Time-Resolved Charge Movements in the Sarcoplasmatic Reticulum Ca-ATPase

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ABSTRACT The time-resolved kinetics of the Ca^{2+}-translocating partial reaction of the sarcoplasmatic reticulum Ca-ATPase was investigated by ATP-concentration jump experiments. ATP was released by an ultraviolet light flash from its inactive precursor and charge movements in the membrane domain of the ion pumps were detected by the fluorescent styryl dye 2BITC. Two oppositely directed cation movements were found, which were assigned to Ca^{2+} release and H^+ binding. The faster process with a typical time constant of 30 ms reports the rate-limiting process before Ca^{2+} release, probably the conformation transition $E_1 \rightarrow E_2$. The following, slow uptake of positive charge had a pH-dependent time constant, which was 1 s at low pH and ~3 s at pH > 8. This process is assigned to an electrically silent conformational relaxation of the state P-E_2 preceding H^+ binding. This interpretation is in agreement with the observation that the fast process was independent of the substrate concentrations (i.e., when $[Ca^{2+}] > 200$ nM, and [ATP] > 20 μM). The slow process was independent of the Ca^{2+} concentration. The activation energy of the resolved processes was between 80 kJ/mol and 90 kJ/mol, which is comparable to the activation energy of the enzymatic activity (92 kJ/mol) and these high values point to conformational changes underlying rate-limiting steps of the pump cycle.

INTRODUCTION

The purpose of the Ca-ATPase of the sarcoplasmatic reticulum (SR) Ca-ATPase is to promote muscle relaxation by pumping Ca^{2+} ions back into the SR until a cytoplasmic concentration of 100 nM is maintained, representing the resting state of the muscle fibers. Because the relaxation processes occur in 50 ms (or faster), a high transport capacity is required. The Ca^{2+} flux through the pump, $\Phi_{Ca} = 2 \times n \times \nu_p$, is controlled by the number of Ca-ATPases, n, and by the turnover rate, ν_p. The factor of 2 comes from the stoichiometry of the SR Ca-ATPase, which is 2 Ca^{2+}/2 H^+/1 ATP so that 2 Ca^{2+} ions are transported per turnover into the lumen of the SR (Yu et al., 1993; de Meis, 1985; Yu and Inesi, 1995). To provide a large enough Ca^{2+} flux for the fast relaxation process two different ''strategies'' are possible: either the number of ion pumps, n, or the turnover rate, ν_p, or both must be high. Analysis of the SR membranes showed that >70% of the membrane proteins are Ca-ATPases. They reach a density of ~30,000 μm^{-2} (Franzini-Armstrong and Ferguson, 1985), a density much higher than that found for other P-type ATPases, such as the Na,K-ATPase in the plasma membrane or the H,K-ATPase in parietal cells.

Based on enzyme activity and on the known molar mass of the protein the turnover rate of the Ca-ATPase can be calculated. Turnover rates were found to be 10 s^{-1} at 25°C (Inesi et al., 1982) or between 6 s^{-1} and 36 s^{-1} at 37°C for the different preparations used in this work. With these numbers in mind it has to be proposed that the rapid Ca^{2+} uptake is caused by the high number of pumps rather than by their speed.

To optimize the energetics not only the counter transport of H^+ helps but also the high leak conductance of the SR membranes for monovalent ions (Hasselbach and Oetliker, 1983). Such a leak conductance abolishes any electric membrane potential across the SR membrane. This property is energetically required to enable the generation of the observed high chemical-potential gradient for Ca^{2+} across the SR membrane, $\Delta\mu_{Ca}$, with the Gibbs free energy provided by ATP hydrolysis, $-\Delta G_{ATP} \geq \Delta\mu_{Ca}$ (Walz and Caplan, 1988). On the other hand, a consequence of the high leak conductance is that the SR Ca-ATPase is not accessible to direct electrophysiological investigations.

An alternative, successful method to study electrophysiological properties became available by the use of fluorescent styryl dyes that allow the detection of charge movements within the membrane domain of ion pumps, including the SR Ca-ATPase (Butscher et al., 1999; Pedersen et al., 2001; Peinelt and Apell, 2002). By these investigations it was found that all ion-binding and release steps in the pump cycle are electrogenic, i.e., the ions move through the membrane dielectric. This is in agreement with a position of the ion-binding sites deep inside the membrane domain as confirmed by the crystal structure of the SR Ca-ATPase recently resolved with atomic resolution (Toyoshima et al., 2000; Toyoshima and Nomura, 2002). When the enzyme conformations between the state Ca_2E_1 and the Tharpsigargin-stabilized E_2 state are compared, significant rearrangements of α-helices in the membrane domain can be detected. Such a movement will most probably occur when the Ca^{2+} ions are leaving their sites inside the pump or immediately after. Diffusion-controlled ion movements would occur in a submicrosecond range and hardly could be analyzed in

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a time-resolved manner, but the conformational relaxations between Ca^{2+} release and the subsequent H^+ binding are expected to happen in a significantly slower time window and, therefore, may be detectable.

When SR membrane preparations were kept in buffer containing 4.5 μM Ca^{2+} at pH 7.2 and in the absence of ATP, the Ca-ATPase is stabilized preferentially in the state Ca_2E_1 (Peinelt and Apell, 2002). In this buffer condition, SR membranes were equilibrated with 200 nM styryl dye 2BITC and a constant fluorescence level is maintained. Addition of ATP produces a biphasic fluorescence change (Fig. 1 *B*). According to the mechanism of the styryl dyes (Pedersen et al., 2001) the fluorescence increase reflects a release of positive charges from the membrane part of the ion pumps. The rising phase in Fig. 1 *B* cannot be resolved in this experiment; it was controlled by the stirring process after 5 μl ATP were added to 2-ml buffer in the fluorescence cuvette. The subsequent fluorescence decrease, however, is significantly slower and could be resolved well. Typically, the time course can be fitted with two time constants that are pH dependent and are in the order of 1 s and <10 s at pH 7.4 and Ca^{2+} concentrations below 1 mM. The fluorescence decrease is caused by the uptake of positive ions into the membrane domain of the proteins.

