Carbonic anhydrase inhibition and calcium transients in soleus fibers*

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We simultaneously measured cytoplasmic Ca^{2+} transients using Fura-2 and isometric force in rat soleus fiber bundles In the presence of the carbonic anhydrase inhibitor, chlorzolamide, we observed a decreased amplitude and retarded decay of the Ca^{2+} signal. This corresponded with a decreased isometric force and a retarded muscle relaxation We conclude that muscle carbonic anhydrase participates in excitation-contraction coupling, possibly by rapidly providing protons that are exchanged for Ca^{2+} across the sarcoplasmic reticulum membrane.

Muscle carbonic anhydrase; Calcium ion, Fura-2, Sarcoplasmic reticulum, Rat soleus fiber

1. INTRODUCTION

Studies of Geers and Gros [1,2] have shown that inhibition of muscle carbonic anhydrase (CA) in the isolated rat soleus and extensor digitorum longus results in a decrease in isometric force and an increase in relaxation time. These results seemed to indicate that muscle CA may be involved in some step in excitation-contraction coupling. In this study, we wanted to examine whether CA influences Ca^{2+} mobilization from the sarcoplasmic reticulum (SR) and its reuptake. Therefore, we simultaneously recorded single-twitch force of muscle fiber bundles and cytoplasmic Ca^{2+} transients using Fura-2 in the presence and absence of CA inhibitors.

2. MATERIALS AND METHODS

A fiber bundle prepared from the soleus of female Wistar rats is mounted in a chamber which is perfused with oxygenated Ringer solution that is buffered to a pH value of 7.5 with 25 mM HCO_3^-/5% CO_2. The muscle bundle on one side is connected to a force transducer (Grass, model FT03) and is directly stimulated by pulses of 1 ms duration and supramaximal voltage using platinum wires The length of the fiber bundle is adjusted to give maximal isometric twitch tension. The muscle fibers are loaded with the membrane-permeant ester of Fura-2 [3] (Molecular Probes, Eugene, OR, USA) for 90 min at 21°C in a solution containing a concentration of 7×10^{-7} M Fura-2,AM with 0.0004% Pluronic F-127 [4]. Fura-2 is alternatively excited by light of a wavelength of 340, 360, or 380 nm (UV filter, Schott, Mainz, FRG) The intensity of the emitted light is measured by a photomultiplier (type HTV R 928) of a Zeiss fluorescence microscope using a 500–530 nm band-pass filter. Light intensity recording and control of UV light shutter and stimulator are accomplished by a personal computer.

Fig. 1 shows the changes in Fura-2 fluorescence after electrical stimulation of the muscle bundle. The rapidly increasing cytoplasmic Ca^{2+} concentrations as they occur during muscle activation lead to an increase in Fura-2 fluorescence at an excitation wavelength of 340 nm and to a decrease in light emission at 380 nm. The excitation wavelength of 360 nm is the isosbestic point, and recordings at this wavelength were used to check for any artifacts caused by the movement of the fiber bundle during contraction. In the results reported here, as in Fig. 1, such artifacts were not present.

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* This paper is dedicated to Professor Heinz Bartels on the occasion of his 70th birthday

3. RESULTS

3.1. *Effects of CA inhibitor chlorzolamide on twitch and Ca^{2+} transient*

Fig. 2 shows force recordings of single-twitches and simultaneous measurements of Fura-2 fluorescence in the absence and presence of 1.7×10^{-3} M chlorzolamide (CLZ). After 20 min CLZ exposure single-twitch force is reduced from a control value of 2.09 mN to 1.08 mN, and 6 min later to 0.59 mN. This decrease in force is paralleled by a decrease in the amplitude of the Fura-2 signal. The amplitude decreases from 0.4 (control) to 0.17 (20 min CLZ) to 0.06 (26 min CLZ). Thus, the decrease in force is associated with a decrease in the amount of calcium released from the SR. Single-twitch force and amplitude of the Fura-2 signal show a rapid recovery: 5 min after removing the CLZ, single-twitch force as well as amplitude of the Fura-2 signal are back to their control values.

