Relationship between Myoplasmic Calcium Transients and Calcium Currents in Frog Skeletal Muscle

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ABSTRACT Ca^{2+} currents (I_{Ca}) and myoplasmic Ca^{2+} transients were simultaneously recorded in single muscle fibers from the semitendinosus muscle of *Rana pipiens*. The vaseline-gap voltage-clamp technique was used. Ca^{2+} transients were recorded with the metallochromic indicator dye antipyrylazo III. Ca^{2+} transients consisted of an early fast rising phase followed by a late slower one. The second phase was increased by experimental maneuvers that enlarged I_{Ca}, such as augmenting $[Ca^{2+}]_o$ (from 2 to 10 mM) or adding (−)-Bay K 8644 (2 μM). When $[Ca^{2+}]_o$ was increased, the second phase of the Ca^{2+} transients and I_{Ca} showed an average increase at 0 mV of 2 ± 0.9 μM (4) and 1.4 ± 0.3 mA/ml (4), respectively. (−)-Bay K 8644 increased the late phase of the Ca^{2+} transients and I_{Ca} at 0 mV by 0.8 ± 0.3 μM (3) and 6.7 ± 2.0 mA/ml (4), respectively. The initial fast rising phase of the Ca^{2+} transients was not modified. (−)-Bay K 8644 slowed the time constant of decay of the transients by 57 ± 6 ms. In other experimental conditions, Ca^{2+} release from the sarcoplasmic reticulum (SR) was impaired with repetitive stimulation in 1 mM $[EGTA]_i$-containing fibers. Under those circumstances, Ca^{2+} transients directly followed the time integral of I_{Ca}. Pulses to 0 mV caused a large Ca^{2+} transient that became suppressed when large pulses to 100 mV were applied. In fibers with functioning SR, pulses to 100 mV elicited somewhat smaller or similar amplitude Ca^{2+} transients when compared with those elicited by pulses to 0 mV. The increase in I_{Ca} after raising $[Ca^{2+}]_o$ or adding (−)-Bay K 8644 cannot directly explain the change in Ca^{2+} transients in fibers with functioning SR. On the other hand, when Ca^{2+} release from the SR is impaired Ca^{2+} transients depend on I_{Ca}.

INTRODUCTION

Several studies have addressed the role of external Ca^{2+} in excitation-contraction coupling in skeletal muscle. It is accepted that external Ca^{2+} reduction decreases the force elicited by K^+ contractures, (Frank, 1960; Lüttgau, 1963; Caputo and Giménez, 1967; Stefani and Chiarandini, 1973; Lüttgau and Spiecker, 1979; Cota and Stefani, 1981). However, muscle fibers can contract after virtually eliminating

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all extracellular Ca^{2+} (Armstrong et al., 1972; Lüttgau and Spiecker, 1979), indicating that extracellular Ca^{2+} is not essential during excitation-contraction coupling. Accordingly, Miledi et al. (1977) have demonstrated that the extracellular Ca^{2+} does not have an important role in the generation of intracellular Ca^{2+} signals during a single twitch.

Studies with simultaneous recordings of the time course of myoplasmic free Ca^{2+} concentration (Ca^{2+} transients) and Ca^{2+} currents (I_{Ca}) have been performed only recently. No direct correlation between the magnitude of the voltage-dependent Ca^{2+} entry and the voltage dependence of the myoplasmic Ca^{2+} transients was found. I_{Ca} decreased for voltages more positive than +10 mV, while myoplasmic Ca^{2+} transients and corresponding Ca^{2+} input fluxes to the myoplasm were not reduced for voltages up to +70 mV (Brum et al., 1987). In agreement with this, the decrement in myoplasmic Ca^{2+} transients caused by a reduction in external Ca^{2+} could not be explained by the reduction of the tubular Ca^{2+} current. The decrement in Ca^{2+} current could not account for >5% of the reduction of the input flux into the myoplasm. Therefore it was concluded that in low external Ca^{2+} a weakened Ca^{2+} release from the sarcoplasmic reticulum (SR) is the major cause for the Ca^{2+} transient reduction (Brum et al., 1988*b*).

We have further studied the contribution of the inward calcium current to the myoplasmic Ca^{2+} concentration measured with the metallochromic indicator dye antipyrylazo III (APIII) in single skeletal muscle fibers. In these experiments we studied the effects of increasing I_{Ca} on intracellular Ca^{2+} transients in normal conditions and after impairing Ca^{2+} release from the SR. Ca^{2+} transients increased in parallel to Ca^{2+} current enhancement after raising the extracellular Ca^{2+} concentration from 2 to 10 mM or after adding the Ca^{2+} channel agonist (−)-Bay K 8644. However, the increment of the inward Ca^{2+} currents was too small to account for the Ca^{2+} transient potentiation. Preliminary results have been reported elsewhere (Stefani et al., 1988; Amador et al., 1989).

METHODS

Experiments were performed in single skeletal muscle fibers isolated from the semitendinosus muscle of the frog (*Rana pipiens*). We followed the procedures recently described by Brum et al. (1988*b*). I_{Ca} and myoplasmic Ca^{2+} transients were recorded simultaneously.

