Effect of the Calcium Buffer EGTA on the "Hump" Component of Charge Movement in Skeletal Muscle

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ABSTRACT Three manifestations of excitation–contraction (E–C) coupling were measured in cut skeletal muscle fibers of the frog, voltage clamped in a double Vaseline gap: intramembrane charge movements, myoplasmic Ca^{2+} transients, and changes in optical transparency. Pulsing patterns in the presence of high [EGTA] intracellularly, shown by García et al. (1989. *J. Gen. Physiol.* 94:973–986) to deplete Ca^{2+} in the sarcoplasmic reticulum, were found to change the above manifestations. With an intracellular solution containing 15 mM EGTA and 0 Ca, 10–15 pulses (100 ms) to −20 mV at a frequency of 2 min^{-1} reduced the "hump" component of charge movement current. This effect was reversible by 5 min of rest. The same effect was obtained in 62.5 mM EGTA and 0 Ca by pulsing at 0.2 min^{-1}. This effect was reversible by adding calcium to the EGTA solution, for a nominal $[Ca^{2+}]_i$ of 200 nM, and was prevented by adding calcium to the EGTA solution before pulsing. The suppression of the hump was accompanied by elimination of the optical manifestations of E–C coupling. The current suppressed was found by subtraction and had the following properties: delayed onset, a peak at a variable interval (10–20 ms) into the pulse, a negative phase (inward current) after the peak, and a variable OFF transient that could be multi-phasic and carried less charge than the ON transient. In the previous paper (Csernoch et al., 1991. *J. Gen. Physiol.* 97:845–884) it was shown that several interventions suppress a similar component of charge movement current, identified with the "hump" or Q_γ current (I_γ). Based on the similarity to that component, the charge movement suppressed by the depletion protocols can also be identified with I_γ. The fact that I_γ is suppressed by Ca^{2+} depletion and the kinetic properties of the charge suppressed is inconsistent with the existence of separate sets of voltage sensors underlying the two components of charge movement, Q_β and Q_γ. This is explicable if Q_γ is a consequence of calcium release from the sarcoplasmic reticulum.

INTRODUCTION

The physiological release of calcium from the sarcoplasmic reticulum (SR) is controlled in skeletal muscle by a device in the transverse (T) tubules, presumably an integral membrane protein, that responds to changes in transmembrane voltage with conformational changes. These conformational changes are accompanied by measurable currents of "intramembrane charge movement" (Schneider and Chandler, 1973). The existence of a causal relationship between charge movement and Ca^{2+} release has been supported by the demonstration that interventions intended to inhibit the voltage sensor (Ca antagonist drugs, low extracellular $[Ca^{2+}]$, voltage-dependent inactivation) depress both charge movement and Ca^{2+} release, to a similar extent and with similar kinetics (Chandler, Rakowski, and Schneider, 1976; Ríos and Brum, 1987; Brum, Fitts, Pizarro, and Ríos, 1988a; Brum, Rios, and Stefani, 1988b; Feldmeyer, Melzer, and Pohl, 1990).

In the first paper of this series (Csernoch, Pizarro, Uribe, Rodríguez, and Ríos, 1991), the relationship between charge movement and Ca^{2+} release was probed with a reverse approach, the application of interventions expected to block or diminish the phenomenon of Ca^{2+} release. Even though such interventions should not primarily affect the voltage sensor, they were shown to selectively affect a delayed component of intramembrane charge movement (I_γ). These observations are explicable if Q_γ is a consequence of Ca^{2+} release.

One of the interventions in the previous paper was a conditioning pulse protocol intended to cause depletion of Ca^{2+} in the SR. The success with this protocol was limited, as it probably inhibited release through both depletion and Ca^{2+}-dependent inactivation, and in general did not have large effects. In this paper a more radical approach is used to cause depletion. High concentrations of EGTA are added to the internal solution, thus lowering the $[Ca^{2+}]_i$ beyond 10^{-10} M and greatly increasing the buffering capacity of the myoplasm. Under similar conditions (with less extreme buffering) García, Amador, and Stefani (1989) demonstrated that the SR ceased to contribute to the Ca^{2+} transients, as if it was completely depleted. Here we show that this treatment leads to selective and reversible loss of a delayed component of charge movement, with all the properties of the intervention-sensitive current described in the previous paper.

