J Membrane Biol (2008) 221:7–13
DOI 10.1007/s00232-007-9079-z

Effects of Verapamil and Gadolinium on Caffeine-Induced Contractures and Calcium Fluxes in Frog Slow Skeletal Muscle Fibers

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Received: 3 September 2007 / Accepted: 8 October 2007 / Published online: 25 November 2007

Abstract In this work, we tested whether L-type Ca^{2+} channels are involved in the increase of caffeine-evoked tension in frog slow muscle fibers. Simultaneous net Ca^{2+} fluxes and changes in muscle tension were measured in the presence of caffeine. Isometric tension was recorded by a mechanoelectrical transducer, and net fluxes of Ca^{2+} were measured noninvasively using ion-selective vibrating microelectrodes. We show that the timing of changes in net fluxes and muscle tension coincided, suggesting interdependence of the two processes. The effects of Ca^{2+} channel blockers (verapamil and gadolinium) were explored using 6 mM caffeine; both significantly reduced the action of caffeine on tension and on calcium fluxes. Both caffeine-evoked Ca^{2+} leak and muscle tension were reduced by 75% in the presence of 100 μM $GdCl_3$, which also caused a 92% inhibition of net Ca^{2+} fluxes in the steady-state condition. Application of 10 μM verapamil to the bath led to 30% and 52% reductions in the Ca^{2+} leak caused by the presence of caffeine for the peak and steady-state values of net Ca^{2+} fluxes, respectively. Verapamil (10 μM) caused a 30% reduction in the maximum values of caffeine-evoked muscle tension. Gd^{3+} was a more potent inhibitor than verapamil. In conclusion, L-type Ca^{2+} channels appear to play the initial role of trigger in the rather complex mechanism of slow fiber contraction, the latter process being mediated by both positive Ca^{2+}-induced Ca^{2+} release and negative (Ca^{2+} removal from cytosol) feedback loops.

Keywords Slow muscle fiber · Caffeine contracture · Calcium flux · Verapamil · Gadolinium

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Introduction

Skeletal muscles possess two different types of muscle fibers: twitch and slow (or tonic) fibers. Twitch fibers give a transient contracture in high potassium. In contrast, slow fibers generate prolonged contracture under similar conditions (Kuffler & Vaughan-Williams, 1953; Huerta, Muñiz & Stefani, 1986).

It has been shown that addition of caffeine to the external solution evokes contractions in twitch and slow skeletal muscles of different species (Axelsson & Thesleff, 1958; Caldwell & Walster, 1963; Huerta & Stefani, 1981; Fryer & Neering, 1989; Muñiz et al., 1992; Hoock, Steinmetz & Schmidt, 1996). Caffeine penetrates muscle fiber membranes (Bianchi, 1962; Brunder et al., 1992; Konishi & Kurihara, 1993) and opens Ca^{2+} channels in the membrane of the sarcoplasmic reticulum (SR) (Su &

Hasselbach, 1984; Weber & Herz, 1968, Luttgau & Oetliker, 1968; Delay, Ribalet & Vergara, 1986; Rousseau et al., 1988). When caffeine was injected in skeletal muscle fibers, Caldwell & Walster (1963) and Hannon et al. (1992) observed a contractile response. However, Axelsson & Thesleff (1958) and Hoock et al. (1996) did not observe a visible or measurable contraction in twitch and slow muscle fibers of the frog. In that case, an external site of action was postulated for the caffeine-evoked contracture of frog skeletal muscle fibers (Hoock et al., 1996).

On the other hand, extracellular Ca^{2+} modifies the caffeine-evoked contractures of frog and chicken twitch and slow fibers (Luttgau & Oetliker, 1968; Huerta & Stefani, 1981; Muñiz et al., 1992). The exact mechanism of this modification remains elusive. The opening of the L-type Ca^{2+} channel and increasing Ca^{2+} release from the SR in these slow muscle fibers was suggested as the most likely mechanism (Huerta & Stefani, 1986; Huerta et al., 1986; Muñiz et al., 1992). In this work, the above hypothesis was further tested by studying the effects of the Ca^{2+} channel blockers verapamil and gadolinium on caffeine-evoked muscle contracture and concomitant net Ca^{2+} fluxes in slow muscle fibers of the frog.

