Contraction Threshold and the "Hump" Component of Charge Movement in Frog Skeletal Muscle

G. SZŰCS, L. CSERNOCH, J. MAGYAR, and L. KOVÁCS

From the Department of Physiology, University Medical School, Debrecen, Hungary, H-4012

ABSTRACT The delayed component of intramembranous charge movement (hump, I_γ) was studied around the contraction threshold in cut skeletal muscle fibers of the frog (*Rana esculenta*) in a single Vaseline-gap voltage clamp. Charges (Q) were computed as 50-ms integrals of the ON (Q_{ON}) and OFF (Q_{OFF}) of the asymmetric currents after subtracting a baseline. The hump appeared in parallel with an excess of Q_{ON} over Q_{OFF} by ~2.5 nC/μF. Caffeine (0.75 mM) not only shifted the contraction threshold but moved both the hump and the difference between the ON and OFF charges to more negative membrane potentials. When using 10-mV voltage steps on top of different prepulse levels, the delayed component, if present, was more readily observable. The voltage dependences of the ON and OFF charges measured with these pulses were clearly different: Q_{ON} had a maximum at or slightly above the contraction threshold, while Q_{OFF} increased monotonically in the voltage range examined. Caffeine (0.75 mM) shifted this voltage dependence of Q_{ON} toward more negative membrane potentials, while that of Q_{OFF} was hardly influenced. These results show that the delayed component of intramembranous charge movement either is much slower during the OFF than during the ON, or returns to the OFF position during the pulse. Tetracaine (25 μM) had similar effects on the charge movement currents, shifting the voltage dependence of the ON charge in parallel with the contraction threshold, but to more positive membrane potentials, and leaving Q_{OFF} essentially unchanged. The direct difference between the charge movement measured in the presence of caffeine and in control solution was either biphasic or resembled the component isolated by tetracaine, suggesting a common site of caffeine and tetracaine action. The results can be understood if the released Ca plays a direct role in the generation of the hump, as proposed in the first paper of this series (Csernoch et al. 1991. *J. Gen. Physiol.* 97:845–884)

INTRODUCTION

Skeletal muscle activation is brought about by an increase in the intracellular free calcium concentration (Ebashi, Endo, and Ohtsuki, 1969) when calcium enters the myoplasmic space through the release channels of the sarcoplasmic reticulum (SR).

Address reprint requests to Dr. G. Szűcs, Department of Physiology, University Medical School, Debrecen, Hungary, H-4012.

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The opening of these channels is believed to be controlled by the membrane potential of the transverse (T) tubular system via a protein, presumably the high affinity dihydropyridine receptor (Ríos and Brum, 1987) in the T tubule walls. The change in transmembrane potential leads to a conformational change of this molecule, causing a displacement of its charged subunit(s) that can be detected as a nonlinear capacitive current or charge movement (Schneider and Chandler, 1973).

There is a delayed component (the hump, or Q_γ current; Adrian and Peres, 1979) present on these currents at or close to the contraction threshold (Horowicz and Schneider, 1981*b*). It was extensively studied as it shows a steep voltage dependence resembling that of the tension development (Huang, 1982; Hui, 1983) and calcium concentration increase (Vergara and Caputo, 1983). The hump is also tetracaine sensitive in intact (Huang, 1981) and cut fibers (Csernoch, Huang, Szűcs, and Kovács, 1988).

When described in cut fibers, humps were suggested to be a consequence of calcium release (see Horowicz and Schneider, 1981*b*). The model implies that an increase in triadic calcium concentration, through binding to fixed charges on the inner face of the T tubular membrane, causes extra nonlinear charges to move. This model has been supported by results in the two previous papers (Csernoch, Pizarro, Uribe, Rodríguez, and Ríos, 1991; García, Pizarro, Ríos, and Stefani, 1991). Though the actual calcium concentration in the triad is unknown, if neither the diffusion properties of calcium nor its binding to troponin-C and other binding sites in the myoplasm are modified after an intervention, then the same free $[Ca^{2+}]_i$ (as derived from dye signals) should correspond to the same myoplasmic calcium concentration near the triad. On the basis of this model one would expect that changes in the contraction threshold (which corresponds to a certain $[Ca^{2+}]_i$ since the same troponin saturation is reached [Kovács, Szűcs, and Csernoch, 1987]) should result in a shift of the delayed component of charge movement.

Interventions studied so far (e.g., perchlorate [Csernoch, Kovács, and Szűcs, 1987] seemed to shift both the contraction threshold and the hump component of charge movement in the same direction on the voltage axis. The only contradictory finding was that caffeine, the commonly used twitch potentiator (Axelsson and Thesleff, 1958; Sandow, Taylor, Issacson, and Seguin, 1964), which was also shown to enhance calcium-induced calcium release from the SR (Endo, 1975; Su and Hasselbach, 1984), was reported to have no effect on the voltage dependence of charge distribution (Kovács and Szűcs, 1983). This latter result, however, was derived from measurements where the depolarizing pulses applied were 20 mV apart and might have overlooked events visible only in a narrow voltage range.

