with ADP is thus more likely due to ATP synthesized by myokinase which contaminates the SR preparation (24). The impurities in AMPPNP could possibly induce Ca\(^{2+}\) transport.

Fig. 6 summarizes the effects of all three nucleotides, showing that they decreased Ca\(_{50}\) at acidic pH, with or without added Mg\(^{2+}\). Note the specific effect of AMPPNP in the presence of 3 mM Mg\(^{2+}\) which increased the Ca\(^{2+}\) affinity at all pH values.

The experiments described above show that ADP and ATP analogues undoubtedly bind to the various forms of ATPase which participate in the Ca\(^{2+}\) binding reaction and that the binding of these nucleotides at acidic pH results in an increase in the affinity for Ca\(^{2+}\). The next step was thus the evaluation of the affinity of this nucleotide site.

The apparent affinity for AMPPCP was measured at equilibrium and at various pH values. These experiments are shown in Fig. 7. They were conducted by measuring the Ca\(^{2+}\) binding to ATPase at various AMPPCP concentrations, keeping the free Ca\(^{2+}\) concentration fixed at a value close to Ca\(_{50}\). For example, at pH 6 and without added Mg\(^{2+}\), the free Ca\(^{2+}\) concentration was fixed at 6 \(\mu\)M which gives half-saturation. Addition of AMPPCP induced an increase in the amount of bound Ca\(^{2+}\) up to saturation. This curve thus reflects the increase in the affinity for Ca\(^{2+}\) due to AMPPCP addition. Its shape is that of noncooperative binding curve, corresponding to the binding of AMPPCP at a single site with an apparent dissociation constant of 10 \(\mu\)M.

This experiment was repeated at various pH values, with and without Mg\(^{2+}\) (Fig. 7). The curve was flat at pH 7, as expected from the absence of effect of 0.3 mM AMPPCP under similar conditions in Fig. 6. The curve was sigmoidal at pH 5.5, and the corresponding apparent dissociation constant was 1 \(\mu\)M. The effect of Mg\(^{2+}\) is illustrated at pH 6.3, where the apparent dissociation constant for AMPPCP was 15 \(\mu\)M without Mg\(^{2+}\). 2 pH 5.5 was chosen as being the most acidic pH that does not induce ATPase precipitation. An equilibrium Ca\(^{2+}\) binding curve was monitored by intrinsic fluorescence measurements and yielded a value of 50 \(\mu\)M for Ca\(_{50}\) at pH 5.5.
To analyze the pH sensitivity of the nucleotide affinity which modifies the Ca\(^{2+}\) binding reaction, we also needed to determine whether the nucleotide affinity was pH-sensitive in the absence of Ca\(^{2+}\). These measurements were monitored by measuring the intrinsic fluorescence response to nucleotide addition. They are summarized in Fig. 8, which shows N\(_{1/2}\), the nucleotide concentration yielding half-saturation of the fluorescence signal, as a function of pH. As already shown for ATP (5), neither of the two ATP analogues we used was found to have a pH-sensitive affinity in the absence of Ca\(^{2+}\). Note that the affinities of these nucleotides are close to that of ATP and that ADP is the only nucleotide displaying a pH-sensitive affinity in the absence of Ca\(^{2+}\) and Mg\(^{2+}\).

In studying the effects of ADP and ATP analogues on the Ca\(^{2+}\) binding reaction, we have found that (i) ADP, AMPPCP, and AMPPNP increased the rate of Ca\(^{2+}\) binding 10–30-fold at pH 6 and to a smaller extent at pH 7 and 8 (Figs. 5–7), (ii) FITC labeling induced a 2-fold increase in the Ca\(^{2+}\) binding rate at pH 6, and nucleotides had no additional effect (Fig. 4), (iii) ADP, AMPPCP, and AMPPNP increased the Ca\(^{2+}\) affinity at acidic pH, whereas they had no effect at neutral or alkaline pH, except for AMPPNP in the presence of Mg\(^{2+}\) (Figs. 5–7), (iv) the affinities of ATP, AMPPCP, and AMPPNP for Ca\(^{2+}\)-deprived ATPase were not sensitive to pH, at variance with that of ADP (Fig. 8).

A few papers have reported the existence of a pH-dependent equilibrium between Ca\(^{2+}\)-deprived forms of ATPase and suggest that this explains the inhibition of Ca\(^{2+}\) binding by protons at equilibrium (22, 32–34). The Ca\(^{2+}\) binding kinetics are also pH-dependent, and this is illustrated by Scheme 2, where the Ca\(^{2+}\)-deprived ATPase exists under different protonated forms. At pH 6, where Ca\(^{2+}\) binding is slow, this model predicts that all ATPase is in the EH₂ form. At pH 8, where Ca\(^{2+}\) binding is fast, half the ATPase is in the EH form and the other in the E form, and, at pH 9, where Ca\(^{2+}\) binding is fast, all ATPase is in the E form. Thus, the deprotonation steps are thought to be rate-limiting, probably because they induce some slow conformational changes which were not explicitly described in the model (22, 23).