Materials and Methods

Mechanical Recording

Isometric tension was recorded from small bundles with a few tonic fibers dissected from the tonic fascicle of the *cruralis* muscle of *Rana pipiens* (Gilly, 1975; Huerta et al., 1986). All other general aspects of the methods were as described by Huerta et al. (1986). Isometric tension measurements were done with an isometric mechanoelectrical transducer (400A; Cambridge Technology, Lexington, MA). Initial control of the K^+ contracture in each experiment was done for additional identification of the type of muscle fibers. The bundles were allowed to rest for 15 min between contractures. Experiments were performed at room temperature (20–22°C).

Solutions

The normal solution was (mM) NaCl 117.5, KCl 2.5, $CaCl_2$ 1.8. The pH was adjusted to 7.4 using imidazole chloride. The solutions with caffeine were prepared by adding an appropriate volume of 0.1 M caffeine (Sigma, St. Louis, MO) stock solutions. Preparing the stock solution required heating the solutions to dissolve the alkaloid. Pharmacological studies were performed using 10 μM verapamil and 100 μM Gd^{3+}. Ca^{2+} channel blockers were added 5 min prior to caffeine addition.

Noninvasive Ca^{2+} Flux Measurements

Net fluxes of Ca^{2+} were measured non-invasively using ion-selective vibrating microelectrodes (the MIFE® [A1]technique, University of Tasmania, Tasmania, Australia), generally as described in our previous publications (Shabala, Newman & Morris, 1997; Shabala & Shabala, 2002; Shabala, Shabala & Van Volkenburgh, 2003; Shabala et al., 2006). The theory of noninvasive ion flux measurements was recently reviewed in detail (Newman, 2001; Shabala et al., 2006). Briefly, if Ca^{2+} is taken up by muscle cells, its concentration in the proximity of the cell surface will be lower than that farther away. Vice versa, if Ca^{2+} is extruded across the plasma membrane, there will be a pronounced electrochemical potential gradient directed away from the cell surface. The magnitude of the gradient will be proportional to the rate of Ca^{2+} movement across the plasma membrane, i.e., to the net Ca^{2+} flux. The MIFE technique measures the magnitude of this gradient by moving a Ca^{2+}-selective microelectrode probe (tip diameter $\sim$3 μm) in a slow square-wave (5-s half-cycle) manner between two positions, close to (position 1) and distant from (position 2) the muscle surface. Recorded at two positions, voltage characteristics are converted into concentration parameters using the calibrated Nernst slopes of Ca^{2+} electrodes. Net Ca^{2+} fluxes (nmol m^{-2} s^{-1}) are then calculated by the specialized MIFEFLUX software, assuming cylindrical diffusion geometry. More details are available in Newman (2001).

Ca^{2+} Microelectrode Fabrication and Experimental Procedure

The complete experimental procedure for ion-selective microelectrode fabrication has been described elsewhere (Shabala et al., 1997, 2003, 2006). Briefly, microelectrodes were pulled from borosilicate glass capillaries, oven-dried and salinized with tributylchlorosilane. Dried and cooled electrode blanks were back-filled with back-filling solution (500 mM $CaCl_2$). Immediately after back-filling, the electrode tips were front-filled with a commercially available calcium ionophore cocktail (21048; Fluka Chemical, Buchs, Switzerland). The electrodes were calibrated to a set of three known standards to cover the concentration range to be measured. A reference electrode was fabricated in a similar way from a glass microcapillary and filled with 1 M KCl in 2% agar.

Microelectrodes were held in E45P-F15PH electrode holders (CDR Clinical Technology, Middle Cove, Australia) mounted on a three-dimensional micromanipulator. An open-type perfusion chamber mounted on the microscope stand allowed easy access to both sides to measure net flux

from frog muscle. Isometric tension recording was obtained as described in our previous publication (Muñiz et al., 1992). Muscles were bathed in normal saline solution, described above, minus caffeine. Calcium electrodes were positioned 15–20 μm above the frog muscle tissue. During measurements, a computer-controlled stepper motor moved the electrodes 30 μm from the tissue surface and back in a square-wave manner with 0.05 Hz frequency. Data were taken every 0.1 s and averaged for 5-s intervals. The recorded potential differences were converted into electrochemical potential differences using the calibrated Nernst slope of the electrodes. Ion fluxes were calculated assuming cylindrical diffusion geometry (Shabala et al., 1997, 2006). In this work, we maintain our previous sign convention that net influx into tissue is positive.