The effects of caffeine applied in a low concentration were thus studied at and around the contraction threshold and were compared with those of tetracaine. Here we show that caffeine, while shifting the mechanical activation to more negative membrane potentials, also moves the hump to the new contraction threshold, leaving the rest of the charge movement unaltered. We also demonstrate that the component affected by caffeine is (all or a part of) the tetracaine-sensitive charge.

Short communications of these results have been published (Kovács, Csernoch, Magyar, and Szűcs, 1989; Kovács, Magyar, Csernoch, and Szűcs, 1990).

METHODS

Preparation and Solutions

Single fibers from the m. semitendinosus of the frog (*Rana esculenta*) were dissected in Ringer's solution ([in mM] 115 NaCl, 2.5 KCl, 1.8 $CaCl_2$, and 2 Tris sodium maleate), cut at one end after changing to relaxing solution ([in mM] 120 K-glutamate, 2 $MgCl_2$, 0.1 EGTA, and 5 Tris sodium maleate), and mounted in a single Vaseline-gap chamber (Kovács and Schneider, 1978). The relaxing solution was replaced with external solution ([in mM] 75 TEA-sulfate, 10 Cs_2SO_4, 8 $CaSO_4$, 3.1×10^{-4} tetrodotoxin, and 5 Tris sodium maleate) in the closed end pool and with internal solution ([in mM] 108 Cs-glutamate, 5.5 $MgCl_2$, 0.1 EGTA, 0.0082 $CaCl_2$, 4.5 Tris sodium maleate, 13.2 cesium maleate, 5 ATP, and 6.5 glucose) in the open end pool. The compositions are designed to minimize ionic currents that might be activated at the contraction threshold. The estimated free $[Ca^{2+}]_i$ is 43 nM using a 0.49 μM dissociation constant for the calcium EGTA reaction. Tetracaine and caffeine were applied extracellularly at concentrations of 25 μM and 0.75 mM, respectively. Experiments were carried out at low temperatures (4–6°C).

Charge Movements and Calcium Transients

The working segment of the fiber was under voltage clamp control Kovács and Schneider, 1978; Kovács and Szűcs, 1983). From a holding potential of −100 mV depolarizing and hyperpolarizing pulses were applied and the accompanying currents and changes in light absorbance were recorded. Data were acquired every 200 μs with upper frequency cutoffs at 10 and 1 kHz for the current and the optical records, respectively. Five consecutive points were then averaged to obtain the corresponding data point for each millisecond.

Charge movement was calculated from the current records as in Horowicz and Schneider (1981*a*) by subtracting first the "linear capacitive" component, then a sloping baseline. As in the other papers of this series, the records shown are corrected only for the linear capacitance that was derived from the OFF parts of 30-mV hyperpolarizing pulses. The sloping baseline, subtracted in the calculation of charge moved by the voltage change, together with the interval where the fitting was done, is shown superimposed on the traces. All nonlinear currents were normalized to total linear capacitance.

Changes in intracellular free calcium concentration were determined from changes in fiber absorbance at 720 nm using the metallochromic indicator antipyrylazo III, as described by Kovács, Ríos, and Schneider (1983) after correcting for intrinsic absorbance changes measured at 850 nm (Melzer, Ríos, and Schneider, 1986). The dye was added into the internal solution in 1 mM concentration, and reached the terminated segment by diffusion.

Determination of the Contraction Threshold

The contraction threshold was determined with depolarizing pulses of 100 ms in duration and of increasing amplitude. Movement was checked visually with 400× magnification using a compound microscope with a water immersion objective. Further details are given elsewhere (Kovács and Szűcs, 1983).

Movement Artifacts

Charge movement currents sometimes show movement artifacts when measured in single Vaseline-gap chambers. This phenomenon, however, is not present unless the fiber is depolarized at least 10 mV more positive than the contraction threshold (see, for example,

Horowicz and Schneider, 1981*a*). Consequently, in these measurements pulses not exceeding the threshold by more than 5 mV were applied.

Statistics

When calculating statistical significance, the Student's paired *t* test was used. Averages are expressed as mean ± SEM.

RESULTS

Delayed Component in Caffeine

Intramembrane charge movements, when measured at the contraction threshold, show a distinct slow component which is not present if the fiber is depolarized to a ~10 mV more negative level. The left column of Fig. 1 shows a typical example in

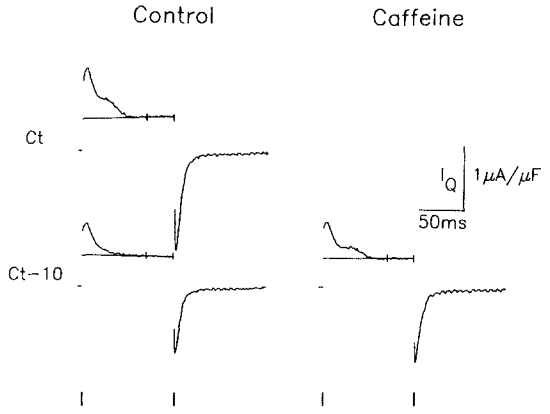

FIGURE 1. Charge movement currents recorded at the contraction threshold (Ct = −41.5 mV) in control solution and at a 10 mV more negative membrane potential (Ct − 10) in the absence and in the presence of 0.75 mM caffeine (the pulse was suprathreshold by 2.3 mV when caffeine was present). Here and in all subsequent figures the records shown are corrected only for the linear capacitance. Sloping baselines were then fitted and subtracted to calculate the charge moved by the pulses, these are shown superimposed on the traces. The vertical bars placed on the current records represent the beginning and the end of the interval for baseline fit, while those below the traces mark the onset and the end of the depolarizing pulse. Fiber 81104A. Sarcomere length, 2.1 μm. Temperature, 5.9°C.