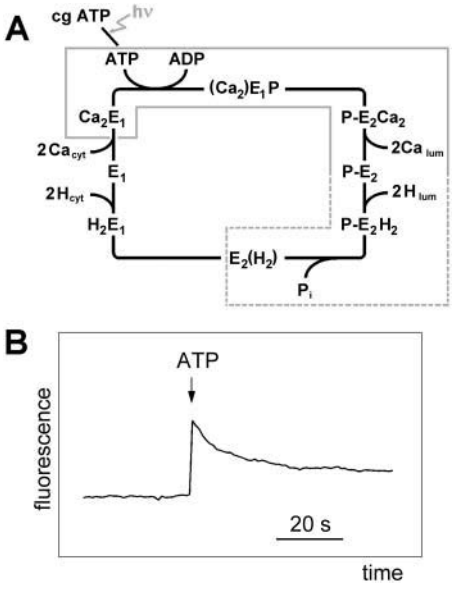

FIGURE 1 Overview of the ATP-concentration-jump-induced partial reaction of the SR Ca-ATPase. (*A*) Post-Albers scheme of the pump cycle under physiological conditions. In the presence of a few μM Ca^{2+} most of the enzyme is stabilized in its Ca_2E_1 state. Upon addition of ATP phosphorylation of the enzyme, the conformation transition E_1 to E_2 and release of the two Ca^{2+} ions occurs because the binding affinity of the ion sites is reduced by orders of magnitude. Subsequent binding of H^+ ions (and continuation through the pump cycle) depends on the buffer pH. (*B*) Part of a low time-resolution fluorescence experiment with 2BITC shows the fluorescence response of addition of 1 mM ATP to enzyme in the presence of 4.5 μM free Ca^{2+} at a buffer pH of 8. According to the mechanism of the styryl dye 2BITC fluorescence increase represents the removal of positive charge from the interior of the membrane domain of the ion-pump protein.

The aim of this article is to study and analyze the time course of the fluorescence transients and to identify the underlying mechanism with respect to the partial reaction of the Post-Albers cycle that is induced by ATP-concentration jump experiments (Fig. 1 *A*).

MATERIALS AND METHODS

Materials

Phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase, NADH, the Ca^{2+} carrier A23187, and ATP (disodium salt, special quality) were from Boehringer (Mannheim, Germany). Caged ATP (P^3-1-(2-nitrophenyl)ethyl) ester of ATP, A1048) and the chelator BAPTA (1,2bis(2-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′ tetrasodium salt, B1214) were ordered from MoBiTec, Göttingen, Germany. Tharpsigargin was purchased from Sigma (Munich, Germany). The styryl dye 2BITC was a gift from Dr. H.-D. Martin, University of Düsseldorf, Düsseldorf, Germany (Pedersen et al., 2001). All other reagents were the highest grade commercially available.

Enzyme preparations and reconstitution

Ca-ATPase was prepared by a slight modification of the method of Heilmann et al. (1977) from psoas muscles of rabbits. The whole procedure was performed at temperatures below 4°C. The determination of the protein content of the membrane preparation was performed according to Markwell et al. (1978). The most active fractions of the final density gradient separation had a protein content of 2–3 mg/ml. The enzyme activity was determined by the linked pyruvate kinase/lactate dehydrogenase assay (Schwartz et al., 1971). Background enzyme activity of the isolated preparation was obtained by addition of 1 μM tharpsigargin that blocks the SR Ca-ATPase completely. The Ca-ATPase-specific activity was ~175 μM P_i/mg protein and h at 37°C and could be increased up to 310 μmol P_i/mg protein and h in the presence of A23187 to short-circuit the vesicles forming membranes for Ca^{2+}. With a molar weight 110,000 g/mol and a specific activity of 5.2 units/mg the turnover rate of the pump is 9.5 s^{-1} in this preparation. In control experiments the effect of the styryl dye 2BITC on the enzymatic activity was checked. Up to dye concentrations of 1.2 μM no changes of the enzymatic activity could be observed.

Detection of partial reactions with 2BITC

Fluorescence measurements in ''low-speed'' experiments were performed with a self-constructed setup by using a HeNe laser with a wavelength of 543 nm (Laser 2000, Wessling, Germany) to excite the fluorescence of the dye 2BITC. The emitted light was collected perpendicularly to the incident light, filtered by a narrow-band interference filter (λ_{max} = 589 nm, halfwidth 10.6 nm) and detected by a head-on photo multiplier (R2066, Hamamatsu, Japan). The photo current was amplified by a Keithley current amplifier 427 (Keithley Instruments, Cleveland, OH) and collected by a data-acquisition board of a PC (PCI-T112, Imtec, Backnang, Germany) with sampling frequencies between 1 and 10 Hz, displayed on the monitor and analyzed on the computer. The temperature in the cuvette (2 ml) was maintained by a thermostat at 20°C.

For high-resolution recording of time-dependent fluorescence signals a setup was modified, whose design was published earlier (Stürmer et al., 1989). A cylindrical quartz cuvette (internal diameter, 7.8 mm) contained 300 μl buffer (layer height, ~5 mm) and was placed in the upper focus of an ellipsoidal mirror (Melles Griot, Zevenaar, Netherlands) whose opening was directed downward (Fig. 2). The buffer contained 600–650 nM 2BITC, 18 μg Ca-ATPase preparation, 100 μM caged ATP, and defined concentrations of $CaCl_2$; pH was adjusted by HCl. The fluorescence of the dye was excited by a 543-nm HeNe laser from the top of the setup. A quartz lens was

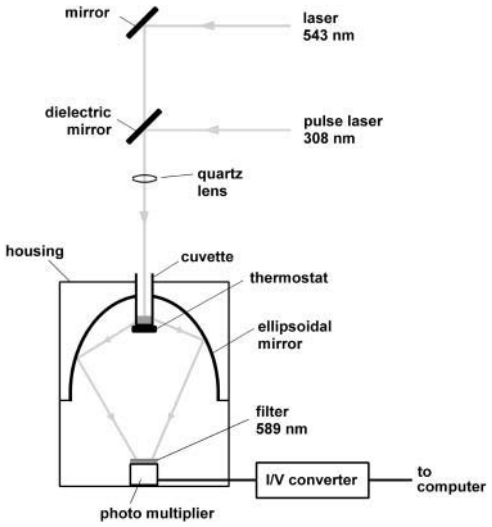

FIGURE 2 Schematic representation of the experimental setup for fluorescence experiments with high time resolution. The excitation light (543 nm) and the 10-ns UV-light flash from an excimer laser (308 nm) are directed into a cylindrical quartz cuvette mounted on a thermostated metal console. The light emitted by the sample is collected by an ellipsoidal mirror and focused onto the cathode of the photo multiplier. The light-emitting solution in the cuvette is located in the upper focal point of the mirror and the photocathode in the lower. The photomultiplier signal is amplified, digitized by an analog-to-digital converter board and processed in a computer.

adjusted to illuminate the whole solution almost homogeneously. The light emitted by the dye in the solution was collected by the ellipsoidal mirror and focused into the second focus of the mirror. At this point was placed the entrance window of a photo multiplier (PM, R928, Hamamatsu Photonics, Hamamatsu, Japan) and an interference light filter (589 ± 10 nm) to select the light of the specific emission of the styryl dye. The PM-output current was amplified by an I/V converter and fed into a 12-bit data-acquisition board of a PC with sampling frequencies between 1 and 500 kHz. The bottom of the cuvette was in contact with a thermostated copper socket (that also stopped the incident light). To release ATP from caged ATP a light flash (wavelength, 308 nm; total energy, 150 mJ; duration, 10 ns) was generated by an EMG 100 excimer laser (Lambda Physics, Göttingen, Germany) and directed into the cuvette. Typically 15–25 μM ATP were released by single flash (Stürmer et al., 1989).