Effects of caffeine on net Ca^{2+} fluxes and muscle contraction were assessed by monitoring changes in muscle contraction and net Ca^{2+} fluxes simultaneously after changing the experimental solution in the chamber for solutions of the same composition containing different concentrations of caffeine (2, 4, 6, 8, 10 mM). The rate of solution flow through the perfusion chamber was 6 ml/min. No effects of Ca^{2+} channel blockers on the Ca^{2+} electrode sensitivity and characteristics were found in preliminary measurements for the range of concentrations used (*data not shown*).

Data Analysis

The values are given as mean ± standard error of the mean (SEM). The number of observations is found in parentheses. Standard EXCEL® (Microsoft, Redmond, WA) tools were used to calculate correlation coefficients between different data series. The significance of the differences between means was calculated using the *t*-test, considering $P < 0.05$.

Results

Effect of Caffeine on Kinetics of Net Ca^{2+} Flux and Muscle Tension

Net Ca^{2+} fluxes and muscle tension were measured concurrently during the experiments (Fig. 1). Kinetic experiments were crucial to evaluate the time constants of the mechanisms underlying the effect of caffeine on muscle tension. Addition of 6 mM caffeine to the bath led to a sharp increase in net Ca^{2+} extrusion for 2–3 min, to the value of about -80 nmol m^{-2} s^{-1} (Fig. 1, open circles). This coincided with a dramatic increase in muscle tension, to the value of 1.49 g (Fig. 1, closed circles). Transient Ca efflux

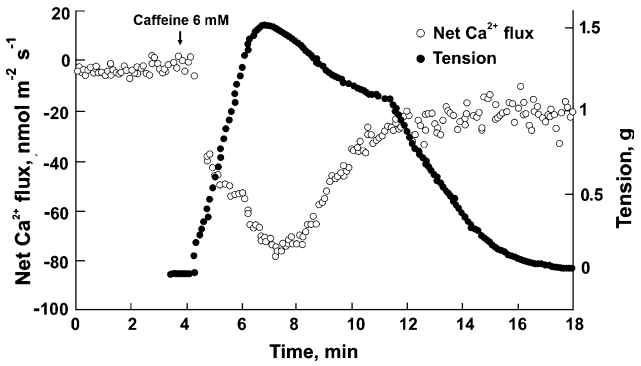

Fig. 1 Effect of caffeine on kinetics of net Ca^{2+} fluxes (*empty circles*) and associated muscle tension (*filled circles*). Caffeine (6 mM) was added at 4 min. Negative values for Ca^{2+} flux correspond to net Ca^{2+} efflux, positive values to net Ca^{2+} influx. Data were taken every 5 s

was followed by Ca reabsorption and reached a steady-state value (approximately -20 nmol m^{-2} s^{-1}) about 8 min after caffeine addition. After reaching a peak 2.5 min after caffeine was added, the caffeine-evoked tension relaxed to the control level in about 10 min.

Dependence of Response on Caffeine Concentration

Immediately after addition of 6 mM caffeine to the bath, calcium ions were extruded from the muscles (Fig. 2). This extrusion coincided with the increase in the concurrently measured muscle tension. Evoked calcium extrusion depended on the external caffeine concentration, with the maximum net Ca^{2+} extrusion occurring at 8 mM caffeine in the bath (-85 nmol m^{-2} s^{-1}), and decreased at higher caffeine concentrations (Fig. 2a). The same trend was observed for the difference between the peak and the stationary values of net Ca^{2+} flux (Fig. 2b). Similar to calcium changes, the rate of the caffeine-evoked tension change was proportional to the caffeine concentration in the bath (Fig. 2c). The threshold of caffeine's effect was below 2 mM caffeine, and a maximum tension of 1.8 g was reached at 8 mM caffeine.