control solution where the charge movements were recorded at the contraction threshold (Ct) (Ct = −41.5 mV) and at a 10 mV more negative membrane potential (Ct − 10). The exponential decay of the current seen at the smaller depolarization is clearly interrupted by a secondary rising phase on the record measured at the contraction threshold. On adding 0.75 mM caffeine to the external solution, the contraction threshold was shifted toward the resting membrane potential (to −53.8 mV in this case) as reported earlier (Kovács and Szűcs, 1983). Though caffeine is believed to act specifically on SR calcium release (Kumbaraci and Nastuk, 1982), an obvious change is seen on the charge movement measured with a depolarization to Ct − 10 mV (Fig. 1, right column): the appearance of the secondary rising phase. That is, caffeine not only shifted the threshold for mechanical activation but also moved the delayed component of intramembrane charge movement to more nega-

tive membrane potentials (the depolarization in Fig. 1 in caffeine is slightly suprathreshold, by 2.3 mV).

ON and OFF Inequality in Caffeine

It was mentioned in the first paper of this series (Csernoch et al., 1991) that there is an apparent difference between the amounts of ON and OFF charge at moderate depolarizations (around −50 to −40 mV) associated with the delayed component. This difference was also present in the charge movement currents in Fig. 1: Q_{ON} = 18.0 nC/μF and Q_{OFF} = 13.8 nC/μF in control solution at contraction threshold, while Q_{ON} = 14.0 nC/μF and Q_{OFF} = 9.9 nC/μF in the presence of caffeine.

The charge moved during the ON (Q_{ON}) and during the OFF (Q_{OFF}), and their difference ($Q_{ON} - Q_{OFF}$), is compared at and near the contraction threshold in Table I. The average values (n = 5) at the reference threshold (Ct) and at the threshold

TABLE I

The Effect of 0.75 mM Caffeine on the Nonlinear Charges Moved at and Near the Contraction Threshold

	Reference				0.75 mM caffeine			
	Membrane potential	Q_{ON}	Q_{OFF}	Q_{ON-OFF}	Membrane potential	Q_{ON}	Q_{OFF}	Q_{ON-OFF}
	mV	*nC/μF*	*nC/μF*	*nC/μF*	*mV*	*nC/μF*	*nC/μF*	*nC/μF*
A								
Mean	−40.8	16.34	13.95	2.50	−48.5	12.98	10.61	2.39
±SE	±1.4	±0.97	±0.61	±0.59	±2.2	±1.22	±0.91	±0.47
B								
Mean	−41.9	16.44	14.29	2.16				
±SE	±1.9	±1.27	±1.13	±0.66				
Mean	−51.9	8.84	8.55	0.30	−51.9	12.36	10.44	1.92
±SE	±1.9	±0.69	±0.63	±0.59	±1.9	±1.43	±0.53	±1.20

Part *A* presents the average (n = 5) of the nonlinear charge moved at the contraction threshold determined in reference solution and after the addition of caffeine. Part *B* shows the results from measurements (n = 5) done at the reference contraction threshold (top row) and at a 10 mV more negative potential (bottom row).

measured in the presence of 0.75 mM caffeine are shown in part *A*. Q_{ON} was significantly greater ($p < 2\%$) than Q_{OFF}, both in control solution and in the presence of caffeine. Furthermore, the $Q_{ON} - Q_{OFF}$ values, as determined in the two solutions, did not differ statistically from each other.

Part *B* of Table I shows the results of experiments (n = 5; three fibers are the same as in part *A*) that followed the strategy presented in Fig. 1. The measurements were done first at the contraction threshold (upper row in part *B*) as determined in control solution, then at a 10 mV more negative level (lower row) both before and after the addition of caffeine. The excess of Q_{ON} over Q_{OFF}, which is present at the threshold, seems absent (the excess is not significant; $p > 60\%$) at the more negative voltages in reference solution. The caffeine treatment moves the difference of Q_{ON} and Q_{OFF} to

this more negative potential; that is, the $Q_{ON} - Q_{OFF}$ values determined in caffeine are significantly greater ($p < 5\%$) than those measured in reference solution. The results show that the difference between Q_{ON} and Q_{OFF} is tightly coupled to the voltage range where the movement appears.

