Effect of Magnesium on the Calcium-dependent Transient Kinetics of Sarcoplasmic Reticulum ATPase, Studied by Stopped Flow Fluorescence and Phosphorylation*

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At pH 7 and 20 °C and in the absence of potassium and magnesium, the intrinsic fluorescence rise after addition of calcium to a calcium-deprived enzyme was monoeponential. On the other hand, when the calcium-deprived enzyme was preincubated with magnesium, this fluorescence rise was clearly biphasic at high calcium concentrations. For a constant magnesium concentration (higher than millimolar), the rate constant of the slow phase and the amplitude of the fast phase rose for the same range of calcium concentrations, between pCa 5 and 4. At pH 6, fluorescence signals were monoeponential even with 20 mM Mg²⁺.

We also found that at pH 7, phosphorylation of the enzyme after simultaneous addition of calcium, magnesium, and ATP was faster when sarcoplasmic reticulum was originally calcium-deprived in the presence of magnesium. At pH 6, on the contrary, preincubation with magnesium did not influence the phosphorylation time course.

It is concluded that (a) magnesium produces an enzyme conformation which is able to react with calcium and ATP as required by the kinetics of the ATPase reaction; (b) the magnesium-enzyme complex presents one readily accessible site of relatively low affinity (Kₐ ≈ 25 μM) for calcium; (c) occupancy of this site by calcium triggers a relatively slow (4-6 s⁻¹) conformational change unmasking a second high affinity site; (d) interaction between and occupancy of these two calcium sites occur with positive cooperativity and yield enzyme activation; and (e) the kinetic difference between the low time constant of the second component of fluorescence rise following addition of calcium (in the absence of ATP) and the relatively fast phosphorylation obtained in the same conditions (but in the presence of ATP) is attributed to an Mg²⁺-dependent accelerating effect of ATP on enzyme isomerization.

Calcium binding to sarcoplasmic reticulum ATPase induces a major event in the catalytic center: the transition from a configuration in which this ATPase reacts with inorganic phosphate (*E form) to a configuration in which phosphorylation from ATP takes place (E form) (Masuda and de Meis, 1973; Champeil et al., 1976; Dupont, 1976; Murphy, 1976; Carvalho et al., 1976). This * E → E transition has been assigned a molecular significance of major importance for the calcium pump, i.e. reorientation of the calcium binding site(s) from the internal to the external side of the vesicles (for review, see de Meis and Viana, 1979). To our knowledge, there is no direct evidence for this attractive hypothesis, but previous kinetic data were in fact tentatively analyzed in reference to this hypothesis (Guillain et al., 1980, 1981; Dupont, 1982). Furthermore, biphasic progress curves were recently obtained for calcium binding, measured directly (Dupont, 1982) or indirectly (Ikemoto et al., 1981). In all cases, these phenomena have been related to a cooperative mechanism of calcium binding which was first demonstrated in equilibrium conditions (Inesi et al., 1980).

The kinetics of the Ca-induced events are not easy to measure. Information has generally been derived from the rates of appearance or disappearance of the various phosphoenzymes formed from either ATP or Pi, (see Guillain et al., 1981, Ikemoto et al., 1981, and references therein). Only recently was the rate of calcium binding itself directly measured by Millipore filtration, after slowing down the reaction by lowering the temperature below 0 °C in the presence of glycerol (Dupont, 1982). Meanwhile, the intrinsic fluorescence of SR vesicles has emerged as a reliable index of the * E → E transition, both under equilibrium conditions (Dupont, 1976; Guillain et al., 1980; Verjovski-Almeida and Silva, 1981; Silva and Verjovski-Almeida, 1982) and transient conditions (Dupont and Leigh, 1978; Guillain et al., 1981; Dupont, 1982).

Further investigations of these calcium-induced events should allow more detailed discussion of their molecular significance with respect to cooperative interactions of binding sites. We therefore extended our previous fluorescence measurements to include new experimental conditions and found that at pH 7 and in the absence of potassium, the kinetics of the calcium-induced transition at high calcium concentration could be either monoeponential or biphasic, depending on the magnesium concentration. We also measured by chemical quenching the time course of ATPase phosphorylation after simultaneous addition of ATP and calcium to an enzyme

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1 The abbreviations used are: SR, sarcoplasmic reticulum; EGTA, ethylene glycol bis(β-aminoethyl ether)N,N,N′,N′-tetraacetic acid; Mes, 2-(N-morpholino)ethanesulfonic acid; Mops, 4-morpholinopropanesulfonic acid; cutoff filter, long wavelength pass filter.
preincubated with or without magnesium, and thereby checked the evolution of the enzyme to a fully activated state following addition of the divalent cations.