Single fibers were dissected free in a chamber containing dissecting solution (see below) and then transferred to the experimental chamber with mounting solution. The rationale of using these two different solutions is that any damage in the fiber membrane was detected in the dissecting solution, since it contained ~1 mM Ca^{2+}. Once the fiber was positioned in the experimental chamber, both ends were attached to mobile pedestals and the fiber was stretched to long sarcomeric spacing (3.5–4.1 μm). Two vaseline strands divided the external surface of the fiber in three segments and the seals were made with a coverslip put over the fiber. At the same time, the chamber was divided in three compartments. The mounting solution was then replaced by the extracellular solution in the middle pool, and by the intracellular solution in the end pools. The intracellular solution containing the APIII diffused into the fiber via notches cut near the vaseline seals. The pools were connected with agar bridges to wells containing 3 M KCl. Ag/AgCl pellets provided the electrical connection between the

wells and the amplifier. Linear components of the membrane current were subtracted by appropriate scaling of control pulses ($P/-4$).

Solutions

The following solutions were used (in millimolar): dissecting solution: 101 K_2SO_4, 2 $CaSO_4$, 2 $MgSO_4$, 5 K-*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate (K-HEPES); mounting solution: 120 K-glutamate, 2 $MgCl_2$, 0.1 Na-ethyleneglycol-bis (β-aminoethylether) *N'N'*-tetra-acetate (Na_2-EGTA), 5 K-HEPES; extracellular solution: 105 tetraethylammonium-methane-sulfonate (TEA-CH_3SO_3), 10 Ca-$(CH_3SO_3)_2$, 5 TEA-HEPES, 10^{-3} tetrodotoxin, 1 3,4-diami-nopyridine; intracellular solution: 108 Cs-glutamate, 0.1 or 1 Cs_2-EGTA, 0.0082 $CaCl_2$, 5.5 $MgCl_2$, 5 Cs-HEPES, 5 glucose, 5 Na_2-ATP, 0.5 APIII. In all solutions pH was adjusted to 7.0. Solutions were filtered through 0.45-μm pore filters (Millipore Co., Bedford, MA) immediately after their preparation. The temperature (15–17°C) was monitored with a thermistor probe placed in the middle pool of the chamber.

Optical Measurements

The procedures and calculations of Brum et al. (1988*b*) were followed. In brief, a light beam from a 100 W quartz, tungsten-halogen lamp (FCR 100; Barbizon, Woburn, MA) was focused on the fiber where a slit was formed occupying two-thirds of the fiber in the field (~50 × 250 μm). Once the beam emerged, it was split and passed through narrow bandwidth filters centered at 700 or 850 nm (57630 and 57670; Oriel Co., Stratford, CT). The transmitted light was then monitored by two photodiodes adapted to a compound microscope. The intrafiber dye concentration was obtained from its absorbance at 550 nm (53890; Oriel Co.). The voltage output from the photodiodes and the calcium currents were simultaneously sampled at 1 kHz by a 12-bit Axolab-1 A/D converter (Axon Instruments, Inc., Burlingame, CA) and stored for deferred analysis. Fibers were stimulated with a single pulse every 1–2 min. Pulses delivered at +100 mV were bracketed by pulses to 0 mV.

Ca^{2+} *Transients Processing*

To derive myoplasmic Ca^{2+} concentration and Ca^{2+} input flux from photodiode output voltage, we calculated the absorbance transients at both monitored wavelengths (Abs_{700} and Abs_{850}). Although the Ca-$APIII_2$ complex has a maximum absorbance at 700 nm, the light sensed at this wavelength has an additional component that is due to changes in intrinsic optical properties of the fiber (Kovács et al., 1983). On the contrary, the light measured at 850 nm is purely an intrinsic signal since neither APIII nor its metal complexes absorb at this wavelength. The absorbance transient due exclusively to the formation of the Ca-$APIII_2$ complex (corrected absorbance) was calculated upon subtraction of Abs_{850} from Abs_{700} after proper scaling. The scaling factor was found by measuring the ratio of signals at both wavelengths at the beginning of the experiment, when there is no dye in the fiber (Melzer et al., 1986). The corrected absorbance transient at 700 nm was then processed to derive the time course of the Ca-$APIII_2$ complex concentration using the molar extinction coefficients previously published (Ríos and Schneider, 1981). Finally, assuming instantaneous equilibrium of the Ca-dye reaction (Kovács et al., 1983), we calculated the time course of myoplasmic free Ca^{2+} concentration from the APIII and Ca-$APIII_2$ complex concentrations. The value of the dissociation constant for the Ca-dye reaction was linearly interpolated between the published values of 40,000 μM^2 at 21°C (Ríos and Schneider, 1981) and 17,500 μM^2 at 5°C (Kovács et al., 1983). The records shown are single sweeps.

RESULTS

Extracellular Ca^{2+} Concentration and Ca^{2+} Transients Amplitude

Fig. 1 shows records of I_{Ca} (*lower traces*) and Ca^{2+} transients (*upper traces*) obtained with 2 mM $[Ca^{2+}]_o$ (*A*) or 10 mM $[Ca^{2+}]_o$ (*B*). Command pulses of 250 ms duration were delivered to the values indicated by the numbers between the traces. Ca^{2+} transients have a complex time course since they reflect two opposing processes: input flux and removal (Melzer et al., 1986). Ca^{2+} transients usually had an early fast rising phase followed by a much slower one. At the end of the pulse Ca^{2+} transients decayed following a quasi exponential time course. Current traces have an initial upward deflection that corresponds to the charge movement (Schneider and Chandler, 1973). This is followed by the inward Ca^{2+} current. At the end of the pulse the Ca^{2+} tail current and the OFF charge movement current are added. When