Low Tetracaine Concentration

Similar effects on the slow component were seen with 25 μM tetracaine, but the shift in this case was toward more positive membrane potentials. Fig. 2 demonstrates that the hump, present in control solution at the contraction threshold (left column), disappears after tetracaine treatment at this voltage, while the mechanical threshold is shifted from −50.5 mV to −43.9 mV. The difference between Q_{ON} and Q_{OFF}, 14.7 ± 1.22 and 11.4 ± 0.98 nC/μF, respectively ($n = 5$) is not present after tetracaine treatment (11.9 ± 1.89 and 11.0 ± 1.33 nC/μF). Charge movements at the new threshold showed less distinct humps (though the falling phase of the currents was not a single exponential), like that in Fig. 2 (right column, lower trace); nevertheless,

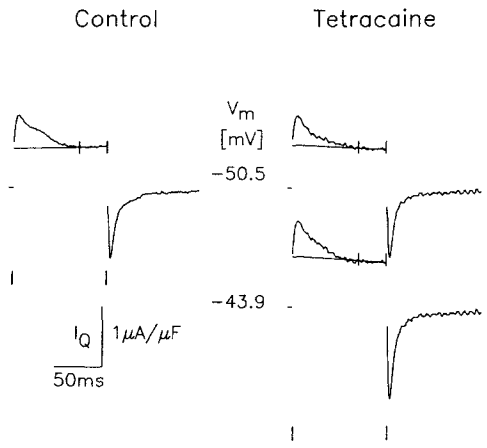

FIGURE 2. Charge movement currents measured in control solution and in the presence of 25 μM tetracaine at the contraction threshold determined in control solution (*upper traces*) and after treatment with the drug (*lower trace*). The membrane potentials during the pulses are indicated next to the corresponding traces. Fiber 81130. Sarcomere length, 2.5 μm. Temperature, 5.5°C.

the ON and OFF inequality reappeared: Q_{ON} = 17.8 ± 1.09 nC/μF, Q_{OFF} = 15.1 ± 1.02 nC/μF ($n = 5$).

Prepulse Experiments

The study of humps with pulses directly to the contraction threshold requires the kinetic resolution of a component carrying ~2.5 nC/μF of charge from the rest of the charge movement, which is ~14 nC/μF (see values for $Q_{ON} - Q_{OFF}$ and Q_{OFF} in Table I, under Reference at the contraction threshold). It would therefore be useful to find a pulse protocol that maximizes the I_γ/I_β ratio. One such protocol is suggested by Adrian and Huang (1984), showing that a subthreshold prepulse substantially reduces the magnitude of I_β but not of I_γ moving during a subsequent test pulse. This possibility may also be surmised from Fig. 1, which suggests that if a prepulse to a 10 mV more negative level than the contraction threshold precedes a 10-mV test pulse, an appreciable I_β current and little I_γ is moved during the prepulse. That is, the

subsequent 10-mV test pulse will result in a current having substantially less I_β and most of I_γ if compared with that moved by a depolarization done in one step.

Fig. 3 shows the effect of a prepulse on the charge movement and on the calcium transient elicited by a just threshold test pulse. During the prepulse the current decays essentially with a single exponential time course, and only a slight increase in $[Ca^{2+}]_i$ is seen. A pronounced calcium release and the delayed component of charge transfer appear when the membrane is further depolarized to the contraction threshold. The charge moved during the prepulse and the test pulse was 8.57 and 7.86 nC/μF, respectively; that is, ~60% of Q_β (using Q_{OFF} = 14.47 nC/μF as an upper limit for Q_β) was removed from the test pulse.

The time course of the calcium release from the SR cannot be correctly estimated from the calcium transients measured on moving fibers since pulses that would be big

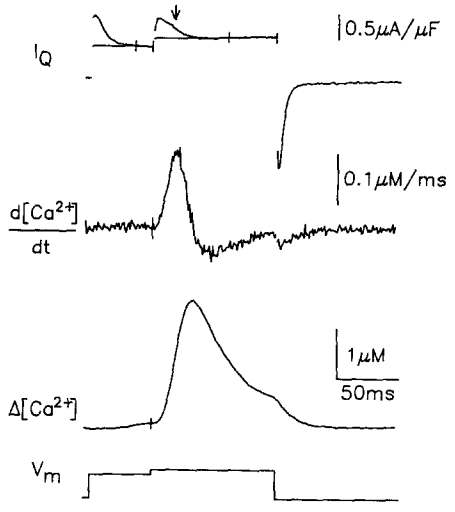

FIGURE 3. The effect of a prepulse (to −52.4 mV) on the charge movement current (I_Q) and on the calcium transient ($\Delta[Ca^{2+}]$) measured at the contraction threshold (−42.4 mV). The pulse protocol (V_m) is shown below the traces. To represent the time course of the calcium release from the sarcoplasmic reticulum at early times, the first derivative of the calcium transient ($d[Ca^{2+}]/dt$) is also shown. For kinetic comparison with the delayed component, an arrow is aligned at the charge movement current with the peak of the first derivative. Vertical bars are also placed on the calcium transient and on its first derivative to show the onset of the test pulse. Fiber 60325A. Sarcomere length, 2.3 μm. Temperature, 4.5°C.

enough to saturate the uptake system would have movement artifacts. Hence, for kinetic comparison with the charge movement record, the first derivative of the calcium transient is shown (Fig. 3, middle trace), which correctly describes the time course of the calcium release at early times. It can be seen from Fig. 3 that though the charge movement current during the 10-mV test pulse starts earlier than the calcium concentration change (which is expected from the kinetics of I_β), the delayed component becomes evident at about the time when the first derivative reaches its peak. This is consistent with the kinetic comparisons of I_γ and Ca release flux in the first paper of this series (Csernoch et al., 1991).