METHODS

The experiments were conducted on cut segments of fast twitch fibers of the semitendinosus muscle of leopard frogs (*Rana pipiens* and *Rana sphenocephala*). The fiber segments were voltage clamped in double Vaseline-gap devices (Kovacs, Ríos, and Schneider, 1979) where they were held at a steady potential of −90 mV and subjected to various patterns of pulse depolarization while recording intramembrane charge movements and, in some cases, optical signals of excitation–contraction (E–C) coupling. The experimental setup is described in detail in Francini and Stefani (1989) and in García et al. (1989). In this setup the dimensions (and diffusion distances) in the Vaseline-gap chamber are optimized for rapid equilibration of the interior of the cell with the solutions in the end compartments. They are somewhat smaller than in the original design of Kovacs et al. (1979).

Intramembrane charge movement currents were recorded by conventional methods. The internal and external solutions had impermeant ions substituted for the permeant, physiologi-

cal ones; asymmetric currents $(\Delta I_a(t))$ were obtained as differences between total current during a depolarizing test pulse and current during a control pulse. The control pulse was also depolarizing, but started from a prepulse level of -120 mV and had an amplitude not greater than 30 mV. The control current was scaled to match control and test pulse amplitudes. $\Delta I_a(t)$ records are in all cases single sweeps, not corrected for sloping baseline.

In addition to this conventional protocol, a second one was used (protocol 1, Csernoch et al., 1991), shown in the previous paper to permit the isolation of a delayed component of charge movement current. Protocol 1 involves the subtraction of total current during two pulses to the same voltage, with the second one preceded by a conditioning pulse (cf. schematic of protocol in Fig. 5).

Ca^{2+} transients (time course of the change in free intracellular calcium concentration) were recorded from absorbance changes in the presence of the metallochromic dye antipyrylazo III (ApIII) by methods described in published works (Kovacs, Ríos, and Schneider, 1983; Brum et al., 1988*b*; Csernoch et al., 1991). In other experiments in which no dye was present, the intrinsic optical (scattering) signal of E–C coupling (Hill, 1949; Barry and Carnay, 1969; Kovacs and Schneider, 1977; Ríos, Melzer, and Schneider, 1983; Ríos, Brum, and Pizarro, 1990) was used as an indication of E–C coupling activity, roughly monitoring the time integral of $[Ca^{2+}]_i$ (Ríos et al., 1983, 1990).

All experiments were carried out in cut fibers mounted at a sarcomeric space of 2–2.5 µm in the double gap chambers. Since all internal solutions contained at least 15 mM EGTA, contractile movement was not expected and was rarely observed. Diameter of the fibers was measured as the distance between both edges.

Solutions

The composition of the external solution was always (in mM): 105 TEA-methanesulfonate, 2 $CaCl_2$, 8 $CoCl_2$, 5 TEA/HEPES, 1 3,4-diaminopyridine, 5 glucose, and 0.0005 TTX. Three internal solutions were used. 15 EGTA (in mM): 15 $(Cs)_2EGTA$, 6.9 $MgCl_2$, 50 Cs-glutamate, 20 Na-pyruvate, 20 Cs-HEPES, 5 glucose, 5 Na_2-phosphocreatine, and 5 Na_2-ATP. High EGTA, 0 Ca (in mM): 62.5 $(Cs)_2EGTA$, 6.9 $MgCl_2$, 10 Cs-HEPES, 5 glucose, 5 Na_2-phosphocreatine, and 5 Na_2-ATP. High EGTA, 200 nM Ca (in mM): 62.5 $(Cs_2)EGTA$, 6.9 $MgCl_2$, 23.6 $CaCl_2$, 10 Cs-HEPES, 5 glucose, 5 Na_2-phosphocreatinine, and 5 Na_2-ATP. In all the solutions, the pH was adjusted to 7.0. Temperature was kept at 12°C and monitored with a thermistor probe placed near the fiber in the central pool. The calculated K_d for Ca-EGTA at this pH and temperature was 0.428 µM. The osmolarity was checked and corrected to 260 mosM with sucrose when necessary.