Mitigation of the Caffeine Effect by Ca^{2+} Channel Blockers

Pharmacological experiments using the Ca^{2+} channel inhibitors verapamil and gadolinium were employed to investigate the role of L-type Ca^{2+} channels in fiber contraction (Huerta et al., 1986; Huerta & Stefani, 1986). The effects of Ca^{2+} channel blockers were explored using 6 mM caffeine (Figs. 3 and 4). Typical examples of response kinetics are shown in Figure 3. Both of the blockers significantly ($P < 0.01$) reduced the effect of caffeine on

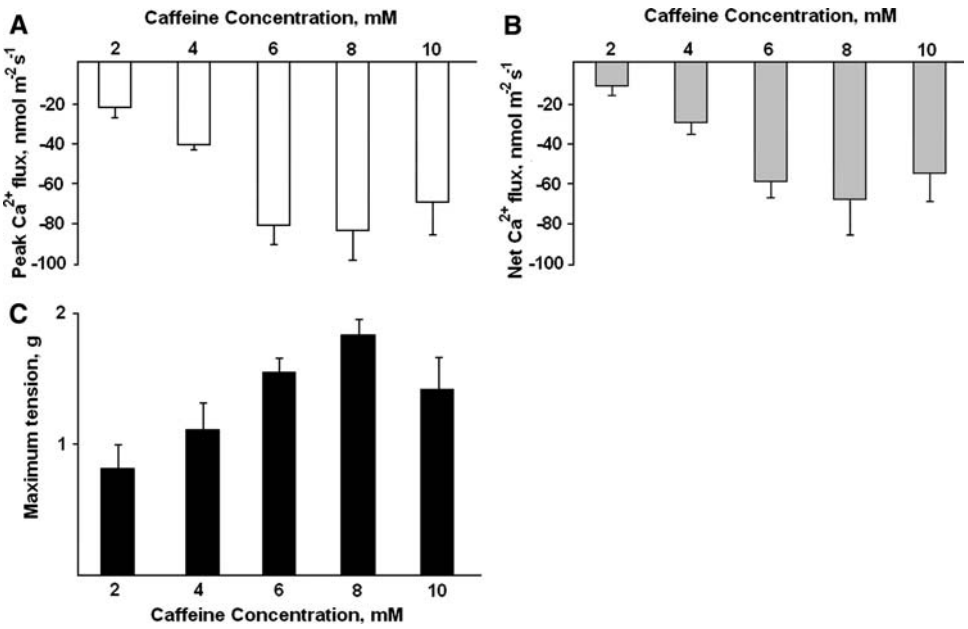

Fig. 2 Peak values for net Ca^{2+} flux (**a**) and muscle tension (**c**) and the difference between peak and steady-state values of Ca^{2+} flux (**b**) at different caffeine concentrations. Error bars are SEM (n = 6–8)

calcium fluxes and on muscle tension. Gd^{3+} was a more potent inhibitor than verapamil, reducing both the magnitude of tension evoked by caffeine and Ca^{2+} leak in the slow muscle fibers of the frog, as well as the time required for their recovery (Fig. 3). The average data for changes in net Ca^{2+} fluxes and muscle tension are given in Figure 4. Application of 10 μM verapamil to the bath led to 30% and 52% reductions in the Ca^{2+} leak caused by the presence of caffeine for the peak (Fig. 4b) and steady-state (Fig. 4a) values of net Ca^{2+} fluxes, respectively. Verapamil also caused a 30% reduction in the maximum values of muscle tension evoked by 6 mM caffeine (Fig. 3c). Under similar conditions, the level of inhibition caused by Gd^{3+} was almost two times higher than that induced by verapamil. Both the caffeine-evoked Ca^{2+} leak and muscle tension were reduced by 75% in the presence of 100 μM $GdCl_3$ (as judged by changes in the appropriate peak values). Furthermore, the calcium leak was almost completely eliminated shortly after caffeine application (97% inhibition of net Ca^{2+} fluxes in steady-state condition, Fig. 4a).