The prolongation of the decay times of single-twitch force and Fura-2 signal in the presence of CLZ, which is also apparent from Fig. 2, is illustrated more detailed in Fig. 3A and B. Fig. 3A shows a marked increase in the 50%, 75% and 90% decay times of single-twitch force which rapidly appears after <5 min of CLZ exposure. After 1 h of CLZ incubation, all decay times

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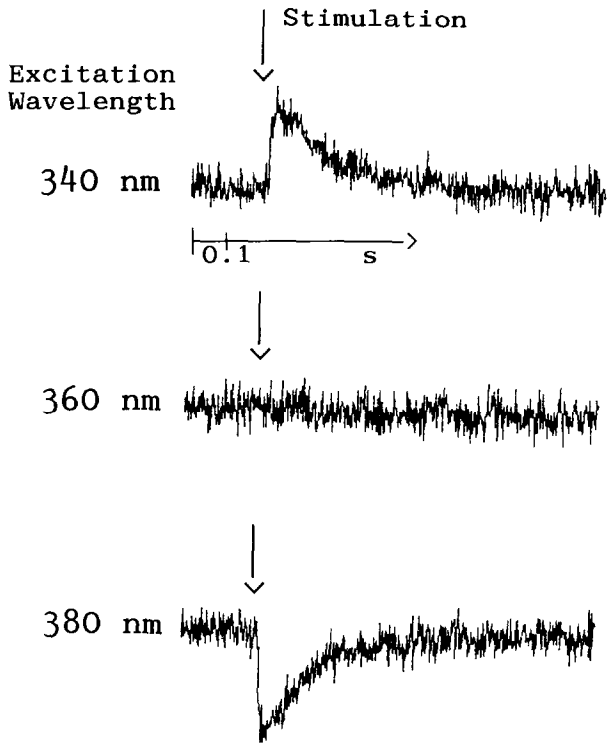

Fig. 1. Recordings of Fura-2 fluorescence at excitation wavelengths of 340, 360 and 380 nm after electrical stimulation of the muscle bundle.