**EXPERIMENTAL PROCEDURES**

Fragmented sarcoplasmic reticulum was prepared from rabbit skeletal muscle and tested as already described (Champell et al., 1978). Free Mg$^{2+}$ and Ca$^{2+}$ concentrations were calculated according to the following apparent equilibrium dissociation constants at pH 7: $K_{Ca} \times \text{free} = 5.94 \times 10^{-7}$ at $K_{Ca} \times \text{free} = 2.53 \times 10^{-5}$ (Guillain et al., 1980). Care was taken to adjust the EGTA and total calcium concentrations so that the traces of Ca always present in water, buffer, membrane suspension, and Mg stock solutions did not result in an excessive error in the final pCa. Unless otherwise specified, the reaction temperature was 30 °C and the medium contained 2 mM EGTA and 150 mM Mes-Tris (pH 6) or Mops-Tris (pH 7) buffer.$^5$ General procedures for both equilibrium (Perkin-Elmer MFP44A) and kinetic (Durrum-D-110) fluorescence measurements were as previously described (Guillain et al., 1980, 1982). The excitation wavelength was 290 nm.

We used two filters to detect fluorescence in the stopped flow measurements: either a Schott WG 320 cutoff filter with half-maximum transmission T at 324 nm and less than 0.001% T below 300 nm, purchased from MTO, Massy, France, referred to here as "MTO324," or a Balzer interference filter with peak transmittance at 332 nm, referred to as "B330." It must be realized that (i) the incident light is never totally monochromatic and (ii) scattered light at 290 nm gives rise to a significant fluorescence from the Schott filter itself. Use of the cutoff filter must therefore be restricted to situations where the scattered light is not expected to vary. The change in light scattering by the vesicles in a given stopped flow experiment can in fact be checked simply by changing incident light wavelength from 290 nm to any new wavelength that passes through the filter, e.g. 330 nm.

Scattering artifacts are minimized when a narrow interference filter is used instead of a cutoff filter to analyze emitted light; for example, Fig. 2 shows a stopped flow experiment involving a large osmotic shock inducing a transient shrinking of the vesicles. The upper trace shows a fast 10% rise in light scattering (≈30 s$^{-1}$) followed by a much slower decrease (Guillain et al., 1982; Kometami and Kasai, 1978). The lower trace shows the fluorescence rise, which is virtually free of any fast process. Therefore, with the interference filter, fluorescence changes can accurately be measured, even under conditions where changes in light scattering do occur. With this filter, however, the signal to noise ratio is poorer, and a larger number of individual traces have to be accumulated.

Phosphorylation from [γ-32P]ATP was measured with a Dionex (Oquebec) 110 preparing apparatus. The reaction was quenched with 7% trichloroacetic acid, 0.2 mM P. Denatured protein was repeatedly washed by centrifugation before solubilization, protein determination, and liquid scintillation counting. SR vesicles for those measurements were prepared as described by Eletr and Inesi, 1972. The method for phosphoenzyme determination was previously described (Inesi et al., 1980).

**RESULTS**

**Kinetics of Ca$^{2+}$-induced Fluorescence Enhancement**

**Magnesium-dependence**—We previously reported that at pH 6, the fluorescence rise which can be observed after addition of calcium to Ca$^{2+}$-depleted SR vesicles can be fitted to a single exponential irrespective of the magnesium concentration (Guillain et al., 1981). At pH 6, the fluorescence spectrum of the calcium-depleted enzyme is insensitive to magnesium; at pH 7, on the other hand, the fluorescence spectrum of the calcium-depleted enzyme is sensitive to magnesium (Guillain et al., 1982). I.e. both calcium and magnesium produce a blue shift, but only calcium increases the fluorescence intensity. We then investigated the kinetics of the Ca$^{2+}$-$^5$ This high buffer concentration was chosen in previous studies because small changes in pH were believed to induce fluorescence changes (Dupont, 1976, 1982; Guillain et al., 1980). This was, however, an incorrect belief, probably due to the fact that Tris buffer was originally neutralized with UV-absorbing maleate.