RESULTS

Association of Q_γ and Ca^{2+} Release

First we extend the observations of close association between I_q and Ca^{2+} release (Csernoch et al., 1991) to unstretched fibers with high EGTA. Fig. 1 shows traces of (from top to bottom) membrane currents, Ca^{2+} transients, calculated Ca^{2+} release flux, and the intrinsic optical signal at 850 nm obtained simultaneously from the same fiber at different potentials. The three recorded signals showed parallel changes. At -50 mV neither Ca^{2+} transients nor intrinsic optical signals were observed, indicating that Ca^{2+} was not released from the SR, and I_γ was not detected. When the fiber was depolarized to a more positive potential (-25 mV) there was Ca^{2+} release from the SR and an I_γ component appeared. At -10 mV the intrinsic signal
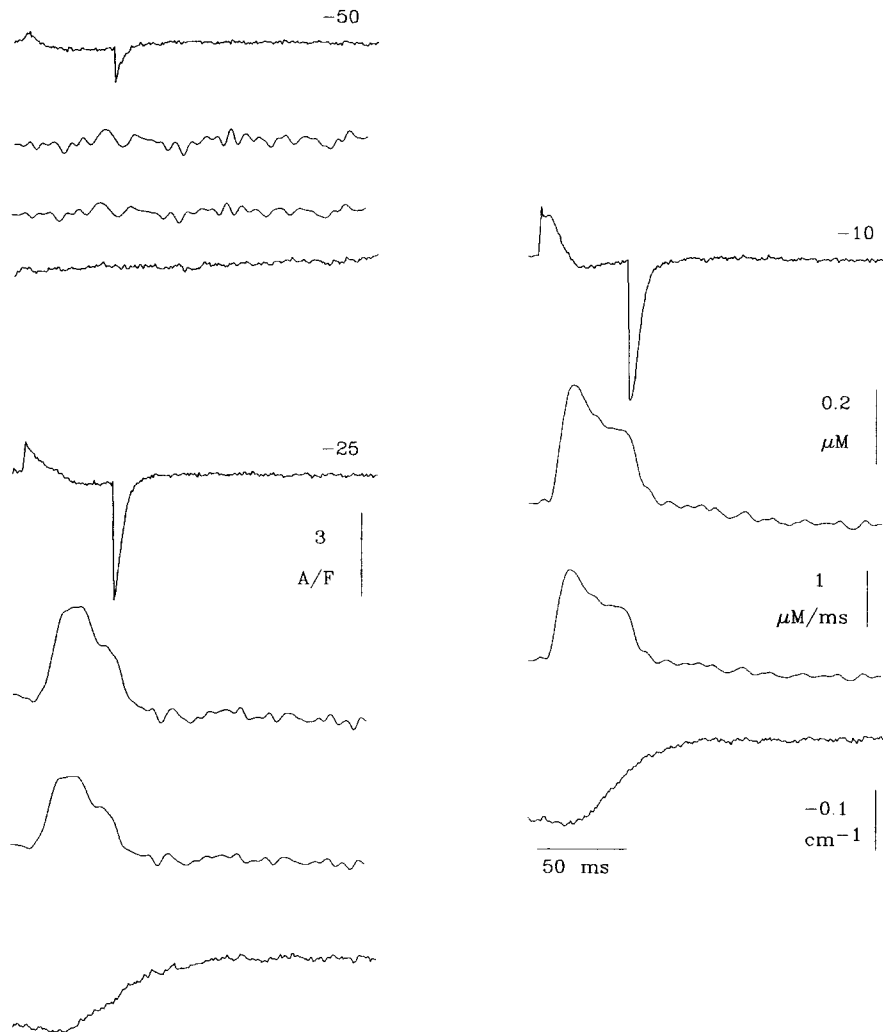
FIGURE 1. Voltage dependence of Q_γ, $[Ca^{2+}]_i$, and intrinsic signal. The first record in each group is the asymmetric current. It shows the difference between current records during pulses from a holding potential of -90 mV to the indicated voltage and a control pulse (one-fourth the amplitude of the corresponding test pulse) from -120 mV. The second record is the Ca^{2+} transient from ApIII signals. The third record is the calculated Ca^{2+} release. The last record is the intrinsic optical signal at 850 nm. Diameter, 90 μm. Middle pool length, 370 μm. Capacitance, 6.8 nF. Dye concentration between 344 and 456 μM (at beginning and end of series). Internal solution, 15 EGTA.