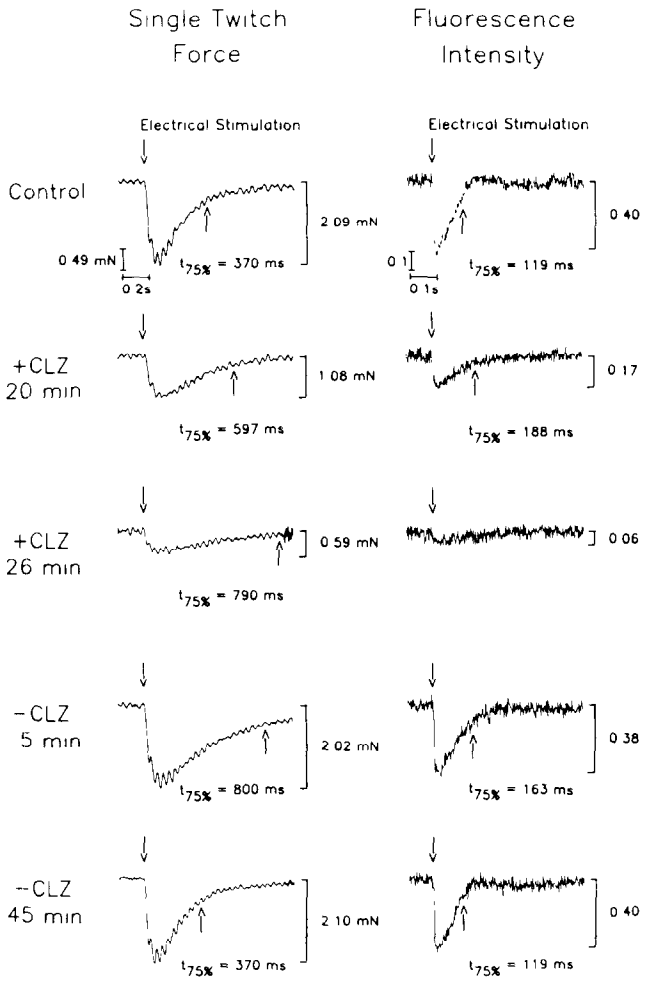

Fig. 2. Recordings of single-twitch force from a rat soleus fiber bundle, and simultaneous measurements of Fura-2 fluorescence at an excitation wavelength of 380 nm. The downwards pointing arrows indicate the time of direct electrical stimulation. After a control period, the muscle bundle is exposed to 1.7×10^{-3} M CLZ for 26 min. In the presence of this CA inhibitor single-twitch force decreases and the time of 75% relaxation (indicated by upwards pointing arrows) is prolonged. The changes in these two contraction parameters are paralleled by a decrease in amplitude and an increase in the time of 75% decay of the Fura-2 signal. 5 min after terminating the CLZ exposure, force and amplitude of the Fura-2 signal show a full recovery, and 45 min after termination of CLZ exposure the decay times of single-twitch force and of Fura-2 signal have also returned to control values.

exhibit a significant increase: the 50% decay time has risen by a factor of 1.7, the 75% decay time by a factor of 1.8, and the 90% decay time by a factor of 2.0. As seen in Fig. 3B, these increases in decay times of single-twitch force are accompanied by significant increases in the decay times of the Fura-2 transients: after 1 h of CLZ exposure, the 50% decay time has risen by a factor of 1.5, and the 75% and 90% decay times by a factor of 1.7. After removing the CA inhibitor, a recovery of the decay times of single-twitch force as well as of the Fura-2 signal is observed (see Fig. 3A and B) which approaches completion in about 15 min. We conclude that CLZ causes four major effects: a decrease in peak amplitude of the Fura-2 signal and of the force, and a prolongation of the decay times of the Fura-2 signal as well as of single-twitch force.

3.2. *Effects of CA inhibitor acetazolamide*

Acetazolamide (ACTZ) was used because, in contrast to the lipophilic chlorzolamide [5], this CA inhibitor is known to have a low membrane permeability [5]. Fig. 3C and D show that ACTZ affects the decay times of single-twitch force and Fura-2 signal only to a small extent, clearly much less than CLZ does. For example, after 1 h exposure to ACTZ, the 90% decay times of single-twitch force and Fura-2 signal have increased only by a factor of 1.2, whereas a two-fold increase is observed in the presence of CLZ (Fig. 3A and B). Not shown is that there was no significant effect of ACTZ on single-twitch force and the amplitude of the Fura-2 signal: after 1 h ACTZ force was 90 ± 14% of control and Fura-2 amplitude was 95 ± 14% of control. We conclude that ACTZ has little or no effect on Ca^{2+} transients and muscle contraction.

3.3. *Effects of metabolic acidosis*

Geers and Gros [2] have reported that in the presence of CLZ the intracellular pH (pH_i) of isolated rat soleus is lowered from 7.16 to 7.05. In order to investigate if