RESULTS

The ATP-induced partial reaction, $Ca_2E_1 \rightarrow (Ca_2)E_1\text{-}P \rightarrow P\text{-}E_2Ca_2 \rightarrow P\text{-}E_2 (\rightarrow P\text{-}E_2H_2)$ contains three principally different types of reaction steps: enzyme phosphorylation, conformation transition and ion release or binding steps. The continuation after the release of the two Ca^{2+} ions depends on buffer pH. Each of the reaction steps may contain charge movements in the membrane and, therefore, may contribute to the time course of the detected fluorescence change. The kinetics of these steps will be investigated in the following.

Effect of ATP concentration jumps on the fluorescence signal

ATP concentration-jump experiments were performed in buffer containing 25 mM tricine, 50 mM KCl, 1 mM $MgCl_2$, 650 nM styryl dye 2BITC, 18 μg SR Ca-ATPase, pH 7.2. The (free) Ca^{2+} concentration in the buffer was determined to be 14 μM in the absence of a chelator. To remove traces of free ATP from the stock solution of caged ATP (10 mM), a water-soluble ATPase, apyrase, (1.4 units/ml) and 1.4 mM $MgCl_2$ were added. With a 10-ns ultraviolet (UV) flash of a wavelength of 308 nm ~20% of the caged ATP present was released. The ATP-release kinetics is pH dependent (Borlinghaus et al., 1988; McCray and Trentham, 1989; Stürmer et al., 1989) and this property has to be taken into account (see below).

In Fig. 3, the first 800 ms of fluorescence signals induced by release of ATP are shown at three different concentrations of caged ATP. The time course of the fluorescence change could be fitted by the sum of two exponentials,

$$F(t) = F_1 \times \exp(-t/\tau_1) + F_2 \times \exp(-t/\tau_2) + F_\infty. \quad (1)$$

At the lowest concentration (10 μM caged ATP) ~2 μM ATP were released, and the rising phase, controlled by a time constant, $\tau_1 = 96 \pm 2$ ms, was significantly slower than in the presence of 100 μM or 500 μM caged ATP ($\tau_1 = 30 \pm 3$ ms). The slower increase was attributed to rate-limiting ATP binding, $Ca_2E_1 + ATP \rightarrow Ca_2E_1{\cdot}ATP$, due to subsaturating concentrations of the released ATP. At 100 μM caged ATP ~20 (±5) μM ATP were released, which is a saturating concentration. The time constant of the falling phase of the fluorescence signals was the same for all three concentrations ($\tau_2 = 1000 \pm 50$ ms). The only significant difference between experiments performed at 100 μM and 500 μM caged ATP was a smaller maximum of the fluorescence

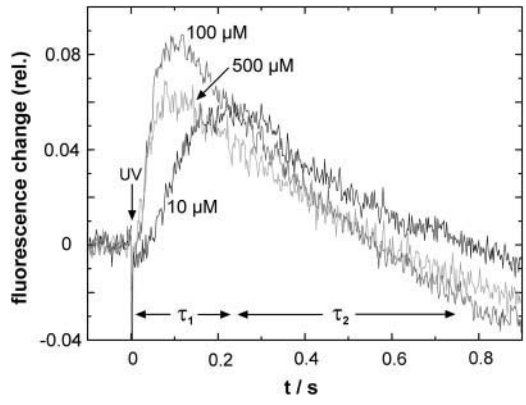

FIGURE 3 Effect of the caged ATP concentration on the 2BITC fluorescence signal upon an ATP concentration jump induced by a 10-ns UV flash at time zero. The concentrations of caged ATP were 10 μM, 100 μM, and 500 μM. A single UV flash had a photochemical yield of 15–25%. The time course of the fluorescence could be fitted by Eq. 1, and the rising phase of the signal was controlled by time constant τ_1, the falling phase by τ_2. (The arrows indicate the range of both phases for trace labeled "10 μM"). The fluorescence intensity of new stationary state at the end of the relaxation process corresponds to the parameter F_∞ in Eq. 1. The maximum fluorescence change was observed at 100 μM ATP. The time constants τ_1 and τ_2 were the same at 100 μM and 500 μM ATP.

transient at 500 μM. To test whether the reduced amplitude was caused by an inhibitory effect of the caged compound that remained after the UV flash (~80%), experiments were performed with various concentrations of caged ATP and by reduction of the UV-light intensity using neutral gray filters in the light path of the laser flash to vary the amount of ATP released. When 500 μM caged ATP were used and the light intensity was reduced to 20%, the amount of ATP released was the same as when 100 μM caged ATP and full light were applied. In the two cases the detected fluorescence transient were the same (not shown). This result indicates that the presence of the residual caged ATP did not significantly affect the observed time dependence in this type of experiments. In the presence of 100 μM caged ATP the UV-light intensity was gradually reduced by gray filters. In these experiments an increase of τ_1 and a decrease of the maximum fluorescence amplitude was found when the light intensity of the UV flash in the cuvette was below 50%. This observation indicates that the concentration of ATP released ($<$10 μM) became limiting (data not shown). In these experiments τ_2 was not significantly affected, similar to the observations presented in the experiments of Fig. 3. These results can be described by a reaction sequence in which the rate-limiting process, which governs the time course of the fluorescence decrease, is independent of ATP, and in the reaction sequence downstream to ATP binding.

In a next series of ATP-concentration jump experiments buffer pH was varied between pH 6.2 and 8.7 (Fig. 4 *A*). The buffer contained 25 mM tricine, 50 mM KCl, 1 mM $MgCl_2$, 650 nM styryl dye 2BITC, 18 μg SR Ca-ATPase; pH was adjusted by addition of aliquots of HCl or KOH and measured by a pH electrode. The (free) Ca^{2+} concentration in the aqueous solution was determined to be 14 μM. When the time course of the fluorescence signals was analyzed according to Eq. 1, it was found that the fluorescence amplitude, F_1, of the fast process, which is controlled by τ_1, was practically pH independent (Fig. 4 *B*, *open squares*), despite the fact that the kinetics of the process (i.e., τ_1) was affected by the ATP-release reaction, as will be shown below. In contrast, the steady-state fluorescence intensity, F_∞, at the end of the relaxation process, which is independent of the kinetics of ATP-release reaction, was pH dependent. At a pH $>$ 8 the fluorescence level increased (Fig. 4 *B*, *solid circles*). This observation may be explained by a reduced H^+ binding to the ion-binding sites of the Ca-ATPase (see discussion).