and the I_γ component occurred earlier in the pulse than at -25 mV. The voltage dependence of Ca^{2+} release and I_γ were both shifted to higher voltages (by some 15 mV) in these experiments as compared with the report in the previous paper. This shift is probably due to the higher concentration of divalent cations in the external solution used here. The calculated Ca^{2+} release flux and the Ca^{2+} transients have a similar shape when using high $[EGTA]_i$ due to the slow reaction rate of the buffer with Ca^{2+}, as has been previously demonstrated by Ríos et al. (1990). From the records in Fig. 1, it is evident that the intrinsic optical signal is recorded when Ca^{2+} is being released from the SR. Thus, as previously shown (Kovacs and Schneider, 1977; Ríos et al., 1983), the intrinsic signal can be used as an indication of the existence of functional SR Ca^{2+} release.

Low Frequency Pulsing in the Presence of 15 mM EGTA

The previous paper, Fig. 2 in this paper, and the work of Horowicz and Schneider (1981a,b) and Hui and Chandler (1988) demonstrate that it is possible to record intramembrane charge movement currents with prominent "humps"—the kinetic signature of Q_γ—in a reproducible way in the presence of intracellular [EGTA] as high as 20 mM. Fig. 2 demonstrates, however, that repeated application of a depolarizing pulse to -20 mV that causes humps, at a frequency sufficiently low that it would not cause any kind of conditioning in the absence of EGTA (Brum et al., 1988a), rapidly eliminates I_γ when EGTA (15 mM) is present.

The family of records shown in Fig. 2 are single sweep asymmetric currents, obtained by pulsing the fiber to -20 mV at 0.5-min intervals. 5 records in a sequence of 14 are shown in the figure. Successive pulses produce charge movement currents with visible humps that become progressively smaller and slower. The OFF transient does not change appreciably. The records at right are the direct differences between the first and every one of the subsequent pulses shown (labeled 1 minus the corresponding trace). These differences have a slow, biphasic ON and a much smaller OFF. The charge blocked increased as the fiber was stimulated. The effect was reversible, and the last record plotted was obtained after the sequence of 14, and a 5-min period of rest. The last record in the right column shows that there is almost no difference between the first and the recovery records. Similar observations were found in five fibers. These experiments clearly indicate that low frequency stimulation ($2\ min^{-1}$) reduces the I_γ component in the presence of 15 mM intracellular EGTA. Based on previous findings that a similar stimulation protocol can impair SR Ca^{2+} release (García et al., 1989) it is most likely that the observed reduction in I_γ can be related to the expected absence of SR Ca^{2+} release. This suggestion was confirmed in the next experiments, where we recorded I_γ and the intrinsic signal in high intracellular EGTA.

Effect of high EGTA

A more radical suppression of the slow component of intramembrane charge movement was attained in experiments in which the solution with 15 mM EGTA was replaced by one with 62.5 mM EGTA (essentially isotonic EGTA-Cs; cf. Methods) and no added Ca^{2+}. Estimating the contaminant Ca^{2+} at 10 μM (Lüttgau and Spiecker, 1979), the nominal $[Ca^{2+}]_i$ was $<10^{-10}$ M. In this medium the intrinsic signal that

accompanies Ca^{2+} release (Kovacs and Schneider, 1977; Ríos et al., 1983) and the charge movement were abolished in minutes, even when the cells were pulsed at a very low frequency.