The leftmost column of Fig. 4 shows how changes in the prepulse level affect the subsequent charge movement, measured with 10-mV test pulses reaching the voltage given next to the traces. The pulse protocol (Fig. 4, *inset*) was slightly modified compared with that in Fig. 3 (a 100-ms prepulse and a 150-ms postpulse to the same

potential bracketed the test pulse), enabling us to also determine the Q_{OFF} corresponding to the test pulse. There is a clear hump during the ON that becomes more and more prominent as the test voltage reaches the contraction threshold (Ct). This delayed component seems to speed up, and is followed by an undershoot at more positive membrane potentials. The OFF transients are single exponential decays, as described earlier (e.g., Adrian and Almers, 1976), and they increase with increasing depolarization.

Caffeine, at 0.75 mM concentration, shifted the appearance of the hump toward more negative membrane potentials (Fig. 4, middle column) without affecting the

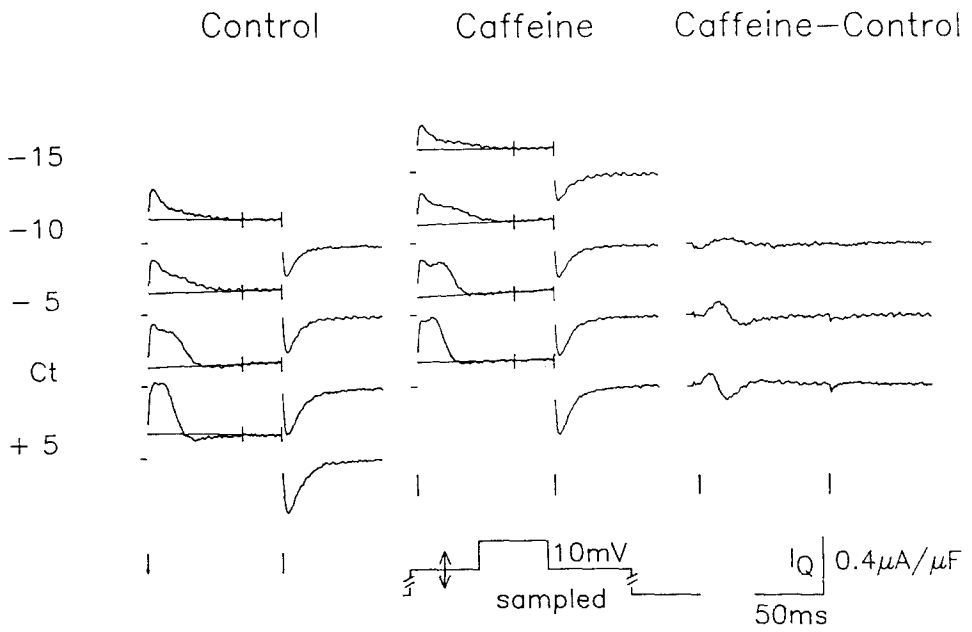

FIGURE 4. Charge movement currents obtained in response to a 10-mV voltage step preceded by a prepulse with varying amplitude (see inset) in control solution and in the presence of 0.75 mM caffeine. The difference between caffeine and control is given in the rightmost column. The membrane potential values during the test pulse, as the difference from the contraction threshold (Ct = −41.5 mV, determined in control solution), are shown next to the corresponding traces. Same fiber as in Fig. 1.

OFF part. The direct difference, caffeine − control (Fig. 4, right column), shows a marked asymmetry between ON and OFF. The ON has the shape of a hump at more negative membrane potentials and resembles that of the tetracaine-sensitive charge (see later) or the component isolated with any of the protocols of the preceding papers. It becomes biphasic at increasing depolarizations, indicating a faster movement in caffeine or the occurrence of an inward phase. The OFF transients are small and very slow and seem to change sign when the negative-going part in the biphasic ON becomes dominant.

The average values (n = 5) of Q_{ON}, Q_{OFF}, and $Q_{ON} - Q_{OFF}$ in control solution and in the presence of 0.75 mM caffeine were plotted vs. voltage in Fig. 5 to show their membrane potential dependence near the contraction threshold. Q_{ON} and the difference between Q_{ON} and Q_{OFF} had a maximum at or slightly above the threshold and decreased with both increasing and decreasing voltage. While only slightly influencing the OFF charge, caffeine shifted this bell-shaped voltage dependence of $Q_{ON} - Q_{OFF}$ to more negative membrane potentials.