Analysis of the rising phase of the fluorescence signal

To analyze the mechanism that produces the time course of the fluorescence changes after an ATP-concentration jump in experiments as shown in Figs. 3 and 4, the signals were fitted by Eq. 1. The time constants of the rising and falling phase of the fluorescence signal, τ_1 and τ_2, were determined for various Ca^{2+} concentrations and buffer pH.

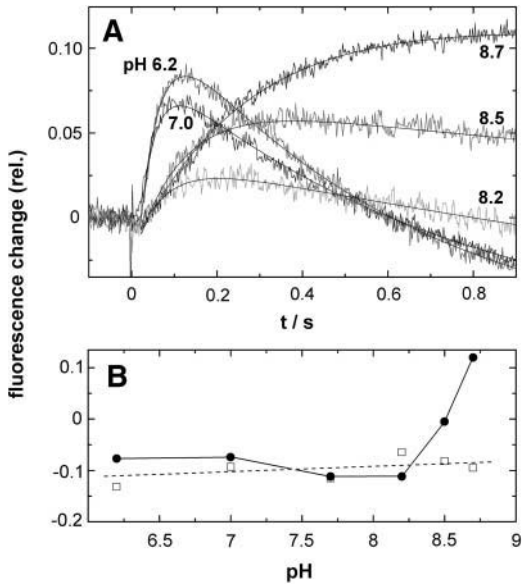

FIGURE 4 Effect of buffer pH on the shape of the fluorescence changes upon a caged-ATP-induced ATP concentration jump. The buffer contained 25 mM tricine, 14 μM free Ca^{2+}, 50 mM KCl, 1 mM $MgCl_2$, 650 nM styryl dye 2BITC, 18 μg SR Ca-ATPase, and 100 μM caged ATP. Buffer pH was adjusted by addition of HCl and was set to the indicated values. (*A*) When fitted with Eq. 1 it was found that some of the parameters were affected by pH. Because the photochemical reaction of ATP release is also pH dependent, the ATP dependence of τ_1 has to be discussed separately. However, the steady-state fluorescence, F_∞, is independent of the kinetics of the ATP-release reaction. (*B*) At pH $>$ 8 the steady-state fluorescence, F_∞, increased (●), indicating a lower occupancy of the ion-binding sites at the end of the relaxation process, whereas the amplitude, F_1, of the faster rising phase showed no significant pH dependence over the whole pH range covered by the experiments (□).

An increase of the 2BITC fluorescence represents a decrease of positive electric charge within the membrane domain of the ion pump (Pedersen et al., 2001). When compared with the reaction scheme in Fig. 1 *A* the partial reaction triggered by an ATP-concentration jump includes: 1), ATP binding, 2), enzyme phosphorylation and ion occlusion, 3), the conformation transition E_1 to E_2, and 4), release of the two Ca^{2+} ions to the luminal aqueous phase. According to the known electrogenicity of this reaction sequence only the last step is accompanied by a charge movement, the release of two Ca^{2+} ions from the membrane part of the protein (Butscher et al., 1999; Peinelt and Apell, 2002). However, depending on rate constants of the preceding reaction steps, in principle, each of them could be rate limiting and, therefore, control the observed time constant, τ_1. The first step, ATP release and binding can be excluded in the pH range below 7.5, because the concentration of ATP released was chosen to be not limiting according to Fig. 3. To test the other substrate-dependent step, Ca^{2+} release to the luminal side, a series of experiments with varying ion compositions was performed.

Fig. 5 shows the dependence of τ_1 on the concentration of Ca^{2+} and H^+, ions that both bind to the ion pump and are

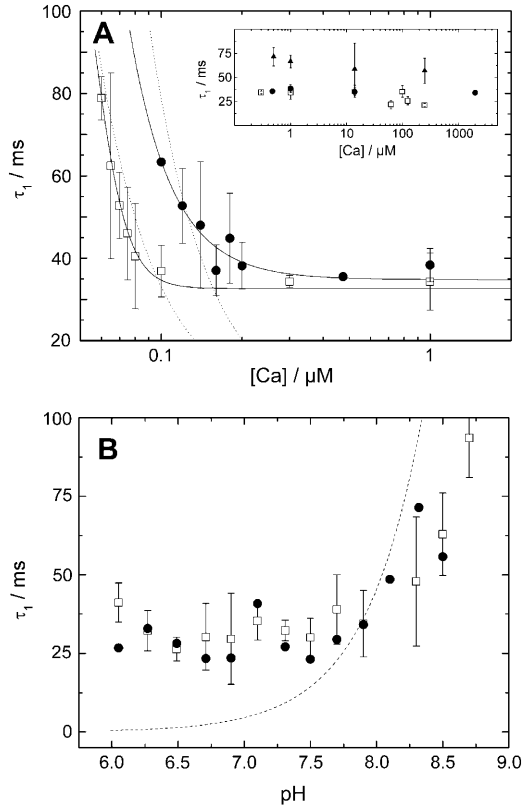

FIGURE 5 Dependence of the characteristic rising time, τ_1, as function of substrate ions. (*A*) Ca^{2+}-concentration dependence between 50 nM and 2 mM at buffer pH of 6.05 (●), 7.1 (□), and 8.2 (▲). The solid lines were drawn to guide the eyes. Both dotted lines show an estimate of τ_1 under the assumption that binding of Ca^{2+} ions would only be the rate-limiting process at low concentrations. (*B*) pH dependence of τ_1 at 14 μM Ca^{2+} (●) and at 100 μM (□). The dashed line represents the published pH dependence of the time constant of the ATP-release reaction from its caged precursor. The data points are mean values of three to seven single measurements, the error bars are the standard error (the error bars of the 14 μM Ca^{2+} data were removed for the sake of clarity).

transported. The experiments were performed in buffer containing 25 mM tricine, 50 mM KCl, 1 mM $MgCl_2$, 400 μM BAPTA, 650 nM styryl dye 2BITC, 18 μg SR Ca-ATPase, and $CaCl_2$ in selected concentrations so that the free Ca^{2+} corresponded to the values shown in Fig. 5 *A*. Free Ca^{2+} concentrations were calculated by the program WinMAXC (Chris Patton, Stanford University, Pacific Grove, CA). pH was adjusted by addition of aliquots of HCl or KOH and measured by a pH electrode.