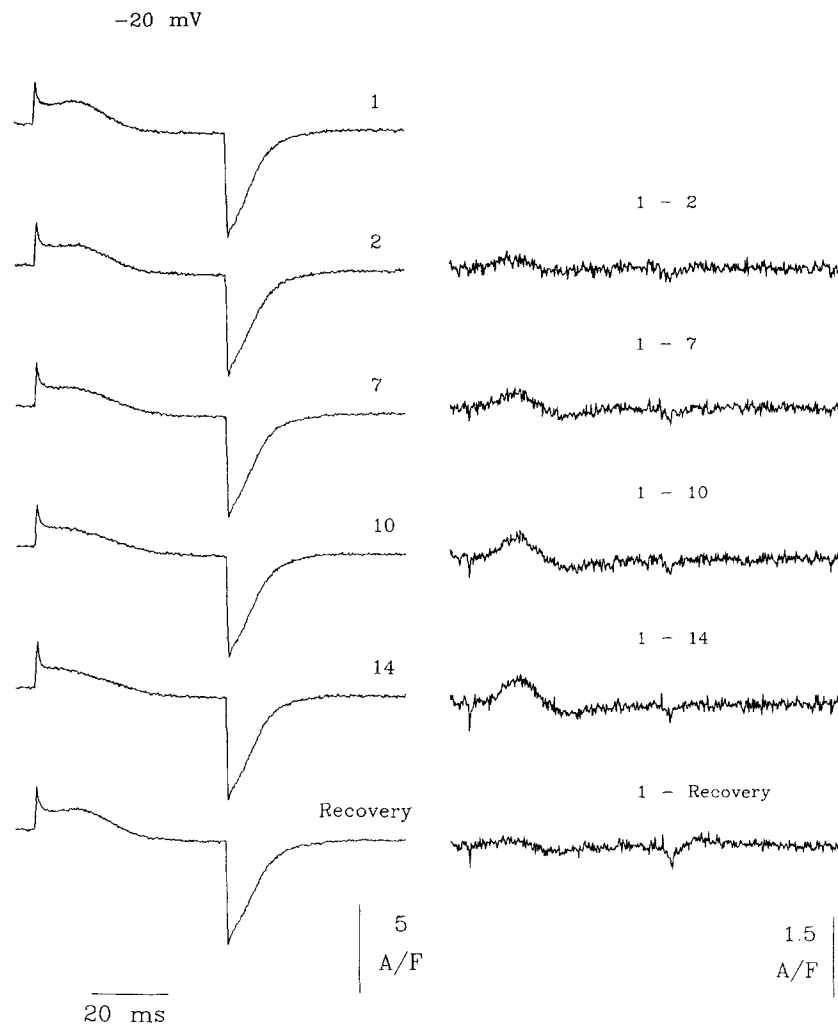Fig. 3 shows traces of intrinsic optical signals recorded at 550 nm (upper traces)

FIGURE 2. Effect of pulsing in 15 mM EGTA. Asymmetric currents as in previous figure legend. Pulses to −20 mV at 30-s intervals; sequence of 14 pulses. The last record on the left was taken after a 5-min rest. (*Right*) Differences between the first record and every one of the records in the sequence. Diameter, 100 μm. Middle pool length, 330 μm. Capacitance, 9.2 nF. Internal solution, 15 EGTA.

and intramembrane charge movement (lower traces) for a pulse to −25 mV. Stable I_γ and intrinsic signal recordings were obtained in the 15 mM EGTA solution at stimulation frequencies lower than 0.5 min^{-1}. The fiber was stimulated at intervals of 0.5–1 min after changing the internal solution to high EGTA, 0 Ca. The record

obtained after 14 min in high EGTA, 0 Ca has no evidence of a hump in the charge movement, and the intrinsic signal has disappeared. In this record, the decay phase of charge movement during the pulse was fitted to a single exponential curve. The same fitted curve is superimposed in the other current traces and the difference between the current record and the fitted curve represents the I_γ current. Records in
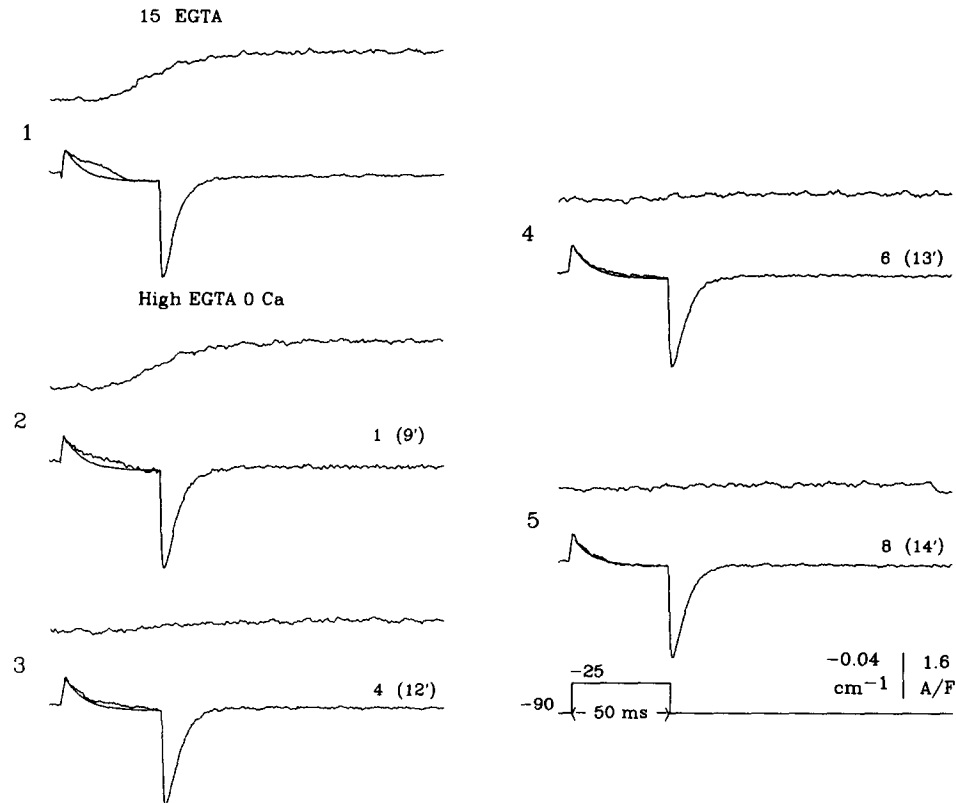