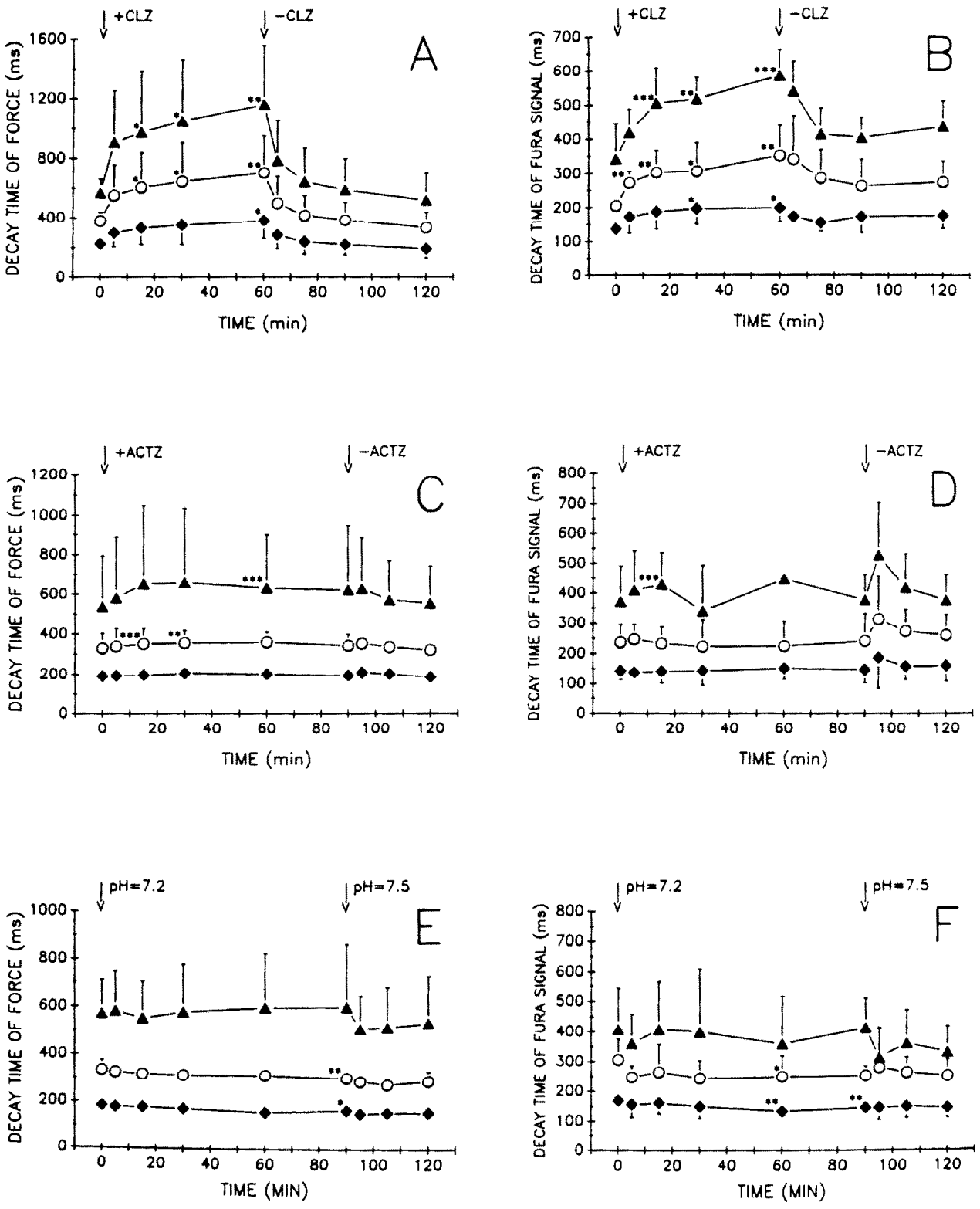

Fig. 3. Time course of various decay times of single-twitch force and Fura-2 signal. All Fura-2 signals shown have been recorded at an excitation wavelength of 380 min. (♦) Time of 50% decay; (○) time of 75% decay; (▲) time of 90% decay. Data are given as mean values of 4 experiments ± SD. The data points at 0 min represent the control values. Levels of significance of differences between control and the corresponding values are indicated by stars: * $P < 0.05$, ** $P < 0.025$ and *** $P < 0.01$ (t-test for paired samples). (A and B) Decay times of single-twitch and Fura-2 signals before, during and after exposure to 5×10^{-4} M CLZ. (C and D) Decay times of single-twitch and Fura-2 signals before, during and after exposure to 1×10^{-3} M ACTZ. (E and F) Decay times of single-twitch and Fura-2 signals before, during and after metabolic acidosis. The pH_e value of the bath solution was reduced from 7.5 to 7.2 for 90 min by applying a 10 mM HCO_3^-/5% CO_2 buffered Ringer solution.