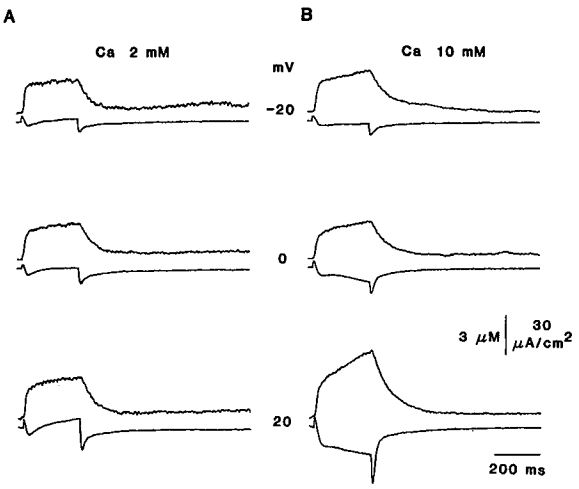

FIGURE 1. Effect of $[Ca^{2+}]_o$ on Ca^{2+} transient. I_{Ca} (*lower traces*) and Ca^{2+} transients (*upper traces*) were recorded with (*A*) 2 or (*B*) 10 mM $[Ca^{2+}]_o$. Numbers between each pair of traces correspond to the command pulse value. Transients consisted of an early fast rising phase and a late slower one in 2 mM $[Ca^{2+}]_o$. Currents and the late phase of transients increased in amplitude with the elevation of the extracellular Ca^{2+}. The early rising phase was not affected in these conditions.

we increased $[Ca^{2+}]_o$ from 2 to 10 mM the second phase of the Ca^{2+} transients became 50–60% larger (four fibers), while the early phase did not show modifications. This percentage represents an average increase of 2.0 ± 0.9 μM (4) for pulses to 0 mV. With the same maneuver, the charge-on was not affected while I_{Ca} had an average increment in its peak and time integral of 1.4 ± 0.3 mA/ml (4) and 0.28 ± 0.07 mC/ml (4) during the pulse to 0 mV.

The increase in the Ca^{2+} transient amplitude after raising $[Ca^{2+}]_o$ may reflect a larger Ca^{2+} entry into the myoplasm through the tubular Ca^{2+} channels. In addition, both increments in I_{Ca} and in Ca^{2+} transient could be just coincidental in their voltage dependency since both events are thought to be initiated by the charge movement (Lamb, 1986; Melzer et al., 1986; Rios and Brum, 1987). However, the fact that modifications in the charge-on were not detected after raising external Ca^{2+} (2–10 mM) makes this last possibility unlikely.

Effect of (−)-Bay K 8644 on Ca^{2+} *Transients*

To further clarify the relationship between I_{Ca} and Ca^{2+} transients, we studied the effects of the Ca^{2+} channel agonist (−)-Bay K 8644. Fig. 2 shows the results obtained with this drug in the presence of 10 mM $[Ca^{2+}]_o$. Records in *A* correspond to control conditions. I_{Ca} and Ca^{2+} transients have the same characteristics described above. After the addition of 2 μM (−)-Bay K 8644 to the extracellular solution, I_{Ca} largely increased (records in *B*). The average increment of I_{Ca} was 6.8 ± 2.0 mA/ml

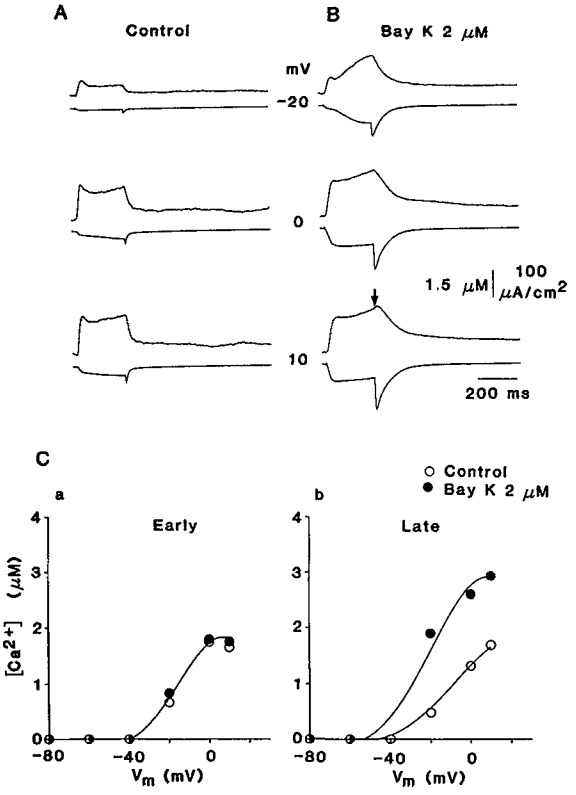

FIGURE 2. Effect of (−)-Bay K 8644 on I_{Ca} and Ca^{2+} transient. I_{Ca} (*lower traces*) and Ca^{2+} transients (*upper traces*) in absence (*A*) and in presence (*B*) of 2 μM $[(-)\text{-Bay K 8644}]_o$. Records were acquired with 10 mM $[Ca^{2+}]_o$. Addition of (−)-Bay K 8644 substantially increased the current amplitude. Transient amplitude showed an increase of the late phase without modifications in the early phase. At the end of the pulse there was a further increase in the Ca^{2+} transient that is coincident with the peak Ca^{2+} tail current. The arrow in *B* marks the end of the pulse. The transient decay was also slower in the presence of the drug. (*C*) Graphs of the early phase (*a*) and the late phase (*b*) amplitudes of the transient as a function of membrane potential. *Open symbols*, control conditions; *filled symbols*, with 2 μM $[(-)\text{-Bay K 8644}]_o$.