![Fig. 1. Stopped flow recordings after addition of a high concentration of magnesium to calcium-deprived SR. These traces are shown to exemplify the fact that under selected conditions, fluorescence measurements can be considered free of light scattering. Excitation wavelength was 290 nm for the fluorescence measurement (lower trace) or 330 nm for the light scattering measurement (upper trace). Emitted light was analyzed through an interference filter (B330, see "Experimental Procedures"). Enzyme syringe contained 150 mM Mops, 2 mM EGTA, 100 μg/ml of SR protein. Substrate syringe contained 50 mM Mops, 2 mM EGTA, 200 mM MgCl. ΔF/F, relative fluorescence change.](http://www.jbc.org/)
that preincubation of the enzyme with magnesium, and not merely a high final Mg concentration, was a prerequisite to obtain a biphasic pattern. In Fig. 4, the upper trace shows the biphasic fluorescence rise observed with 20 mM magnesium present from the beginning in both the enzyme and calcium syringes (same trace as Fig. 2, upper trace). The lower trace shows that when calcium and magnesium were added together to the calcium- and magnesium-deprived enzyme, fluorescence enhancement at pCa 3 was no longer biphasic. Similar results were obtained for different magnesium concentrations (not shown). Unexpectedly, however, the single exponential obtained when magnesium was added together with calcium was slightly faster than the slow phase of the biphasic behavior observed with magnesium in both syringes.

Free Calcium Dependence—The preceding experiments were all performed at a final calcium concentration of 1 mM. We now present data obtained at lower calcium concentrations. These experiments were conducted with the cutoff filter, and Fig. 5 shows the now well established pCa-dependence of SR intrinsic fluorescence, as detected with this cutoff filter, in the absence (a) or presence (b) of 20 mM magnesium under our experimental conditions. The curves in Fig. 5 show a shift to higher calcium concentrations in the presence of magnesium (compare triangles and circles), as described in Guillain et al., 1982. Although there is a slight discrepancy between OFF and ON data (open and closed symbols), all curves are relatively steep, even in the presence of magnesium, suggesting here again positive cooperativity for calcium binding (Inesi et al., 1980; Guillain et al., 1981, 1982; Verjovski-Almeida and

3 Highsmith (1982) reported that a 1-h incubation of SR vesicles in an EGTA solution resulted in a much larger discrepancy between ON and OFF data together with the disappearance of the ON curve cooperativity. Since our ON curves are cooperative and since we always took care to keep vesicles in EGTA for a few minutes only, we believe that technical errors, and not denaturation, are likely to be responsible for the small discrepancy we observed. Some artifacts due to different concentrations of the (Ca-EGTA) complex might also be involved (Andersen & Møller, 1977; Berman, 1982).
**Ca^{2+}-dependent Transient Kinetics of SR-ATPase**

Medium contained 40–50 μg of SR protein/ml and 150 mM Mops-Tris at pH 7 (20 °C), with either no added magnesium (a) or 20 mM total magnesium (b). Closed symbols, right scale: various amounts of calcium were added to an enzyme initially suspended in 2 mM EGTA (ON data, ΔF/F > 0). Open symbols, left scale: various amounts of EGTA were added to an enzyme initially suspended in 100 or 5 μM calcium (OFF data, ΔF/F < 0). Excitation wavelength = 290 nm. Emitted light was analyzed through a cutoff MT0324 filter, with the spectrophotometer emission grating set at the zero order.

Silva, 1981; Murphy et al., 1982; Andersen et al., 1982) irrespective of the presence or absence of magnesium.

We have then designed experiments to measure the kinetics of enzyme preincubated with or without magnesium.