The Ca^{2+} concentration dependence of τ_1 was studied at pH 6.05, 7.1, and 8.1. The results are shown in Fig. 5 *A*. At pH 8.1 and Ca^{2+} concentrations below 100 nM the change of the fluorescence amplitude was so small that the time constants, τ_1 or τ_2, no longer could be determined. At Ca^{2+} concentrations >200 nM (up to 1 mM) the time constant was independent of the concentration: $\tau_1 = 30.7 \pm 2$ ms at pH 6.05 and 7.1, and $\tau_1 = 63.4 \pm 2$ ms at pH 8.1. Only at very low Ca^{2+} the values of τ_1 were affected because of an incomplete occupation of the ion-binding sites by Ca^{2+} ions. Under the assumption that binding of Ca^{2+} (to reach the saturated Ca_2E_1 state) would become the rate-limiting step at low Ca^{2+} concentrations, the two dotted lines are drawn as estimates of the increase of τ_1 with decreasing Ca^{2+} at pH 6.05 and 7.1 (Peinelt and Apell, 2002).

The pH dependence of τ_1 was measured at 14 μM and 100 μM free Ca^{2+} as shown in Fig. 5 *B*. Below a pH ~7.5 the time constant of the rising phase was independent of the H^+ concentration and Ca^{2+} concentration (32.1 ± 0.1 ms); at higher pH the values of τ_1 increased. However, this effect has to be assigned to the pH-dependent release kinetics of ATP from the caged compound, NPE-caged ATP (McCray et al., 1980). For comparison, the pH dependence of the published time constant of ATP release is included in Fig. 5 *B* as a dashed line.

From these findings it can be concluded that under nonlimiting substrate conditions the process that governs the observed fluorescence increase has to be assigned either to enzyme phosphorylation, $Ca_2E_1ATP \rightarrow Ca_2E_1$-P, to the conformation transition, Ca_2E_1-P $\rightarrow$ P-E_2Ca_2, or to the subsequent ion-release steps. At 20°C the rate-limiting reaction step has an averaged characteristic time constant of 31.4 ± 1 ms.

Analysis of the falling phase of the fluorescence signal

Fluorescence decrease of styryl dyes in the SR membranes is generated by import of positive charge into (or, principally, by export or negative charge out of) the apolar interior of the membrane. With respect to the partial reaction investigated, the only reaction step that includes such a charge movement is binding of H^+ ions, P-$E_2 \rightarrow$ P-E_2H_2 (Fig. 1 *A*).

To quantify the observed effects, the characteristic time constant, τ_2, of the fluorescence decrease was plotted as a function of the free Ca^{2+} concentration. The results are shown in Fig. 6 *A*, in which the Ca^{2+} dependence of τ_2 can be seen in the concentration range between 50 nM and 2 mM for experiments in buffer of pH 7.1. In this Ca^{2+} concentration range no significant concentration dependence was observed, and τ_2 was found to be 1.38 ± 0.08 s (pH 7.1). Comparable results were found also for a buffer pH of 6.05 (data not shown). For higher buffer pH > 7.8 the falling phase of the fluorescence had such a small amplitude that the determination of τ_2 became inaccurate (cf. Fig. 6 *B*). In contrast to τ_1, the time constant τ_2 varied with buffer pH. The results in the presence of 100 μM Ca^{2+} are shown in Fig. 6 *B*. Practically identical data were obtained in the presence of 14 μM Ca^{2+} (data not shown). From the pH dependence in Fig. 6 *B* it can be seen that at low and high pH two different processes are limiting. At pH < 7 the H^+ concentration is no longer limiting and the average value of τ_2 was 0.92 ± 0.12 s. At high pH another plateau of τ_2 seems to be reached at ~3.5 s, however, due to the very small amplitude

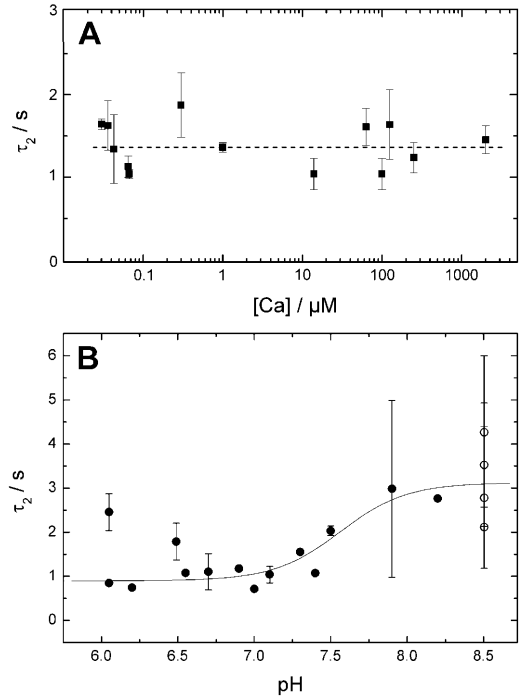

FIGURE 6 Dependence of the time constant of the fluorescence decrease, τ_2, as function of substrate ions. (*A*) Ca^{2+}-concentration dependence in buffer with pH of 7.1. The line through the data is a regression curve that reveals the absence of a concentration dependence. (*B*) pH dependence of τ_2 in the presence of 100 μM free Ca^{2+} ions. The solid data points are mean values of one to seven single measurements, the error bars are the standard error. The open points at pH 8.5 represent the results of fits to single experiments and the error bars are standard errors of the fits. The large errors are caused by the small amplitudes of the fluorescence change of the falling phase at this high pH (cf. Fig. 4 *A*). The solid line represents the pH dependence of the relaxation time of the reaction sequence, $P\text{-}E_2 \rightarrow P\text{-}E_2^* \leftrightarrow P\text{-}E_2^*H_2$, fitted to the data (see text).

of the falling phase the determination is not accurate. (The scattering of the experimental data at pH 8.5 and the standard error of the fits to the fluorescence signal is shown in Fig. 6 *B*).

Because in the ATP-concentration jump experiments the steps preceding the falling phase are fast ($\tau_1 \approx 30$ ms $\ll \tau_2 \approx$ 1 s) the characteristic time, τ_2, of the falling phase may be treated as relaxation time constant of an independent process. To account for the two different limiting cases a two-step model is proposed, which extends the Post-Albers scheme (Fig. 1 *A*) by one additional step (see Discussion),

$$P\text{-}E_2 \overset{k_2'}{\rightarrow} P\text{-}E_2^* \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} P\text{-}E_2^*H_2. \qquad (2)$$

The additional step is a pH-independent reaction, a conformation relaxation, preceding the binding of two H^+ ions. For the sake of simplicity the electrogenic binding of the protons was not resolved in two steps, because the data are not accurate enough. (To resolve the kinetics of H^+ binding we plan to perform pH-jump experiments with caged proton that hopefully will reveal more kinetical details.) Due to the low Ca^{2+} concentration back binding of Ca^{2+} can be neglected and, therefore, the conformation relaxation was treated as virtually irreversible reaction.