(5) and that of its time integral was 1.63 ± 0.41 mC/ml (5) during a pulse to 0 mV.

Ca^{2+} tail currents in skeletal muscle fibers have two time constants of deactivation, fast and slow. The fast one is thought to correspond to the closure of the slow Ca^{2+} channels. (Almers and Palade, 1981; Sánchez and Stefani, 1983). The time constant of this component for pulses from 0 to −90 mV increased from 13.6 ± 3.5 ms (4) to 48 ± 7 ms (4) after the addition of 2 μM (−)-Bay K 8644. On the other hand, the slow component remained unmodified (193.5 ± 30.8 ms [4] and 158 ± 18 ms [4]). As expected, only the amplitude of the fast component increased

with (−)-Bay K 8644. The time constant of deactivation was similar for tail currents with different amplitudes with pulses ranging from −20 mV (50 ms) to 10 mV (68 ms). This makes it unlikely that the slowing of the fast tail component by (−)-Bay K 8644 is due to a lack of voltage-clamp control. Instead, it is consistent with the observations that this compound increases the open probability of Ca^{2+} channels by prolonging the mean open time (Hess et al., 1984; Kokubun and Reuter, 1984), or by increasing the probability of reopening of closed channels (Brown et al., 1984).

The initial fast rising phase of the Ca^{2+} transients was not modified, whereas the late slower one was increased by (−)-Bay K 8644. For pulses to 0 mV, this increment ranged between 20–30% and represents an average increase of ~1 μM (0.8 ± 0.3 μM [3]). The potentiation of the Ca^{2+} transients by (−)-Bay K 8644 was more evident at potentials where a larger effect was observed on I_{Ca} (see Fig. 2, pulse to −20 mV). In addition, immediately after the end of the pulse there is a further increase in the myoplasmic Ca^{2+} transient (Fig. 2 *B*, the arrow indicates the end of the pulse) that is coincident with the peak Ca^{2+} tail current. Finally, (−)-Bay K 8644 (2 μM) slowed by 57 ± 6 ms (3) the decay of the myoplasmic Ca^{2+} transients measured after the pulse was terminated. Graphs in Fig. 2 *C* show the myoplasmic Ca^{2+} concentration during the initial phase (Fig. 2 *Ca*) and the late phase (Fig. 2 *Cb*) of the transient, obtained under control conditions (*open symbols*) and after the addition of 2 μM (−)-Bay K 8644 (*filled symbols*). In conclusion, (−)-Bay K 8644 potentiates the slower late phase of the Ca^{2+} transient without affecting the initial one. Similar effects of (−)-Bay K 8644 were obtained with 2 mM $[Ca^{2+}]_o$ in three other fibers.

These results, as in the previous section, prompted the question whether the increment in the Ca^{2+} transient is related to Ca^{2+} entry from the extracellular solution. It seems unlikely that (−)-Bay K 8644 increases the Ca^{2+} transient through a direct action on charge movement, since it has been previously reported that this dihydropyridine has no effect on charge movement from skeletal muscle, even at concentrations up to 10 μM (Lamb and Walsh, 1987). We have conducted similar experiments in frog skeletal muscle obtaining similar results (García, J., and E. Stefani, unpublished observations).

Ca^{2+} Transients after Reduction of SR Ca^{2+} Release

To study any direct contribution of the Ca^{2+} current to the myoplasmic Ca^{2+} transient, it becomes necessary to abolish or greatly reduce Ca^{2+} release from the SR. Since Ca^{2+} release can be depressed during repetitive pulse stimulation by depleting the SR of Ca^{2+} (Schneider et al., 1987) and reduced by drastically lowering free Ca^{2+} ($\leq 10^{-7}$ M, Meissner et al., 1986), we equilibrated the fibers with 1 mM intracellular EGTA and repetitively stimulated at 0.03 Hz with 250-ms pulses to 0 mV. Fig. 3 shows one of these experiments. Myoplasmic Ca^{2+} transients (*a–d*), I_{Ca} (*e*), and voltage pulse (*f*) obtained at different times are shown. The first pulse (*a*) elicited the largest Ca^{2+} transient. The decay phase decreased monotonically to the basal level in 1–2 s. Ca^{2+} transients showed the following modifications as the fiber was successively stimulated: (*a*) the rate of rise was progressively slower, (*b*) the initial fast component and (*c*) the peak Ca^{2+} concentration became progressively smaller, (*d*) as the transient was reduced, an inflection during its rising phase became evident that was coincident in time with the activation of I_{Ca}, (*e*) the increase in intracellular Ca^{2+}