These results confirm that there is an excess of Q_{ON} over Q_{OFF} around the mechanical threshold, which is tightly coupled to the presence of the delayed

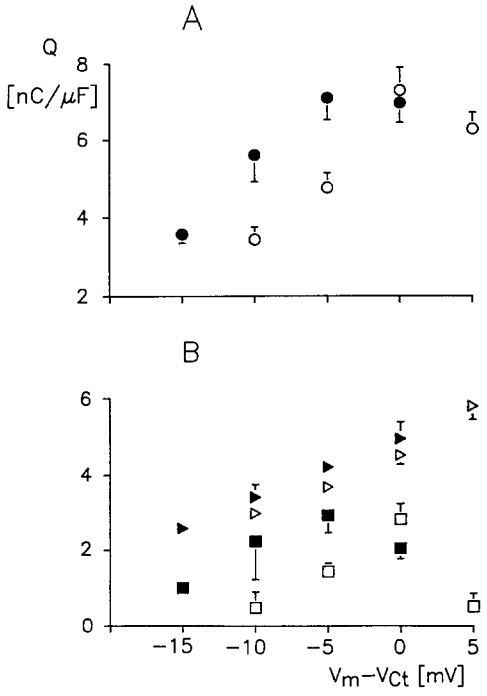

FIGURE 5. Voltage dependence of the nonlinear charges moved around the contraction threshold measured with pulses 10 mV in amplitude (protocol as in Fig. 4) in control solution (*open symbols*) and in the presence of 0.75 mM caffeine (*filled symbols*). *A* shows the membrane potential dependence of Q_{ON}, while *B* gives the corresponding values of Q_{OFF} (▷, ▶) and of $Q_{ON} - Q_{OFF}$ (□, ■). The plotted values are the mean; vertical bars represent the SEM (n = 5).

component. Caffeine seems to affect only this part of the charge movement, suggesting that it is a consequence of calcium release.

Prepulses in Tetracaine

Measurements similar to those in Fig. 4, using the prepulse protocol, were carried out in the presence of 25 μM tetracaine to test whether the component affected by caffeine is the same as the tetracaine-sensitive charge. Fig. 6 shows a typical experiment where tetracaine shifted the Ct from −51.4 mV to −43.9 mV. The slow components present in control solution (left column) disappear after tetracaine treatment (middle column) if the membrane is depolarized to the same potential. Though decays more complex than single exponentials seem to be present at more

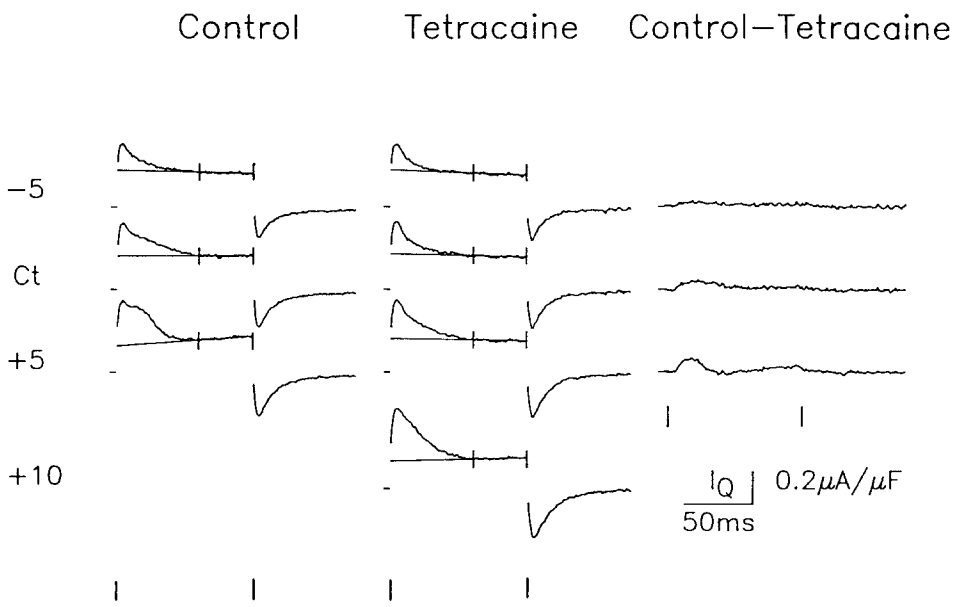

FIGURE 6. Charge movement currents in control solution and in the presence of 25 μM tetracaine measured with pulses 10 mV in amplitude (for protocol see Fig. 4). The charge component suppressed by tetracaine is shown in the rightmost column. Same fiber as in Fig. 2.

positive membrane potentials in the presence of the drug (see Ct + 10), they do not appear as distinct humps, as in control solution.

The tetracaine-sensitive charge isolated in this way (right column) shows a bell-shaped ON and only hints at an OFF transient. Though less biphasic, these records are strikingly similar to those obtained with caffeine, suggesting that the hump isolated with caffeine is more or less equal to Q_γ (or at least to the hump isolated with this concentration of tetracaine).