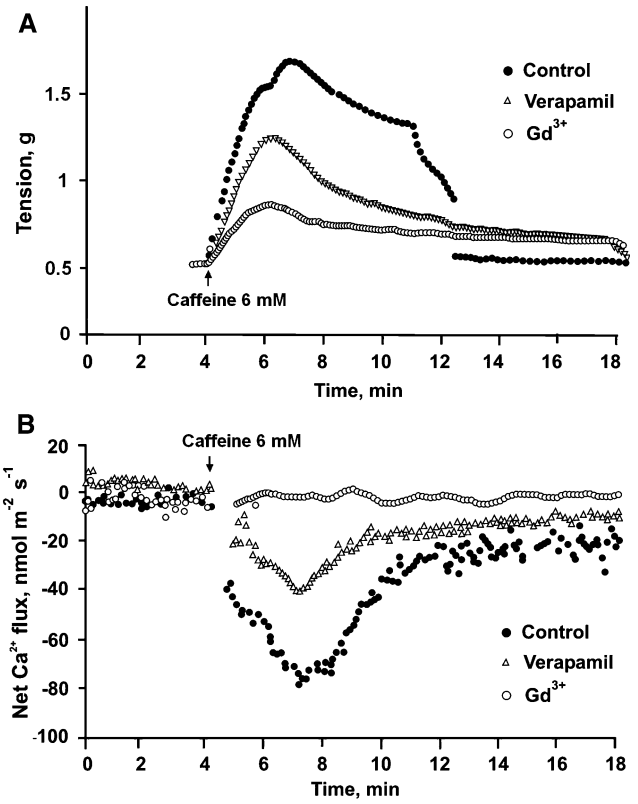

Fig. 3 Typical examples of kinetic changes in muscle tension (**a**) and net Ca^{2+} flux (**b**) for 6 mM caffeine in the presence of 10 μM verapamil and 100 μM $GdCl_3$. Verapamil and $GdCl_3$ were added 5 min prior to caffeine addition. Caffeine was applied at 4 min. Data for Ca^{2+} flux were taken every 5 s

Discussion

In this work, we tested the hypothesis that L-type Ca^{2+} channels are involved in the increase in muscle tension evoked by caffeine in slow muscle fibers. For this, we simultaneously measured net Ca^{2+} fluxes and changes in the muscle tension of frog muscle fibers before and after adding caffeine to the bath. A high level of temporal resolution of the MIFE technique (approximately 5 s) enabled us to study the kinetics of the process. Here, we show that the timing of changes in net Ca^{2+} fluxes and muscle tension coincided (Fig. 1), suggesting interdependence of the two processes. Indeed, peak values of frog muscle tension occurred at the point of maximum net Ca^{2+} extrusion after

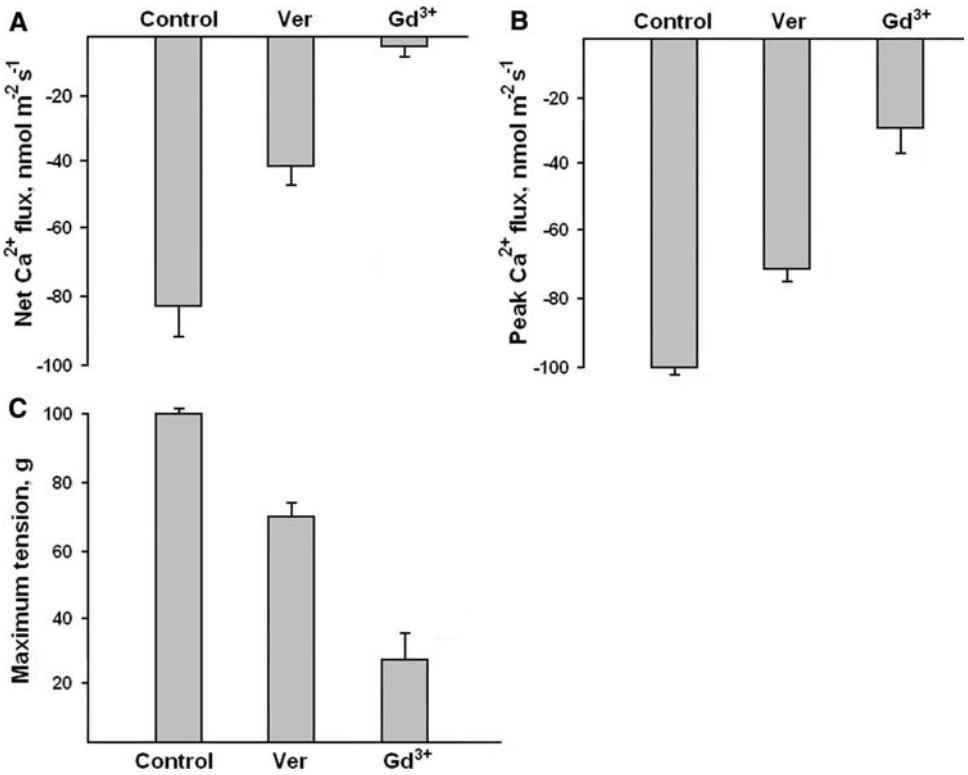

Fig. 4 Effects of calcium channel blockers verapamil and gadolinium on net Ca^{2+} fluxes and muscle tension evoked by caffeine addition. Steady-state values of Ca^{2+} flux (**a**), peak values of net Ca^{2+} flux (**b**) and maximum values of muscle tension (**c**) were measured in response to addition of 6 mM caffeine to the bath. Verapamil (10 μM) and $GdCl_3$ (100 μM) were added 5 min before caffeine application. Error bars are SEM (n = 4–6)