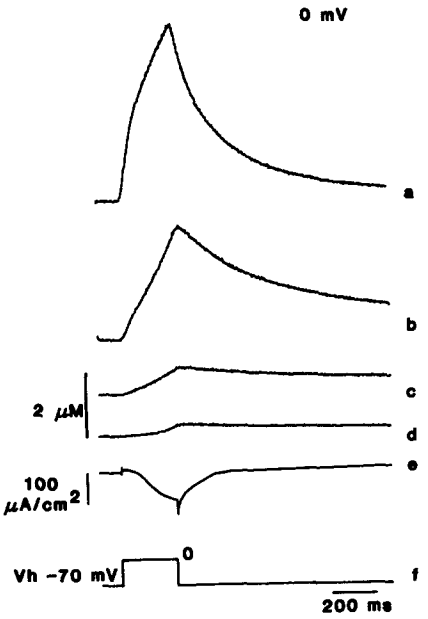

FIGURE 3. Ca^{2+} transients after reduction of Ca^{2+} release by the SR. Ca^{2+} transients recorded at different times during repetitive stimulation with pulses to 0 mV and 250 ms at 0.02–0.03 Hz. The intracellular solution contained 1 mM EGTA. The first trace corresponds to the transient at the beginning of the experiment (*a*). With further stimulation the transients became smaller, and the rate of rise and the decay after the pulses were slower (records *b–d*). I_{Ca} (record *e*) was recorded simultaneously. Voltage pulse (record *f*) is represented schematically.

associated with the Ca^{2+} tail current was more pronounced as the transient became smaller (records *b–d*), and (*f*) the decay phase became progressively slower, probably due to a less efficient removal by the SR. Once these changes were reached, the transient remained stable at this level. I_{Ca} persisted with similar characteristics along the experimental maneuver.

As the fiber was stimulated and less Ca^{2+} was released from the SR, the time course of the Ca^{2+} transients resembled the time integral of I_{Ca} (Fig. 4 *B*), the Ca^{2+}

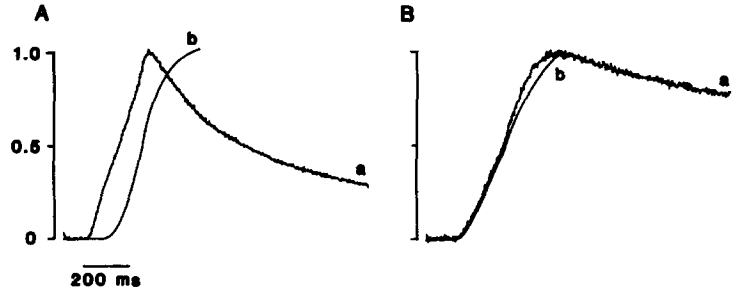

FIGURE 4. Comparison between Ca^{2+} transient and integral of I_{Ca}. Noisier traces correspond to Ca^{2+} transients (*a*) and the integral of I_{Ca} is represented by the superimposed traces (*b*). Records were obtained during the Ca^{2+} release impairment process from the SR. (*A*) Traces obtained before the Ca^{2+} release was completely exhausted. The Ca^{2+} transient signal and the integral of the current still show different temporal courses. (*B*) Traces acquired after Ca^{2+} release impairment and with the addition of 2 μM (−)-Bay K 8644. Note that the transient follows the temporal course of the integral more closely.

transient amplitude followed the I_{Ca} amplitude (see Fig. 6 below) and, as expected, during large depolarizing pulses that diminished the Ca^{2+} driving force, Ca^{2+} transients were suppressed (see Figs. 6 and 8 *B* below). SR Ca^{2+} release was fully abolished in one out of eight fibers tested. In the remaining seven fibers a small fraction of the Ca^{2+} release process remained active since the reported changes were not complete. Nevertheless, in these fibers a clear relationship was found between Ca^{2+} entry and Ca^{2+} transients. The average amplitude of the transient was reduced from 4.1 ± 1.4 μM to 0.7 ± 0.2 μM (8) upon repetitive stimulation.

Fig. 4 shows normalized Ca^{2+} transients and the integral of I_{Ca} at different times after stimulation. In *A*, after 20 min, the Ca^{2+} transient signal rises much earlier than the integral of the current. As the release was impaired towards the end of the stimulation (*B*), the transient followed more closely the temporal course of the integral.

In conclusion, these results show that after Ca^{2+} release from the SR has been greatly reduced, a close relationship was found between I_{Ca} and myoplasmic Ca^{2+} transients which favors the idea that the measured intracellular Ca^{2+} is the result of the voltage-dependent Ca^{2+} entry.

Myoplasmic Ca^{2+} Transients Due To I_{Ca} Are Enhanced by (−)-Bay K 8644

After the Ca^{2+} release from the SR had been greatly reduced, the relationship between the inward Ca^{2+} current and the myoplasmic transient was confirmed by increasing I_{Ca} with (−)-Bay K 8644 (2 μM). Fig. 5 *A* illustrates the records from a fiber whose Ca^{2+} transient is mainly due to Ca^{2+} entry. For the pulse to −20 mV there is neither I_{Ca} nor Ca^{2+} signal. In the next pulses to 0 and 20 mV, I_{Ca} was elicited and an associated transient could be recorded. Note the increase in $[Ca^{2+}]_i$ after the termination of the pulse and its association with the Ca^{2+} tail current. The arrow indicates the end of the pulse. After the addition of 2 μM (−)-Bay K 8644, I_{Ca} became apparent at −20 mV and so did the transient (see Fig. 5 *B*). For the larger pulses, to 0 and 20 mV, current and transient increased accordingly. For example, for the fiber represented in the figure at 0 mV, I_{Ca} increased from 23 mA/ml to 32 mA/ml by the end of the pulse and the associated Ca^{2+} transients from 0.2 to 0.4 μM in control conditions and with (−)-Bay K 8644, respectively. Note that the maximum amplitude of the transient was attained after the pulse during the large inward tail current. Fig. 6 shows the voltage dependence of both Ca^{2+} transient (*circles*) and Ca^{2+} current (*squares*) in control conditions (*open symbols*) and with (−)-Bay K 8644 (*filled symbols*). Values were taken before the end of the pulse for each potential. Ca^{2+} transients and I_{Ca} had a similar voltage dependence with or without the agonist.