The membrane potential dependence of Q_{ON} and Q_{OFF} before and after tetracaine treatment is shown in Fig. 7. Similar to caffeine, tetracaine at this concentration leaves the amount of charge moved during the OFF unaltered, while the voltage

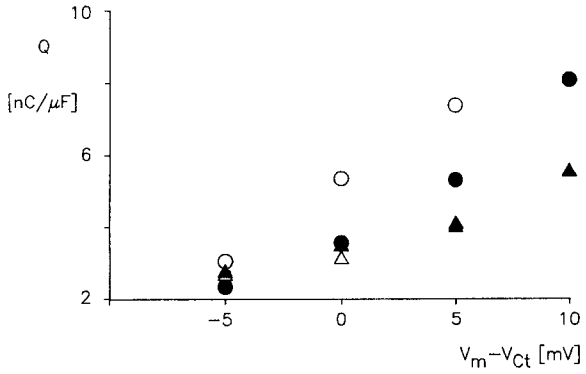

FIGURE 7. Voltage dependence of the charges moved during the ON (○, ●) and OFF (△, ▲) of the nonlinear charging currents obtained in response to 10-mV voltage steps measured in control solution (*open symbols*) and in the presence of 25 μM tetracaine (*filled symbols*) and in the presence of 25 μM tetracaine (*filled symbols*). Same fiber as in Fig. 6.

dependence of Q_{ON} is shifted in parallel with the contraction threshold, that is, toward more positive membrane potentials.

Tetracaine- and Caffeine-sensitive Charge

For direct comparison, the charge movement components affected by tetracaine and caffeine are shown together in Fig. 8. The records were obtained by directly subtracting the traces shown in Fig. 1 (Ct − 10) for caffeine − control and in Fig. 2 (−50.5 mV, the control contraction threshold) for control − tetracaine (Fig. 8 *A*). Fig. 8 *B* pulls records from the measurements with prepulses, that is, from Fig. 4 (the row labeled Ct − 10) and from Fig. 6 (the row labeled Ct).

Though recorded on different fibers, the similarity of the two components is obvious. The caffeine-sensitive part of the charge movement record thus seems to be

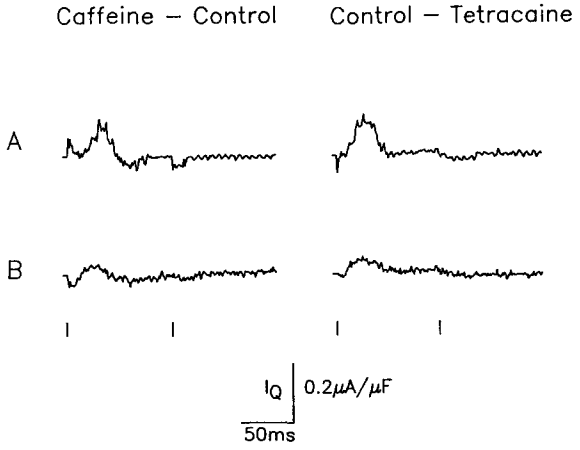

FIGURE 8. Charge movement components affected by caffeine (*caffeine − control*) and by tetracaine (*control − tetracaine*). *A* shows the difference of the charge movement currents from Fig. 1 (*Ct − 10* under *caffeine − control*) and from Fig. 2 (*−50.5 mV* under *control − tetracaine*). *B* repeats the difference records from Fig. 4 (*Ct − 10*) and Fig. 6 (*Ct*) for the caffeine- and tetracaine-sensitive charges, respectively. The depolarization was done in one step (*A*), or using the prepulse protocol (*B*), changing the membrane potential to the reference contraction threshold (control − tetracaine) and to a 10 mV more negative level (*caffeine − control*), which was slightly suprathreshold (by 2.3 mV) in caffeine. Two different fibers for the two columns: the same as in Fig. 1 for *caffeine − control,* and the same as in Fig. 2 for *tetracaine − control.*

the tetracaine-sensitive charge, or part of it (there is indirect evidence that 25 μM tetracaine does not remove all the tetracaine-sensitive charge). These results clearly indicate that caffeine shifts the delayed (Q_γ) component of the charge movement currents to more negative membrane potentials, and together with the finding that this component appears as an excess of Q_{ON} over Q_{OFF} they strengthen the idea (Csernoch et al., 1991) that the released calcium plays a direct role in the generation of the hump.

DISCUSSION

These experiments studied the effects of caffeine on intramembranous charge movement at and around the contraction threshold and compared the changes with those induced by tetracaine. Together with the other papers of this series, we present

new findings indicating that the hump component of charge movement is secondary to calcium release. Details of the model and its possible physiological importance have been discussed elsewhere (Pizarro, Rodríguez, Csernoch, and Ríos, 1990*b*; Csernoch et al., 1991) and a quantitative simulation is presented in the next paper. We are aware that some of the results reported here can be explained in terms of a direct action on a subgroup of charges, though no such effect of caffeine has been published so far. The following discussion, however, shows that the observed effects—the shift of the hump by caffeine, the excess of Q_{ON} over Q_{OFF}, and the caffeine/tetracaine antagonism—are better interpreted assuming that the hump is a consequence of calcium release.

Are the Effects of Caffeine Secondary to Changes in Resting Calcium Levels?

Caffeine is believed to increase the resting free $[Ca^{2+}]_i$ when applied in subthreshold concentrations (Konishi, Kurihara, and Sakai, 1985). This increase, through binding to fixed charges on the inner surface of the T tubular membrane, could shift the voltage-dependent processes to more negative membrane potentials and hence account for most of the reported effects. The shift, however, would be present in the Q vs. V curve; that is, the membrane potential dependence of both Q_{ON} and Q_{OFF} should have been shifted, which was not the case. Moreover, there is indication that in the presence of 20 mM EGTA humps seem to appear at the same voltage as in control solution (see, for example, Horowicz and Schneider, 1981*a*).