addition of caffeine to the bath; also, the time of muscle relaxation coincided with the point of stabilization of calcium fluxes (Figs. 1 and 3). We showed earlier that the magnitude of muscle tension was determined by the caffeine concentration used (Huerta & Stefani, 1981; Muñiz et al., 1992). In the current work, we further show that this is also true for changes in calcium fluxes (Fig. 2). A range of caffeine concentrations was screened, demonstrating (for the first time) that, similar to maximum muscle tension, maximum calcium changes occurred in the presence of 8 mM caffeine in the experimental conditions used (Fig. 2), further supporting our suggestion of the interdependence of muscle tension and calcium fluxes.

Early observations reported sarcomere shortening upon caffeine injection into frog twitch fibers (Hannon et al., 1992; Brunder et al., 1992). However, other authors have shown that injection of caffeine, in contrast to extracellular application, does not produce a visible or measurable contraction in twitch or slow muscle fibers of the frog (Axelsson & Thesleff, 1958; Hoock et al., 1996). In that case, an external site of action was postulated in the caffeine-evoked contracture of frog skeletal muscle fibers (Hoock et al., 1996). Addition of caffeine to the external solution was demonstrated to evoke contractions in twitch and slow skeletal muscles of different species (Huerta & Stefani, 1981; Fryer & Neering, 1989; Muñiz et al., 1992; Hoock et al., 1996). In slow muscle fibers, caffeine has been suggested to open Ca^{2+} channels in the cell plasma membrane and therefore release Ca^{2+} from the SR, leading to an increase in the cytosolic free calcium level (Huerta & Stefani, 1981; Muñiz et al., 1992[A2]). Therefore, it was presumed that addition of caffeine would result in net calcium uptake across the plasma membrane. Surprisingly, our results have shown that caffeine evokes net Ca^{2+} extrusion in muscle fiber cells. The most likely explanation for the observed phenomenon is a complex feedback regulation of cytosolic Ca^{2+} levels under muscle contractures, as illustrated in Figure 5.

We suggest here that both plasma membrane and SR Ca^{2+}-permeable channels mediate caffeine-induced muscle contractures (Fig. 5). During caffeine-evoked contractures, the external solution could be an additional calcium source in the slow type of muscle fibers (Huerta & Stefani, 1981; Muñiz et al., 1992). Caffeine induces intracellular calcium increase by acting directly on L-type Ca^{2+} channels and directly on sarcoplasmic ryanodine receptors (RyRs) or through a second messenger such as cyclic AMP (Huerta & Stefani, 1981; Klein, Simon & Schneider, 1990; Hoock et al., 1996) (Fig. 5, steps 1 and 2). Consequently, the muscle fibers develop tension (Konishi & Watanabe, 1998; Fryer & Neering, 1989). To maintain optimal cytosolic Ca^{2+} homeostasis and to neutralize the (otherwise detrimental) prolonged cytosolic Ca^{2+} elevation, the excess of cytosolic calcium is removed by reuptake, by pumping it into the SR (Fig. 5, step 4) and by the efflux of calcium through Na–Ca exchange (Fig. 5, step 5), causing the relaxation of muscle fibers. It was shown that caffeine activates Na^{+}–Ca^{2+} exchange in twitch muscle fibers