Relationship between Ca^{2+} Transients and I_{Ca} in Fibers with Impaired SR Release

The relationship between Ca^{2+} transients and I_{Ca} can be visualized by expressing I_{Ca} as its time integral per unit volume. The units will be charge per unit volume (millicoulomb/milliliter) or mole per liter (molar). A linear relationship was found between the Ca^{2+} entry in these units and the myoplasmic transient for different pulse amplitudes (Fig. 7 *A*). From the slope a value of 3.85×10^{-3} was obtained for the ratio between the recorded Ca^{2+} transient and the expected Ca^{2+} concentration

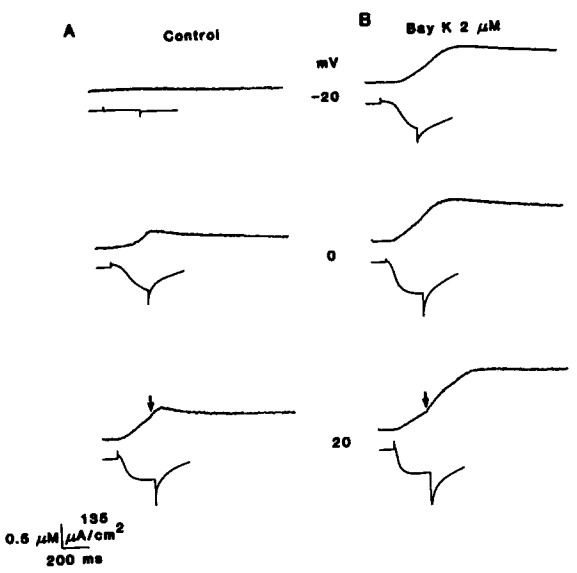

FIGURE 5. Enhancement of Ca^{2+} transient by (−)-Bay K 8644. I_{Ca} (*lower traces*) and Ca^{2+} transients (*upper traces*) obtained in the absence (*A*) and presence (*B*) of 2 μM (−)-Bay-K 8644. Records were acquired after the Ca^{2+} release impairment from the SR was completed. For the pulse to −20 mV in control conditions I_{Ca} was not elicited, nor was the Ca^{2+} transient. With larger depolarizations, the current became evident and the transient appeared. Note the increase in Ca^{2+} transients after the termination of the pulse and its association with the Ca^{2+} tail current. The arrows mark the end of the pulse. After the addition of the agonist, the current amplitude increased and the deactivation of the tail was slower. Associated with this increment, the transient became larger and the decay was also slower. The maximum amplitude of the transient was attained after the end of the pulse during the large inward tail current.

due to Ca^{2+} entry assuming free diffusion into the total fiber volume. The inverse of this slope is proportional to a volume expansion (*E*) for the Ca^{2+} measuring compartment. Assuming a value of 0.7 for the free aqueous intracellular volume, *E* would be ~180 (Kovács et al., 1983). For example, to reach 1 μM of intracellular free Ca^{2+} concentration a Ca^{2+} input flux is required that would produce a total Ca^{2+} concentration of 180 μM or its equivalent in coulombic charge (35 mC/ml). The additional quantity of Ca^{2+} required can be explained by the volume expansion for Ca^{2+} due to the fast cytoplasmic Ca^{2+} buffers (Kovács et al., 1983). In these calculations we did not include the volume expansion by EGTA (1 mM), since it is a relatively slow buffer and is likely to be saturated by the Ca^{2+} released by the SR.

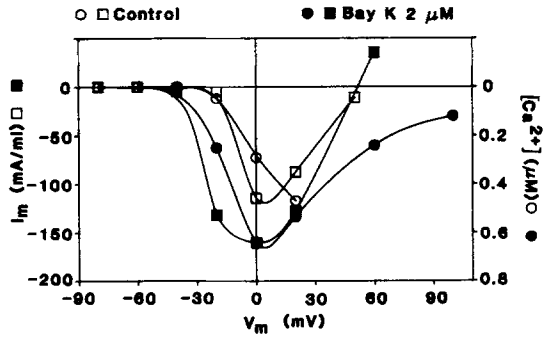

FIGURE 6. Voltage dependence of I_{Ca} and Ca^{2+} transient. *Circles*, peak Ca^{2+} transients; *squares*, I_{Ca}; *open symbols*, control conditions; *filled symbols*, with 2 μM (−)-Bay K 8644. Both I_{Ca} and Ca^{2+} transient were shifted to more negative potentials and increased after the addition of the agonist.