FIGURE 3. Effect of pulsing in high EGTA on the charge movement and the intrinsic signal. The top record in each pair is the increase in light intensity normalized to total light intensity. The lower record is the asymmetric current measured as in the other figures. The first set of records (1) was obtained in 15 EGTA internal solution. The other four pairs of records (2–5) belong to a sequence obtained by pulsing the fiber every 0.5–1 min after changing the internal solution to high EGTA, 0 Ca. Numbers at right list the place of the pulse in the sequence. A single exponential was fitted to the last record (5), in which the hump component disappeared. The same fitted curve was overimposed on the other current records. Pulse is represented schematically. Diameter, 80 μm. Middle pool length, 330 μm. Capacitance, 9.6 nF.

high EGTA, 0 Ca (2–5) clearly demonstrate a progressive reduction of I_γ. Unlike the experiments in Figs. 2 and 4, there was no negative phase during the ON in the current supressed by high EGTA.

In five fibers, Q_γ and the intrinsic signal consistently showed an analogous reduction. Blockade of the slow component of the charge movement and the intrinsic

signal was also observed when pulsing the fibers every 3–4 min with high EGTA, 0 Ca. When pulsing at such a low frequency, the blockade required longer times.

Fig. 4 demonstrates reversibility of the effect of high EGTA. The first record (*A*) was obtained after 58 min in 15 mM EGTA; the fiber had been pulsed every 2–10 min to −25 or −20 mV, and showed a marked and stable hump component. Record *B* was obtained after 40 min in high EGTA, 0 Ca. Record *C* was obtained in the same experiment, 15 min after changing the internal solution to high EGTA, 200 nM Ca. During the 15-min recovery period in high EGTA, 200 nM Ca the kinetics of the currents changed due to modifications in the passive electrical properties. Nevertheless, a hump component obviously reappeared. The recovery of the I_γ was partial. Overimposed to record *C* is a single exponential fitted to the decay of the charge during the pulse in record *B*. As in the previous figure, the difference between the
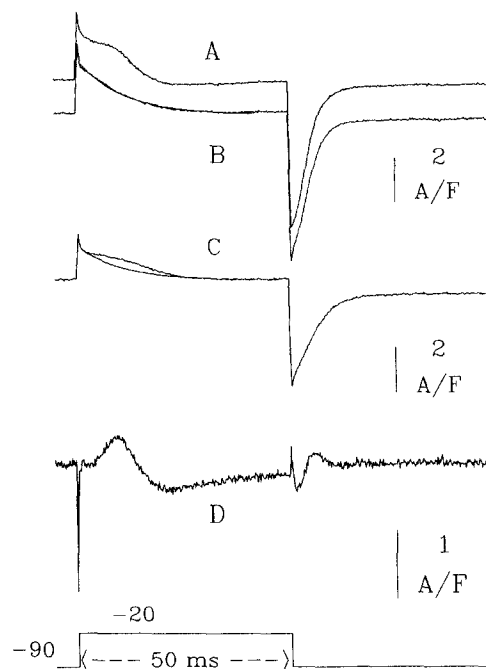