Calcium-induced Kinetics: Slow Calcium-independent Isomerization?—We would like first to recall briefly some of the results we previously reported (Guillain et al., 1982). In the absence of calcium, the interaction of SR-ATPase with magnesium induced a pH-dependent blue shift in intrinsic fluorescence emission. Equilibrium titrations with calcium in the presence or absence of magnesium suggested that the

**Enzyme Phosphorylation following Addition of ATP**

The expression of SR-ATPase activation following addition of calcium is its ability to be phosphorylated by ATP (Ya-mamoto and Tonomura, 1987; Makinose, 1989). It is by now established that the time course of phosphoenzyme formation is significantly faster when ATP is added to enzyme preincubated with calcium than when ATP and calcium are added to enzyme preincubated in the absence of calcium (Sumida et al., 1978; Scofano et al., 1979; Inesi et al., 1980). In the latter case, phosphorylation is delayed due to the *E → E* transition. We have then designed experiments to measure the kinetics of enzyme activation (as revealed by enzyme phosphorylation), using enzyme preincubated with or without magnesium.

This is shown in Fig. 7. At pH 7, phosphorylation was quite rapid (virtually completed in 100 ms) when ATP was added to enzyme preincubated with calcium (closed symbols), irrespective of whether magnesium was added to the enzyme previously to, or simultaneously with ATP; when calcium, magnesium, and ATP were added to enzyme preincubated in the absence of either divalent cation, the phosphorylation curve was slow (open circles). However, when ATP and calcium were added to enzyme preincubated with 20 mM magnesium, but no calcium, a significantly faster phosphorylation was obtained (open triangles). Maximal phosphorylation levels were reached with a halftime of approximately 45 ms.

At pH 6, phosphoenzyme formation was again much faster following addition of ATP to enzyme preincubated with calcium, than following addition of ATP and calcium to enzyme preincubated in the absence of calcium. In either case, however, no difference was noted when magnesium was added to the enzyme previous to or simultaneously with ATP (not shown). This is in agreement with measurements of intrinsic fluorescence showing no effect of magnesium at pH 6.

**DISCUSSION**

Calcium-induced Kinetics: Slow Calcium-independent Isomerization?—We would like first to recall briefly some of the results we previously reported (Guillain et al., 1982). In the absence of calcium, the interaction of SR-ATPase with magnesium induced a pH-dependent blue shift in intrinsic fluorescence emission. Equilibrium titrations with calcium in the presence or absence of magnesium suggested that the
magnesium ion responsible for the fluorescence shift was simply competing for one high affinity calcium binding site; kinetic studies of the magnesium-induced fluorescence changes were consistent with the hypothesis of de Meis and Viana (1979) that the calcium (and magnesium) binding site was not available in the so-called *E conformation of the pump (in the presence of EGTA), but became available after its isomerization into an "E" form (Scheme 1, similar to that of Martin and Tanford, 1981).

Scheme 1 at first appeared to be confirmed by the observation that the calcium-induced fluorescence changes in the absence of magnesium occurred with a rate constant (3-4 s⁻¹) consistent with the rate constants observed for the magnesium-induced fluorescence changes (Guillain et al., 1982). According to this scheme, the biphase behavior observed following addition of calcium in the presence of magnesium (Fig. 2) would then be explained as follows. In the absence of calcium, but in the presence of magnesium, part of the enzyme would be in the E-Mg form and would therefore readily bind calcium, thus giving rise to the fast phase, whereas the rest of the enzyme would still have to go through the slow *E → E transition. The fact that amplitudes but not rate constants depend on the magnesium concentration (Fig. 3) fits into this scheme. The fact that the calcium-induced transition recovered a monoeponential character when magnesium was added together with calcium (Fig. 4) also fits into this scheme.

**Calcium-induced Kinetics in the Presence of Magnesium: Slow Calcium-Triggered Isomerization?**—Three observations, however, cannot be reconciled with Scheme 1. (i) The comparable amplitudes of the two kinetic components in the presence of magnesium would predict an equilibrium constant (E + E-Mg)/E = 1, whereas interpretation of the observed cooperativity (Fig. 5) with Scheme 1 would require that the equilibrium is very much in favor of *E (Tanford and Martin, 1982). (ii) The amplitude of the slow phase was never reduced to zero (Fig. 3, c and d) even at very high Mg²⁺ concentrations likely to drive the *E → E equilibrium fully to the right. (iii) The rate constant of the slow phase was an increasing function of the calcium concentration (Fig. 6a), such a behavior is generally observed when substrate binding takes place before the slow transition. In fact, Fig. 6 shows data strikingly similar to what were previously reported for calcium binding measurements at −10°C in the presence of potassium (Dupont, 1982), namely, that both the rate constant of the slow phase and the amplitude of the fast phase rose for the same range of calcium concentrations (10−100 μM). The simplest explanation of such a behavior is that initial (fast) binding to a relatively low affinity site (10−100 μM) is responsible for the activation of a slow conformational transition (4-6 s⁻¹ in Fig. 6) unmasking a second high affinity site (Scheme 2).