Because at alkaline pH, Ca\(^{2+}\) binding is fast and has a high affinity, the Ca\(^{2+}\) binding acceleration effect of nucleotides at acidic pH could be explained by a nucleotide-induced deprotonation of the Ca\(^{2+}\)-deprived ATPase. That is, if at pH 6 and in presence of a nucleotide the predominant form was E instead of EH₂ in Scheme 2, this would increase both the Ca\(^{2+}\) binding rate and affinity.

Nucleotides Do Not Change the Protonation pK of Ca\(^{2+}\)-deprived ATPase—The affinity of the nucleotides for Ca\(^{2+}\)-deprived ATPase was measured, and no pH dependence could be detected between pH 6 and 8, neither for ATP nor for AMPPNP or AMPPCP (Fig. 8). Thus, none of these nucleotides can induce a deprotonation of the Ca\(^{2+}\)-deprived ATPase.

As reported above, we observed that ADP also accelerates Ca\(^{2+}\) binding, but at variance with the triphosphate analogues, ADP displayed a higher affinity for Ca\(^{2+}\)-deprived ATPase at acidic pH.
pH 8 than at pH 6 (Fig. 8), and this could possibly deprotonate ATPase at pH 6. This assumption was checked using the previously measured pH values of Ca²⁺-deprived ATPase (22). Given these, the ADP affinity should be much more sensitive to pH to induce a significant change in the distribution of the Ca²⁺-deprived ATPase. Thus, according to our model, even in the case of ADP, the changes induced in the affinity are too small to cause deprotonation. We can thus conclude that the pH-dependent equilibrium of Ca²⁺-deprived ATPase is not modified by the presence of ADP, ATP, AMPPNP, or AMPCP.

Nucleotides Increase the Rate of the pH-Dependent Limiting Steps of Ca²⁺ Binding—At pH 6 and without nucleotide, Ca²⁺ binding is slow, whereas with 0.1 mM nucleotide, it is fast (Figs. 1 and 2). At pH 8 and without nucleotide, Ca²⁺ binding is biphasic, whereas with 0.1 mM nucleotide, Ca²⁺ binding is monophasic and fast (Fig. 1). As the nucleotide does not induce deprotonation of the Ca²⁺-deprived ATPase, it is likely to accelerate the deprotonation steps EΗ₂−→EΗ and EΗ →Ε.

Scheme 2.

Scofano et al. (9), Guilain et al. (10), Fernandez-Belda et al. (12), and Stahl and J. encks (11) reported that ATP increases the Ca²⁺ affinity at pH 6. This assumption was checked using the pre-

Nucleotides Increase the Transport Sites Affinity at Acidic pH—Ca²⁺ binding at the transport sites is known to be inhibited by H⁺ (22, 35), and all the nucleotides tested here increased the Ca²⁺ affinity at acidic pH (Fig. 6). The affinity of all these nucleotides for the Ca²⁺-deprived ATPase were not sensitive to pH (Fig. 8), so that they all have similar affinities for EΗ₂−, EΗ, and E (Scheme 2). Ca²⁺ affinity was not modified by ATP analogues at pH 7, showing that at pH 7, the ATP analogue should have similar affinity for the Ca²⁺-deprived species (EΗ₂−, EΗ, and E) and for the Ca²⁺-bound species (EΗCa, EΚCa, EΚCa₂). This confirms what has been shown for ATP by Lacapère and Guilain (36). Thus, to explain the increase in the Ca²⁺ affinity at acidic pH, we have to assume either that there is a different substrate appearing at acidic pH such as a proton-nucleotide complex which would bind EΚCa₂ with a higher affinity than the free nucleotide, or that there is an additional species such as EΗCa₂ with a higher affinity for the nucleotide than the other forms of ATPase (Scheme 3).

The first assumption relies on the fact that ATP has a pK of 6.5 and ADP of 6.4, so that the HATP or HADP complexes appear in the right pH range. For the ATP analogues, the values of the pK are not known. Nevertheless, we measured the dissociation constants of the calcium-nucleotide complexes at various pH and compared them with that of the CaATP complex. From this comparison, it appears that AMPPCP has, as ATP, a dissociation constant sensitive to pH between 6 and 8. However, the CaAMPPNP dissociation constant was found almost insensitive to pH, so that nucleotide protonation should not be responsible for the increase in Ca²⁺ affinity.

The second assumption thus seems more likely. If there were a species such as EΗCa₂ (Scheme 3) with a pK of 6 and a higher affinity for the nucleotide than all the other species, it would almost certainly not contribute to Ca²⁺ binding in the pH 8 to 6 range in absence of a nucleotide. However, with a nucleotide and below pH 7, it would increase the affinity for Ca²⁺ and thus induce the increase in bound Ca²⁺ seen in Fig. 7.

Interactions between the Catalytic and Transport Sites—We have shown here that the Ca²⁺ binding acceleration induced by the nucleotides is eliminated by FITC labeling, which suggests that the acceleration is mediated by the catalytic site. The acceleration of Ca²⁺ binding was also found to be pH-dependent. Our results suggest an acceleration of the conformational changes which follow the deprotonation steps occurring during Ca²⁺ binding. As ATPase has been shown to exchange three protons for two calcium ions (37, 38), these protons are thought to bind to the transport sites, and their dissociation rate constants are sensitive to the presence of a nucleotide at the catalytic site. There is a clear indication of an interaction between the catalytic and transport sites, whether they are occupied by protons or calcium.

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