In the analysis of the average time constant at low pH one data point, $\tau_2 = 2.4$ s at pH 6.05, was not included, because such a high value at the low pH was found only in experiments with one of the enzyme preparations used in this study. Therefore, we considered it as not significant. When all other experimental data are fitted under the boundary conditions described above, k'_2 was determined to be 1.1 $\pm$ 0.1 s^{-1}, $k_2 = (6 \pm 0.3)\cdot 10^{14}$ M^{-2} s^{-1} and $k_{-2} = 0.42 \pm 0.2$ s^{-1} (Fig. 6 *B*, *solid line*).

Temperature dependence

Another approach to obtain information about the kinetics of the pump is to investigate the temperature dependence of a partial reaction. According to the empirical concept of Arrhenius the activation energy of a (chemical) process can be obtained from the equation

$$k = A \times \exp\left(\frac{-E_a}{RT}\right), \qquad (3)$$

and determined from the slope of a so-called Arrhenius plot (Fig. 7). Although we do not have a one-step reaction but a partial reaction with several steps, it is possible to discriminate two phases. The clearly separated, rate-limiting processes are represented by two time constants, τ_1 and τ_2, whose temperature dependence was analyzed separately. To relate a time constant τ to the rate constant k in Eq. 3 the reciprocal values were used, $k = 1/\tau$.

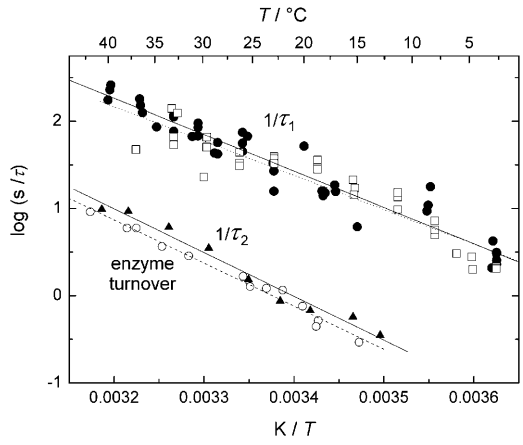

FIGURE 7 Temperature dependence of the ATP-induced partial reactions represented in the Arrhenius plot of the reciprocal time constant of the rising ($1/\tau_1$) and falling ($1/\tau_2$) phase of the fluorescence signal. The rising phase was analyzed in buffer of pH 7.2 (●) and pH 8.2 (□), which could be fitted by activation energies of 81.5 kJ/mol and 78.7 kJ/mol, respectively. The slower, falling phase was measured at pH 7.2 and fitted with an activation energy of 92.3 kJ/mol (▲). The enzyme activity was also investigated in the temperature range between 18°C and 42°C, and analyzed in terms of turnover rate (in s^{-1}). The data (○) could be fitted by an activation energy of 92.8 kJ/mol.

The activation energy of the process generating the time dependence of the fluorescence increase, controlled by τ_1, was determined for two buffer pH values without significant difference: E_a(pH 7.2) = 81.5 ± 3.6 kJ/mol (Q10 = 3.09) and E_a(pH 8.2) = 78.7 ± 5.3 kJ/mol (Q10 = 2.97) (Fig. 7).

The activation energy of the process controlled by τ_2 was determined at pH 7.2. At pH > 7.8 the amplitude of the fluorescence decrease was too small to obtain reliable data (or did not show a falling phase at all). This process has an activation energy E_A(pH 7.2) = 92.3 ± 6.3 kJ/mol (Q10 = 3.58) (Fig. 7, *triangles*), which was significantly higher than that of the activation energy of τ_1. When compared to the respective process in the Na,K-ATPase, P-E_2 + 2 K^+ → P-E_2K_2, the activation energy of this ion-binding step was only 22 kJ/mol (Bühler and Apell, 1995).

In addition, the temperature dependence of the enzyme activity was studied between 15°C and 42°C (Fig. 7), and an activation energy of 92.8 ± 4.0 kJ/mol (Q10 = 3.61) was determined. The enzyme activity is obtained from ion pumps running through the pump cycle, and thus the temperature dependence of turnover rate, v_p, provides the activation energy of the rate constant of the limiting step of the whole pump cycle. All temperature-dependent experiments were performed with the same enzyme preparation.

DISCUSSION

The purpose of the SR Ca-ATPase is to reduce efficiently a Ca^{2+} concentration of ~10 μM in the cytoplasm of muscle fibers during the contracted state to 0.1 μM within a time of ~50 ms to allow relaxation. If we assume in a conservative estimate that the SR pumps of an area of 1 μm^2 SR membrane have to drain a 1-μm-thick layer of cytoplasm above the membrane, then ~6×10^3 ions have to be removed from this volume when the initial (free) Ca^{2+} concentration is 10 μM and the final 0.1 μM. Because the pump density in the SR membrane is ~$3 \times 10^4/\mu m^2$ (Franzini-Armstrong and Ferguson, 1985) and two Ca^{2+} ions are bound to the SR Ca-ATPase, 6×10^4 ions can be transferred per turnover. The comparison of the two numbers of ions pumped shows that the resting-state Ca^{2+} concentration is obtained within one turnover of the pumps, or to be more precise: within the time that the Ca-ATPases need to bind two Ca^{2+} ions in their E_1 state, a process that may be expected to last less long than the 50 ms observed for muscle relaxation. Therefore, the turnover rate of the SR Ca-ATPase is not crucial to maintain fast muscle relaxation under physiological conditions.

Nevertheless, it is interesting to study the time-resolved kinetics of the Ca-ATPase to gain a deeper understanding of the pump mechanism and the underlying molecular processes. In this presentation we studied a partial reaction facilitating Ca^{2+} takeup into the SR, $Ca_2E_1 \rightarrow Ca_2E_1ATP \rightarrow (Ca_2)E_1\text{-}P \rightarrow P\text{-}E_2Ca_2 \rightarrow P\text{-}E_2 \rightarrow P\text{-}E_2H_2$, and analyzed accessible kinetic parameters and their temperature dependence.

In this study we performed ATP-concentration jump experiments and monitored the relaxation into a new steady state of the ion pumps, which depends on substrate conditions and temperature. A limitation of the applied method was set by the time constant of the release reaction of ATP from its inactive precursor, caged ATP. The UV-flash-induced photo reaction is known to be pH dependent (McCray and Trentham, 1989) and affects the time course of the fluorescence signal at pH > 7.7 (Fig. 5 *B*). The fluorescence method itself is based on the electrochromic properties of the styryl dye that responds with time constants lower than microseconds (Ephardt and Fromherz, 1989), and therefore it was always significantly faster than any observed response of the ion pumps.