A similar analysis can be performed by comparing the Ca^{2+} transient with the time integral of I_{Ca} for the whole time course of the depolarization. In this case, the slope of the curve would have the same meaning as in the previous analysis but for a single signal instead of the pool of data. The rate of increase of free Ca^{2+} plus Ca^{2+} bound to fast buffers (Ca_{fast}) is:

$$\frac{dCa_{fast}}{dt} = \frac{I_{Ca}}{2FVa} - \frac{dCa_{rem}}{dt} \quad (1)$$

where F is the Faraday constant, Va is the free aqueous intracellular volume (0.7, Kovács et al., 1983) and dCa_{rem}/dt is the removal rate, Ca_{fast} is approximately

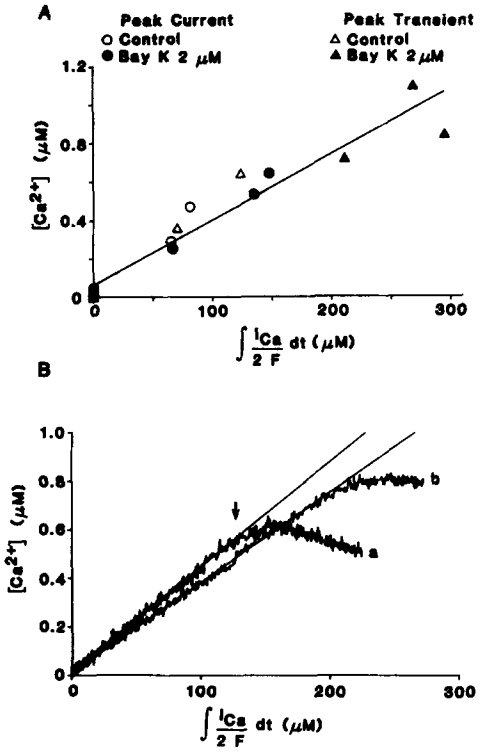

FIGURE 7. Relationship between the time integral of I_{Ca} and Ca^{2+} transient amplitude. (*A*) *Open symbols,* control; *filled symbols,* after 2 μM (−)-Bay K 8644; circles were measured at the end of the pulse while triangles were measured at the peak of the transient. The straight line was fitted to all the points in the graph. (*B*) Plot of the $[Ca^{2+}]_i$ vs. time integral of I_{Ca} for the whole time course of a pulse to 20 mV in control conditions (APIII 1.507 mM) (*a*) and after (−)-Bay K 8644 (APIII 1.635 mM) (*b*) The arrow marks the end of the pulse. The straight lines were fitted until $[Ca^{2+}]_i$ equaled 0.4 mM.

$[Ca^{2+}]_i(1 + E)$. In the event of these small signals, dCa_{rem}/dt is probably proportional to $[Ca^{2+}]_i$ (as indicated by the quasi exponential decay after the pulse) and the equation becomes:

$$\frac{d[Ca^{2+}]_i}{dt} = \frac{I_{Ca}}{2FVa(1+E)} - k[Ca^{2+}] \quad (2)$$

with its integral:

$$[Ca^{2+}]_i = \int \frac{I_{Ca}}{2FVa(1+E)}\,dt - \int k[Ca^{2+}]_i\,dt. \quad (3)$$

From this equation, it can be visualized that the limiting slope is $1/[Va(1 + E)]$ as $[Ca^{2+}]_i \rightarrow 0$.

The plot of such analysis is shown in Fig. 7 *B*. The data correspond to the pulses to 20 mV in Fig. 5. The trace labeled *a* was taken in control conditions and the one labeled *b* after the addition of (−)-Bay K. A linear relationship was found between the Ca^{2+} transient and the corresponding time integral of the I_{Ca}. This linearity was also maintained shortly after the end of the pulse during the Ca^{2+} tail currents (*arrow*). The proportional increase of $[Ca^{2+}]_i$ with I_{Ca} tends to be reduced towards the end and the curve deviates from the straight line as the integral of I_{Ca} grows with time. Such an effect was more pronounced and earlier in control conditions. This represents the second member in the right side of equation (3), and reflects the action of the removal system in the fiber. The calculated slopes of the straight lines and E were 4.47×10^{-3} and 158 for *a*, and 3.81×10^{-3} and 185 for *b*, respectively. These values are very close to the one found with the other method mentioned above. The difference between the values for E in the same fiber obtained at different times are expected because of the increase in intracellular APIII concentration. Interestingly enough, the calculated E is close to the theoretical one ($\approx$130) for the fast Ca buffers (troponin and APIII) (Potter and Gergely, 1975; Ríos and Schneider, 1981). These findings show the adequacy of APIII as a fast intracellular Ca^{2+} indicator.

Ca^{2+} Transients during Large Pulse Potentials

To further test the contribution of I_{Ca} during Ca^{2+} transients, we drastically reduced the Ca^{2+} driving force with 100-mV stimulating pulses. These large pulses were bracketed by 0-mV pulses. The experiment was discarded if the two pulses at 0 mV were different, which could have been due possibly to fiber damage. Fig. 8 *A* illustrates the results obtained from two different fibers with 0.1 mM $[EGTA]_i$. In the fiber represented in Fig. 8 *Aa*, both pulses had almost the same amplitude while for the fiber in Fig. 8 *Ab*, the pulse to 100 mV was smaller than the pulse to 0 mV and the decay of the transients was similar. We never found that the transient at 100 mV was larger than the one at 0 mV. The effect of membrane potential on Ca^{2+} transient was very clear in the fibers where Ca^{2+} release was impaired, as shown in Fig. 8 *B*. In these cases we used 1 mM $[EGTA]_i$ and 2 μM $[(-)\text{-Bay K } 8644]_o$. The pulse to 0 mV elicited a large Ca^{2+} transient while at 100 mV it was barely detectable during the pulse and the rise in Ca^{2+} signal was detected after the pulse was terminated during the large I_{Ca} tail current.