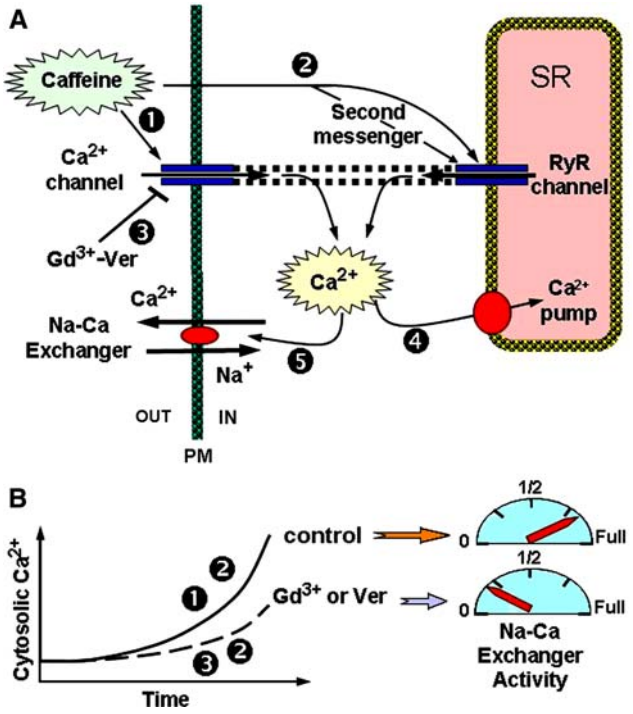

Fig. 5 A model predicting the involvement of the plasma membrane (sarcolemma, *PM*) and the sarcoplasmic reticulum membrane (*SR*) in caffeine-induced muscle tension. Caffeine stimulates Ca^{2+} uptake through the plasma membrane Ca^{2+} channels (*step 1*) and release of Ca^{2+} from the sarcoplasmic reticulum via RyR channels, either directly or indirectly (*step 2*) (**a**). Each of these pathways causes elevation in cytosolic free Ca^{2+} (indicated as continuous curve [control] in **b**). This cytosolic Ca^{2+} elevation activates the Ca^{2+} efflux through the Na^+/Ca^{2+} exchanger located at the plasma membrane (*step 5*), which also initiates the Ca^{2+} uptake by the SR by calcium pump (*step 4*). When Ca^{2+} uptake through the plasma membrane is inhibited by Gd^{3+} or verapamil, the process of free Ca^{2+} elevation occurs more slowly, as indicated by the *dashed curve* (Gd^{3+} or Ver) in **b**, resulting in Ca^{2+} efflux at a much lower rate. Suggested mechanisms of the effect of inhibitors on cytosolic calcium are shown in **b** (control is indicated as a *continuous line*; presence of verapamil and gadolinium, as *dashed lines*)

(Kotsias & Venosa, 2001). Huerta et al. (1991) reported that Na^+–Ca^{2+} exchange might play an important role in the regulation of intracellular calcium concentration. The resultant Ca^{2+} efflux (Fig. 5, step 5) is stronger than initial Ca^{2+} uptake through the plasmatic membrane (Fig. 5, step 1), required to trigger Ca^{2+}-induced Ca^{2+} release (CICR) from the SR, as a substantial portion of increase in cytosolic free Ca^{2+} originated from the internal source. As a result, net Ca^{2+} efflux is measured by the MIFE system.

Specific inhibitors of Ca channels, verapamil and Gd^{3+} (Galizzi, Fosset & Lazdunski, 1984; Glossmann & Striessnig, 1990; Lacampagne, 1994), have been employed to test the involvement of L-type Ca channels. Neither of the Ca^{2+} channel blockers that we used enter through the muscle fiber membrane and, therefore, would not directly affect the SR calcium uptake and release mechanism, under our experimental conditions, as reported elsewhere (Sárközi et al., 2004; Paydar et al., 2005). Application of the inhibitors led to a dramatic reduction of calcium efflux from slow fibers and a decrease in muscle contraction (Figs. 3 and 4). When Ca^{2+} uptake through the plasmatic membrane is inhibited by Gd^{3+} or verapamil, the CICR process occurs more slowly and Ca^{2+} is pumped out of the cell at a much lower rate (Fig. 5b). As caffeine produces the same contractures in slow fibers with widely varying membrane potential, it is unlikely that the decrease in tension caused by caffeine is due to changes of the electric field within the membrane (Elinder & Arhem, 1994; Hoock et al., 1996). This is supported by the absence of detectable changes in the threshold of caffeine contractures (*see* Fig. 3). Therefore, L-type Ca channels appear to play the initial role of trigger in the rather complex mechanism of slow fiber contraction, the latter process being mediated by both positive (CICR) and negative (Ca^{2+} removal from cytosol) feedback loops.

Acknowledgement This work was supported, in part, by a grant from the Consejo Nacional de Ciencia y Tenología (CONACyT-México, ref. 42446-M to M. H.) and by the grant Ramón Alvarez-Buylla de Aldana (FRABA, to M. H. and X. T.). E. S.-P. is a CONACyT-México fellow. The authors thank Drs. I. Pottosin and O. Dobrovinskaya for helpful discussion during the article's preparation.

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