the observed effects of CLZ are possibly mediated by a lowered pH_i, the muscle fiber bundles were exposed to a moderate and to a more pronounced metabolic acidosis. For the moderate acidosis, shown in Fig. 3E and F, the extracellular pH (pH_e) value was reduced from 7.5 to 7.2 by using a 10 mM HCO_3^-/5% CO_2 buffered bathing solution. As is apparent from Fig. 3, no prolongation whatsoever of the decay times of single-twitch force and of the Fura-2 transient occurs. A more pronounced metabolic acidosis (not shown; 2 mM HCO_3^-/5% CO_2 in the bath, pH_e = 6.8, pH_i = 6.7, according to Geers and Gros [2]) also does not induce effects of the direction and size observed under CLZ: after 60 min at pH_e = 6.8, 90% decay time of single-twitch force is 81 ± 11% relative to control, and 90% decay time of the Fura-2 signal is 112 ± 68% relative to control. It may be noted that single-twitch force and Fura-2 transient amplitude are also not affected in any way by either moderate or pronounced metabolic acidosis (after 90 min at pH_e = 7.2: force = 99 ± 7% and amplitude of Fura-2 signal = 106 ± 24%; after 60 min at pH_e = 6.8: force = 98 ± 6% and amplitude of Fura-2 signal = 95 ± 6%; all figures in percent of controls). Thus, mild as well as pronounced metabolic acidosis, inducing an even lower pH_i than that observed under CLZ, cause no prolongation of the decay times and no decrease in amplitude of both signals, and can therefore be excluded as a mechanism by which CLZ exerts its effect.

4. DISCUSSION

4.1. *CLZ-induced changes in Ca^{2+} transients cause altered contractile properties*

During the course of a CLZ inhibition experiment as seen in Fig. 3A and B, force and Fura-2 signal exhibit a variety of decay times. We have utilized this to test whether decay times of force correlate with decay times of the Fura-2 signal. Fig. 4A shows that there are indeed significant correlations for the 75% and 90% decay times ($P < 0.05$). We suggest from this that CLZ causes a slow-down of Ca^{2+} reuptake by the SR and this leads to a retarded relaxation in muscle contraction. To exclude an inhibitory effect of CLZ on SR Ca^{2+}-ATPase, leading to a slow-down of Ca^{2+} reuptake, we measured Ca^{2+}-ATPase activity of isolated SR [6] in the presence and absence of CLZ. There was no inhibition of Ca^{2+}-ATPase of rabbit red skeletal muscle SR vesicles (control: 0.9 ± 0.1, 1.5 mM CLZ: 1.1 ± 0.01, 0.5 mM CLZ: 1.0 ± 0.1 U/mg).

Fig. 4B demonstrates a significant correlation between the amplitude of single-twitch force and the amplitude of the Fura-2 transient: $r = 0.940$, $P < 0.01$. We derive from this correlation that CLZ leads to a decrease in the amount of Ca^{2+} released from the SR which then results in a decrease in isometric peak force.