DISCUSSION

The late slow phase of Ca^{2+} transients increased following the enhancement of I_{Ca} after augmenting extracellular Ca^{2+} concentration or the addition of (−)-Bay K 8644. On the other hand, the onset and the initial peak of the Ca^{2+} transient was not modified by the same experimental maneuvers. The increment in the Ca^{2+} transient can be due to a larger input flux into the myoplasm (via tubular Ca^{2+} channels and/or Ca^{2+} release from the SR) or to a less efficient removal process. Furthermore, a large nonlinear increase in $[Ca^{2+}]_i$ is expected during pronounced Ca^{2+}

input flux, which may saturate the removal sites (Melzer et al., 1986; Brum et al., 1988*a*, *b*). Therefore, one of the questions is to define whether the increase in the Ca^{2+} transient can be directly explained by the extra Ca^{2+} influx during the enhanced I_{Ca}. The following evidence supports the theory that this is the case when Ca^{2+} release from the SR is impaired: (*a*) the close correspondence between the time integral of I_{Ca} and the time course of the Ca^{2+} transient, (*b*) the linear relationship between Ca^{2+} transient amplitude and I_{Ca} integral, and (*c*) the reduction of the Ca^{2+} transient at large positive potentials when the Ca^{2+} driving force is reduced. In view of these observations, the direct contribution of I_{Ca} to the Ca^{2+} transient was directly measured and a calibration factor between the time integral of I_{Ca} and $[Ca^{2+}]_i$ could be obtained. We calculated that 35 mC/ml of Ca^{2+} are needed per

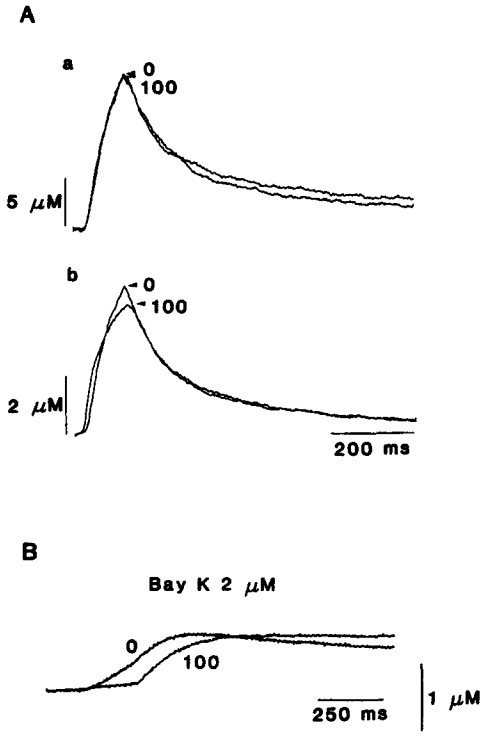

FIGURE 8. Ca^{2+} transients during large pulse potentials. Fibers were stimulated with a pulse to 100 mV, bracketed by pulses to 0 mV. Records in *A* were obtained from two different fibers with 0.1 mM $[EGTA]_i$. Transients recorded at both potentials had almost the same amplitude (*Aa*) or the transient at 100 mV was smaller than the one recorded at 0 mV (*Ab*). The decay of the transients was similar for both potentials. Records in *B* were obtained from another fiber with 1.0 mM $[EGTA]_i$ and 2 μM $[(-)$-Bay K $8644]_o$. Ca^{2+} release from the SR was impaired. Note that the transient amplitude during the pulse is larger at 0 mV. For the pulse at 100 mV, the smaller amplitude is in agreement with a reduction in the electromotive force for Ca^{2+} at this potential.

1 μM of intracellular free Ca^{2+} concentration increase with 1 mM intracellular EGTA. In the reported experiments with functioning SR (0.1 mM intracellular EGTA) the increments in Ca^{2+} transferred via the tubular Ca^{2+} channel (0.28 mC/ml in 10 mM Ca^{2+} and 1.63 mC/ml with (−)-Bay K) are too small to account for the observed large increase in the peak Ca^{2+} transients (2 μM in 10 mM Ca^{2+} and 0.8 μM in (−)-Bay K), even taking into consideration a smaller volume expansion due to the lower EGTA concentration. These calculations strongly suggest that the reported increase in $[Ca^{2+}]_i$ cannot be explained by the associated increase in Ca^{2+} influx via the tubular Ca^{2+} channel, and that an enhancement of the SR release process is taking place together with the potentiation of the Ca^{2+} influx. In accor-

dance, the reduction of Ca^{2+} transients recorded when lowering external Ca^{2+} could not be directly attributed to the smaller I_{Ca} (Brum et al., 1988*b*).

As the primary role of I_{Ca} was discarded, in a speculative way it can be thought that interactions of Ca^{2+} and (−)-Bay K 8644 with specific sites in the membrane regulate the release process from the SR, these sites being associated with the dihydropyridine receptor (Ríos and Brum, 1987). Furthermore, an amplification mechanism such as the Ca^{2+}-induced Ca^{2+} release may also be present (Endo et al., 1970; Ford and Podolsky, 1970).

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