An Inward Component of Asymmetric Current

A distinct undershoot (see, for example, Fig. 4, left column, lower traces) sometimes followed the ON transients, which, if present, appeared at more negative voltages in caffeine. This can either be due to the activation of an outward ionic current or can be a truly inward component of asymmetric current, which can be explained within the framework of the model (Csernoch et al., 1991; Pizarro, Csernoch, Uribe, Rodríguez, and Ríos, 1991). Though we cannot exclude a shift in the voltage dependence of current activation induced by caffeine (Akaike and Sadoshima, 1989) or the presence of a calcium-activated current on the basis of our data, other measurements (Pizarro, Csernoch, and Ríos, 1990*a*) render this interpretation unlikely. If, as we believe, this is an actual inward phase in charge movement, it cannot be explained by schemes in which Q_β and Q_γ are different species of charge responding independently to the step change in potential.

ON/OFF Inequality and Humps

A significant excess of Q_{ON} over Q_{OFF} was observed on the charge movement currents, closely associated with the contraction threshold. This inequality could result from an incorrect estimation of the sloping baseline due to the presence of the undershoot (see the discussion in Csernoch et al., 1991). The baseline used in our correction procedure, fitted over relatively short intervals, may be riding on a slow inward phase during the ON, exaggerating the ON area. On the other hand, the current differences in Fig. 8 suggest that the ON/OFF discrepancy could also be due to a very slow return of part of the charge during the OFF. This slow OFF charge movement is

explained within the model by supposing that the return of the charged particles to their resting state reflects the dissociation of Ca^{2+} from the hypothetical binding sites, and the $[Ca^{2+}]$ remains somewhat elevated after the pulse. The inequality of Q_{ON} and Q_{OFF} is more prominent in the present experiments than in those of García et al. (1991) and Pizarro et al. (1991). This may be due to the use of high intracellular [EGTA] in those studies. High [EGTA] should result in a rapid return of $[Ca^{2+}]_i$ to its very low starting levels, allowing the occupancy of the hypothetical sites to return to normal and permitting equality of Q_{ON} and Q_{OFF}.

ON and OFF inequality at the mechanical threshold, though present in all cases, showed prominent fiber-to-fiber fluctuations (in the range of 1–4 nC/μF) with no direct relation between the observability of the hump and the difference in Q_{ON} and Q_{OFF}. That is, ~2 nC/μF excess could appear as a distinct secondary rising phase or could correspond only to a change in the decay time constant of the current record. Modifications in properties of calcium-removing processes or in the triadic structure, which cause changes in timing of the local $[Ca^{2+}]$ transients, might be the underlying reason. This observation could explain why earlier reports, for example on stretched fibers, overlooked such slow components.

Humps were less distinct in tetracaine at the altered threshold (see Fig. 2), though Q_{ON} was significantly greater than Q_{OFF}. This could be due to the fact that at more positive membrane potentials the distribution depends less strongly on voltage, so the extra potential due to calcium binding moves less charge. On the other hand, this intervention changes the kinetics of calcium release by suppressing the early peak (Csernoch et al., 1991), and this might result in the loosing of fast and especially hump-like slow components.

When shifting the contraction threshold to more negative membrane potentials with caffeine, humps were more readily observable. This speeding effect of caffeine agrees with the observation that the drug potentiates the early peak of calcium release through a calcium-induced calcium release process (Simon, Klein, and Schneider, 1989).

Humps seemed to precede the peak of the first derivative of the calcium transient, which, at that time, describes the kinetics of the release from the SR. This timing is consistent with the Ca^{2+}-binding model, which requires the kinetics of release to be close to $Q(t)$ and not to $I_Q(t)$ (for discussion see Csernoch et al., 1991, and Pizarro et al., 1991).

Antagonistic Effects of Caffeine and Tetracaine

Both tetracaine and caffeine shifted the contraction threshold, in agreement with earlier reports (Lüttgau and Oetliker, 1968), and this was paralleled by a similar shift in the appearance of (*a*) the hump and (*b*) the ON and OFF inequality. Both drugs, studied with the prepulse procedure, moved the peak of the voltage dependence of Q_{ON} approximately to the new contraction threshold, while neither of them influenced the OFF charge. The effects are strikingly similar, though the shifts in the voltage axis are in the opposite direction. In addition, tetracaine at a higher concentration than that used here was shown to prevent caffeine contractures (Lüttgau and Oetliker, 1968). These results strongly suggest a common site of

tetracaine and caffeine action, namely, the SR calcium release channel, where they seem to antagonize each other's effect.

The results presented imply that either caffeine has two sites of action or there is a component of charge movement caused by calcium release. The problem cannot be solved on the basis of these data, but together with the interventions studied in the other papers of this series, our observations further strengthen the idea that calcium released from the SR influences the intramembranous voltage sensors, causing the hump component.

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