**Scheme 2**

This scheme implies that calcium sites are available on the *E conformation of the enzyme (Guillain et al., 1980; Inesi et al., 1980). The permanence of a diphasic pattern even at the highest Ca²⁺ concentrations suggests that the two interacting sites are kinetically nonidentical.

This apparent discrepancy with the original scheme of de Meis and Viana (1979) was tentatively solved by Dupont (1982), who assumed that the low apparent affinity for initial binding was accounted for by an unfavorable pre-existing fast equilibrium between two different conformations of one of the calcium sites. To allow discussion of this suggestion, more experiments are needed under a variety of conditions including Dupont's ones, i.e. in the presence of potassium. In any case, since vesicles preincubated in the presence of magnesium displayed both a fast and a slow phase, one site for low affinity calcium binding must be easily accessible on the outer surface under these conditions. Conversely, from the absence of any fast process in the absence of magnesium, it can be suggested that under those conditions binding sites are initially absent and that their appearance is rate-limiting. It is therefore shown here that preincubation with magnesium drives the ATPase towards a conformation capable of fast calcium binding. The following will suggest that this E-Mg species is the functional enzyme and must be regarded as expressing features required for catalytic and transport activity.

**Enzyme Phosphorylation with ATP—**Phosphorylation following addition of calcium and ATP was accelerated by preincubation of the calcium-deprived enzyme with magnesium at pH 7 (Fig. 7), but not at pH 6. This is consistent with the sensitivity of the SR fluorescence rise to magnesium at pH 7 but not at pH 6. Therefore, a good correlation can be demonstrated between calcium binding phenomena as revealed by fluorescence changes, and enzyme activation as revealed by its ability to be phosphorylated by ATP.

Detailed interpretation of phosphorylation kinetics (obtained in the presence of ATP) with regard to transitions produced by calcium binding (in the absence of ATP) is not, however, straightforward. While addition of calcium to enzyme preincubated in the absence of calcium and in the

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4 Note that the fluorescence enhancement at −10°C also deviated from exponential behavior, although technical problems precluded detailed analysis of the first stage of the reaction (Dupont, 1982).

5 With regard to the effect of potassium, in our early fluorescence investigations, fluorescence changes were in fact measured at pH 7 with 100 mM KCl (Guillain et al., 1980). However, in that work, signal-to-noise ratios were not satisfactory, and possible biphase patterns were deliberately ignored. These experiments are now being resumed in our lab.
presence of 20 mM magnesium at pH 7 produces a bi-exponential rise in fluorescence (Fig. 2), the phosphorylation curve in similar conditions is not resolved in two exponentials, and maximal phosphorylation levels are reached with a half-time of approximately 45 ms (20 °C). This time frame is undoubtedly faster than the slow component of the fluorescence rise (half-time ≈ 170 ms). In this regard, it was already pointed out (Inesi et al., 1980) that the slow rate of calcium-induced fluorescence rise in the absence of ATP was incompatible with the kinetic requirements of rapid enzyme activation by calcium for ATPase phosphorylation with ATP, and it has already been suggested that calcium-induced transitions (and enzyme activation) are accelerated by ATP (Scofano et al., 1979). With regard to this suggestion, a novel finding of our present experimentation is, therefore, that the acceleration by ATP of protein isomerization is fully expressed only if the enzyme has been preincubated with magnesium. Preincubation of calcium-deprived ATPase with magnesium would be a prerequisite for acquisition of an enzyme state most adequate for calcium and ATP binding and activation with time constants consistent with the rapid turnover observed in transient and steady state conditions. Although it has to be further tested under various conditions (see, for instance, Froehlich, 1978), this suggests that the E-Mg enzyme is the species actually involved in the catalytic and transport cycle.

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