As discussed previously (Pedersen et al., 2001), the fluorescence intensity of the styryl dyes reports changes of the amount of electric charge within the membrane domain of the ion pumps. Such changes are generated by ions bound to or released from sites within the membrane domain of the Ca-ATPase but not by conformation transitions of the protein. In recent work we showed that such ''electrogenic'' or charge-translocating steps are mainly the ion-binding and release steps in the Post-Albers cycle (Apell, 2003b; Butscher et al., 1999; Peinelt and Apell, 2002). ''Electrically silent'' steps can be revealed only when they are rate limiting before the (faster) reaction step in which ion(s) are released from or bound to their sites. Due to the mechanism of the styryl dyes a fluorescence increase indicates a release of a cation from the membrane part of the protein (or, in principle an import of an anion, which may be neglected in the case of the Ca-ATPase). A fluorescence decrease is caused by entrance of a cation into the membrane domain of the protein, respectively.

Direction of charge movements

In the ATP-concentration jump experiments two partial reactions with opposite charge movements could be resolved (Figs. 3 and 4). The first process with a time constant, τ_1, in the order of 30 ms (Fig. 5) represents a release of cations, the subsequent slower process (1 s < τ_2 < 3 s) a binding of cations. When compared with the Post-Albers cycle (Fig. 1 *A*), the first process has to be assigned to the partial reaction, $Ca_2E_1 \rightarrow \ldots \rightarrow P\text{-}E_2$. The fluorescence increase is generated by the release of the Ca^{2+} ions, and the time dependence by the rate-limiting step of this reaction sequence. When the Ca^{2+} concentration was >0.3 μM all ion-binding sites in the state E_1 were occupied by Ca^{2+} before the ATP concentration jump (Peinelt and Apell, 2002). In these experiments the amplitude of the rising phase was pH independent (Fig. 4 *B*). At lower Ca^{2+} concentrations, when Ca^{2+} binding became rate limiting, the fast increasing fluorescence amplitude was reduced (data not shown).

The fact that a biphasic fluorescence transient can be observed requires that the second process is slower than and

follows the first one. The fluorescence decrease during the second process reports a cation uptake and, therefore, has to be assigned to the partial reaction, P-E_2 → ... → P-E_2H_2 according to the Post-Albers scheme (Fig. 1 *A*). The subsequent dephosphorylation and ion-occlusion step, P-E_2H_2 → $E_2(H_2)$, occurs spontaneously, but it is electroneutral and cannot be monitored by styryl dyes. It was reported that at ATP concentrations below 50 μM the conformation transition, E_2 → E_1, is the slowest step of the whole cycle (Scofano et al., 1979). Therefore, in the stationary state after the ATP-induced relaxation most of the enzymes will accumulate in state $E_2(H_2)$ at low pH, and in state P-E_2 at high pH, when luminal H^+ binding becomes limiting. This can be derived from the high stationary fluorescence levels in Fig. 4 *B* (*solid circles*).

Rate-limiting process of Ca^{2+} transport

According to the Post-Albers scheme, the Ca^{2+} transporting half cycle consists of at least five steps, E_1 → Ca_2E_1 → Ca_2E_1ATP → $(Ca_2)E_1$-P → P-E_2Ca_2 → P-E_2, which could be discriminated experimentally. In the case of the experiments presented here, conditions could be chosen in a way that no substrate limitation occurred (i.e., [Ca^{2+}] > 200 nM, [ATP] > 20 μM, pH 7). Therefore, the first two processes in this sequence, Ca^{2+} and ATP binding, may be excluded as rate-limiting processes. Due to the low Ca^{2+} concentration, the back reaction in the last step, P-E_2Ca_2 ← P-E_2, Ca^{2+} binding on the luminal side can be excluded ($K_{1/2}$ ≈ 1.8 mM). Ca^{2+} dissociation is expected to be fast (diffusion controlled?), unless a conformational relaxation occurs between the release of the first and second Ca^{2+} ion. Such a process was found in the case of the Na,K-ATPase (Hilgemann, 1994), but even then ion release was in the submillisecond range (Holmgren et al., 2000; Wuddel and Apell, 1995).

In previous analyses of the SR Ca-pump kinetics by phosphorylation/dephosphorylation studies with radioactive phosphate and by biophysical experiments, the time course of the reaction sequence, Ca_2E_1ATP → $(Ca_2)E_1$-P → P-E_2Ca_2 → P-E_2, was modeled, and rate constants were derived from numerical simulations of experiments performed at 21–25°C (Alonso et al., 2001; Froehlich and Heller, 1985; Inesi and de Meis, 1989; Sørensen et al., 2000; Teruel et al., 1987). The rate constants obtained for enzyme phosphorylation ranged from 100 s^{-1} to 750 s^{-1}, for the conformation transition from 320 s^{-1} to 1500 s^{-1}, and in all but one case enzyme phosphorylation was found to be slower than the conformation transition. Only in the most recent paper (Alonso et al., 2001), a theoretical approach based on previously published data (Fernandez-Belda and Inesi, 1986), the phosphorylation reaction was assumed to be faster than the conformation transition (4000 s^{-1} vs. 1000 s^{-1}). In the case of the Na,K-ATPase it was found that the conformation transition was the rate-limiting step in the Na^+-translocating half cycle (Heyse et al., 1994; Sokolov et al., 1998; Wuddel and Apell, 1995). Ca^{2+} release to the lumen of the SR was also deduced to be a fast process with rate constants in the order of 350 s^{-1}–500 s^{-1} (Alonso et al., 2001; Inesi and de Meis, 1989).

From these rate constants, k, it is possible to calculate time constants, $\tau = 1/k$, which have to be compared to the time constant of the fast-rising phase of the fluorescence signal measured here, $\tau_1 = 30$ ms. According to the published data (Alonso et al., 2001; Froehlich and Heller, 1985; Inesi and de Meis, 1989; Sørensen et al., 2000; Teruel et al., 1987), the time constant of the slowest reaction step, the enzyme phosphorylation, $\tau_{\text{phosphorylation}}$, is 1.3–29 ms, which is at least in the same order of magnitude as τ_1.

Additional insight into the processes occurring in the investigated partial reactions may be obtained when we take into account the results of the temperature dependence of the determined time constants. This dependence can be described by the Arrhenius activation energy. Hartung and collaborators found two activation energies, 41 kJ/mol (Q10 = 1.76) and 35 kJ/mol (Q10 = 1.62), in the Ca^{2+} translocation process, which they assigned to enzyme phosphorylation and the conformation transition, respectively (Hartung et al., 1997). In our studies we found only one activation energy governing the time constant τ_1 and it was ~80 kJ/mol. Because the results of the two experimental approaches are significantly different, the question arises whether different reaction steps were detected. An activation energy of as high as 80 kJ/mol indicates that the underlying process should not be a simple chemical reaction, such as the phosphorylation of an amino acid side chain, but would be expected for a conformation transition. On the one hand, such a process could be Ca^{2+} occlusion, which is correlated with enzyme phosphorylation, Ca_2E_1ATP → $(Ca_2)E_1$-P. On the other hand, it could be the conformation transition, $(Ca_2)E_1$-P → P-E_2Ca_2. In both cases the reaction would include structural changes in the membrane domain of the protein, which are related to the ion transport function. The subsequent ion release from the membrane domain of the protein into buffer of low Ca^{2+} concentration is energetically a downhill process. This reaction step may be expected to be fast when compared to the preceding, rate-limiting step, otherwise an effect of the Ca^{2+} concentration on the time constant, τ_1, would have been observed when the concentration was increased to 2 mM.