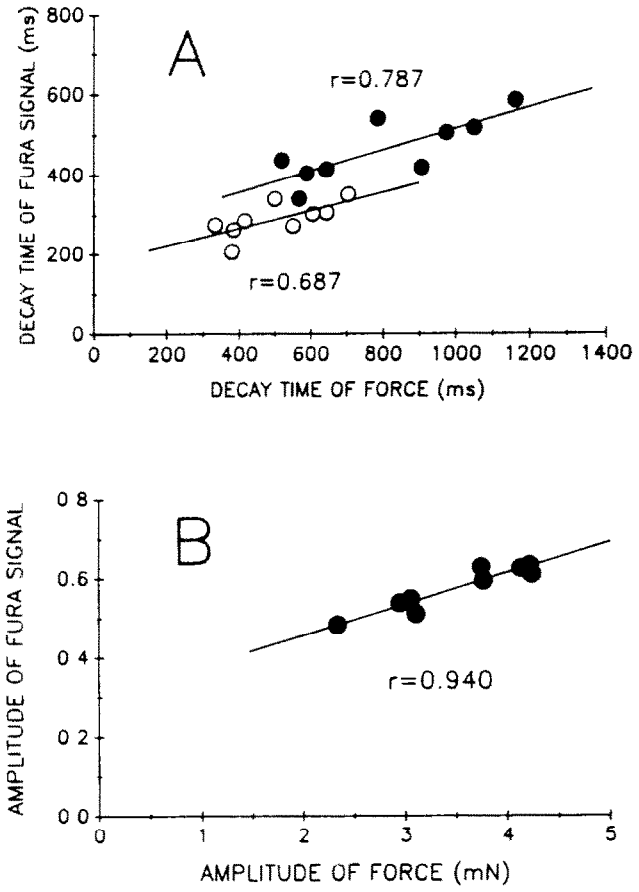

Fig. 4. (A) Plot of 75% decay times (○) and 90% decay times (●) of single-twitch and Fura-2 signals; $r = 0.687$, $P < 0.05$ and $r = 0.787$, $P < 0.05$, respectively. The straight lines represent the calculated linear regressions. Data are mean values from 4 experiments in the presence of 5×10^{-4} M CLZ. Single-twitch and Fura-2 signals were recorded at 0, 5, 15, 30 and 60 min of CLZ exposure and after 5, 15, 30 and 60 min of recovery. (B) Plot of the amplitudes of Fura-2 signals versus single-twitch forces from various phases of CLZ inhibition experiments (Fig. 3A, B). Correlation coefficient r = 0.940, $P < 0.05$.

4.2. *Which muscle CA isozyme is involved in intracellular Ca^{2+} transport?*

CA in mammalian skeletal muscle is found in 3 locations. A cytosolic CA, CA III in the case of soleus [7–9], and membrane-bound CAs in the sarcolemma [10,11] and in the SR [12]. It does not appear likely that CA III is involved in intracellular calcium transport because, as Geers and Gros [2] have shown, CLZ causes qualitatively identical effects on the contractile parameters in the rat extensor digitorum longus, which is low in CA III [8,13], as it does in soleus. Can the effects caused by CLZ be explained by inhibition of sarcolemmal CA? To assess this question, experiments with ACTZ were undertaken. ACTZ readily inhibits the interstitially active sarcolemmal CA [10] but it cannot enter the intracellular space to an appreciable extent within a few minutes. As demonstrated in section 3.2, ACTZ has no effect on the amplitudes of force and

Fura-2 signal, and has very little effect on the decay times of these two parameters. This seems to exclude the sarcolemmal CA, thus it appears likely that it is the SR-CA which influences the Ca^{2+} release and reuptake by the SR.

4.3. *Possible role of SR-CA in Ca^{2+} mobilization and reuptake by the SR*

It has been proposed in several studies [14–19] that protons act as counterions during calcium release and reuptake by the SR in excitation-contraction coupling. The involvement of protons in the fast calcium transport across the SR membrane requires a rapid proton source and sink inside the SR. CO_2/HCO_3^- would be an ideal system for this purpose, but without CA it is rather slow. Bruns et al. [12] have estimated that SR-CA accelerates the CO_2/HCO_3^- reaction within the SR 500–1000-fold. This would reduce the half-time of this reaction from 7 s to about 7 ms, a reaction time that would then appear adequate in view of the kinetics of Ca^{2+} fluxes across the SR membrane. This hypothesis predicts that inhibition of SR-CA will limit the availability of H^+ in the SR and will thus impair the Ca^{2+} movements across the membrane. This may lead to a decrease in Ca^{2+} release from the SR and consequently to a decrease in isometric force, as has been reported here. This would also explain the observed slow-down of Ca^{2+} reuptake and the retardation of force relaxation.

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