From a comparison of the two crystal structures of the SR Ca-ATPase, the Ca_2E_1 state and the tharpsigargin-stabilized E_2 state, it can be seen that major rearrangements occur between the two principal configurations (Toyoshima et al., 2000; Toyoshima and Nomura, 2002). It may be expected that the E_1/E_2 transition needs a high activation energy in the presence of the two bound Ca^{2+} ions. And part of this energy will be dissipated only after the Ca^{2+} ions leave their coordination sites and diffuse into the luminal aqueous phase. Therefore, our proposal is that the high-activation

energy is due to the conformation transition, and, in consequence, the time constant, τ_1, reflects this process.

Rate-limiting process in the P-E_2 conformations

The slow falling phase of the fluorescence signal is produced by H^+ binding to the sites accessible from the luminal side of the membrane. According to the Post-Albers scheme (Fig. 1 *A*) the partial reaction will start with H^+ binding, P-E_2 → P-E_2H_2, and in the presence of typically 20 μM ATP it will be apparently terminated in state $E_2(H_2)$, because at low ATP concentrations the subsequent step is the slowest of the whole cycle (Scofano et al., 1979). (In consequence, the turnover rate determined from the enzymatic activity and the activation energy derived from the temperature dependence of the enzymatic activity (Fig. 7) should be assigned preferentially to this conformation transition $E_2(H_2) \rightarrow H_2E_1$.)

The pH independence of τ_2 at the low and high limit of the experimental pH range (Fig. 6 *B*) indicates that two different processes occur. Such a behavior cannot be described on the basis of the simple Post-Albers scheme as depicted in Fig. 1 *A*. Therefore, an additional reaction step has to be introduced. A first possibility would be to treat H^+ binding in two steps, P-E_2 ↔ P-E_2H ↔ P-E_2H_2. However, the pH independence at pH < 7 excludes H^+ binding as detected process as well as the high-activation energy determined for τ_2 (Fig. 7).

The pH independence of this reaction below pH 7 may be explained by a conformational rearrangement before binding of the first H^+,

$$\text{P-E}_2 \overset{k_2'}{\rightarrow} \text{P-E}_2^* \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} \text{P-E}_2^*\text{H} \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} \text{P-E}_2^*\text{H}_2, \tag{4}$$

or between binding of the first and second H^+,

$$\text{P-E}_2 \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} \text{P-E}_2\text{H} \overset{k_2'}{\rightarrow} \text{P-E}_2^*\text{H} \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} \text{P-E}_2^*\text{H}_2. \tag{5}$$

In the case of the Na,K-ATPase, evidence for conformational relaxations between binding of single ions was found and discussed (Apell, 2003a; Forbush, 1988).

The fact that the H^+-concentration dependence is of second order in the fit of Fig. 6 *B* opposes the mechanism proposed in Eq. 5. The quality of the data at high pH is, however, rather poor, therefore, this argument alone is not convincing. A more reliable argument against the mechanism described by Eq. 5 is that binding of the two H^+ ions is electrogenic and that thus the two ions have to contribute to the fluorescence decrease (Peinelt and Apell, 2002). The amplitude of the fast, rising phase of the fluorescence signal was not affected by pH (Fig. 4 *B*) as would have to be expected by the mechanism in Eq. 5, when the first H^+ binds before the rate-limiting and pH-independent process, P-E_2H → P-E_2*H. An alternative mechanism (Eq. 4) proposes a conformational relaxation after the Ca^{2+} ions left the membrane domain of the ion pump, and before H^+ ions bind. This is a reasonable assumption, because the Coulomb interaction between the two charges of each Ca^{2+} ion and the amino acid side chains of the protein matrix is strong. It will prevent shifts, turns, and bending of the α-helices of the membrane domain, as predicted by the structure of both conformations, until the ions will have left the preferentially apolar interior of the protein. The rate constant obtained for the conformational relaxation, P-E_2 → P-E_2^*, was 1.1 s^{-1} at 20°C. After this rate-limiting process the two H^+ ions bind with a pK in the order of 7.6, as determined from k_2 and k_{-2}. H^+ binding becomes the rate-limiting process at pH > 7.5, and according to the principles of chemical kinetics the value of τ_2 provides information on the rate constant k_{-2}, which was determined to be in the order of 0.4 s^{-1}.

TABLE 1 Rate constants of partial reactions deduced from kinetic experiments on the basis of ATP-concentration jumps

Reaction	Rate constant	Remark
$(Ca_2)E_1P \rightarrow P\text{-}E_2(Ca_2)$	33 ± 2 s^{-1}	Rising phase (pH independent)
$P\text{-}E_2 \rightarrow P\text{-}E_2^*$	1.1 ± 0.1 s^{-1}	Falling phase (low pH)
$P\text{-}E_2^* \rightarrow P\text{-}E_2$	0.4 ± 0.2 s^{-1}	Falling phase (high pH)
$P\text{-}E_2^* + 2H^+ \rightarrow P\text{-}E_2^*H_2$	(6 ± 0.3)×10^{14} $M^{-2}s^{-1}$	Falling phase
$E_2(H_2) \rightarrow H_2E_1$	0.8 ± 0.1 s^{-1}	Turnover rate

The rate constants were calculated as reciprocal values of the experimental time constants, $k = 1/\tau$. The temperature was 20°C.

In summary we can conclude that Ca^{2+} transport through the SR Ca-ATPase is slower than Na^+ transport through the Na,K-ATPase, however, the low rates obtained do not contradict the requirements of a fast Ca^{2+} uptake into the SR, to provide the physiologically observed rates of muscle relaxation. An overview over the rate constants of the pump cycle deduced in this work is shown in Table 1. The high activation energy of the rate-limiting step of the Ca^{2+} translocation led us to the proposal that this process includes a significant conformational relaxation, probably the E_1/E_2 transition of the enzyme. This concept is supported by the significantly different arrangements of the α-helices forming the membrane domain of the ion pump in both conformations. Significant evidence was collected that, before binding of H^+ ions to the state P-E_2 is possible, another conformational relaxation occurs that was found to be the slowest step in the analyzed partial reaction between the state Ca_2E_1 and $E_2(H_2)$.

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