FIGURE 4. Reversibility of the effect of high EGTA. Asymmetric currents as in previous figures, during pulse shown schematically. (*A*) After 58 min in 15 EGTA (pulsing every 2–10 min). (*B*) After 40 min in high EGTA, 0 Ca (same pulsing frequency). (*C*) After 15 min in high EGTA, 200 nM Ca^{2+}. (*D*) Difference $A - B$. An exponential fit is overimposed to record *B*. The same fit was scaled by a factor of 0.6 to match the amplitude in *C*. Diameter, 86 μm. Middle pool length, 350 μm. Capacitance, 9.3 nF.

exponential curve and the current trace in *C* is an indication of the charge movement recovered. Record *D* is the difference $A - B$, showing again the properties of the charge movement suppressed by exposure to high EGTA. In agreement with observations in the other papers of this series, part of the Q_γ component seems to move back (inward) during the ON. Partial reversibility was observed in three experiments. Additionally, in another fiber mounted with high EGTA, 50 nM Ca as the internal solution since the beginning of the experiment, the hump component remained stable after 41 min of stimulating every 2–5 min.

Fig. 5 uses the conditioning pulse protocol 1 (see Methods; cf. also Pizarro, Csernoch, Uribe, Rodríguez, and Ríos, 1991) to monitor the effect of high EGTA. The records shown are differences between test current in the absence and presence of a conditioning pulse to 20 mV, of 200 ms duration, placed 700 ms before the test

pulse (schematically represented in the figure). Record *A* was obtained in 15 mM EGTA, and record *B* after 25 min in high EGTA, 0 Ca. During this time in high EGTA the fiber had been pulsed to −25 mV at 1–2-min intervals. The intrinsic optical signal obtained simultaneously is represented below each current record in *A* and *B*. In the higher [EGTA]$_i$, both the component of the charge movement isolated by this protocol and the intrinsic signal have been lost. Record *C* is the difference between *A* and *B*, and shows the usual properties of the charge suppressed. Confirming the observations in the previous paper, the conditioning pulse suppressed an ionic (net) inward current during the test pulse. This effect is probably unrelated to the suppression of Q_γ, as sometimes remains in high EGTA 0 Ca after
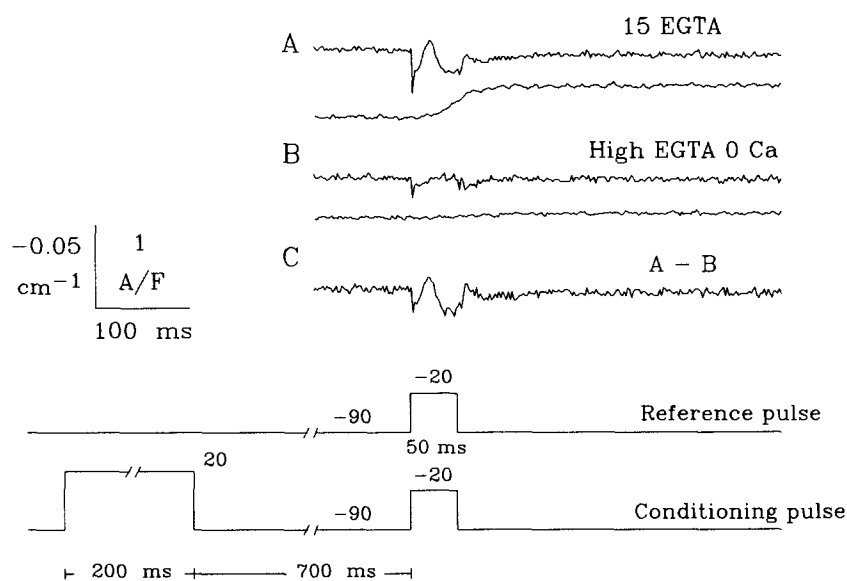

FIGURE 5. Effect of high EGTA on the charge defined by protocol 1. Records are differences between currents obtained according to protocol 1 (Csernoch et al., 1991; cf. pulse schematic). The top record in each pair of traces shows membrane currents. The bottom record in each set is the intrinsic signal. The traces shown, either current or intrinsic signal, are (total response in reference) − (total response in conditioned pulse). (*A*) Internal solution is 15 EGTA. (*B*) After 24 min in high EGTA, 0 Ca. (*C*) *A* − *B*. Diameter, 80 μm. Middle pool length, 330 μm. Capacitance, 9.6 nF.

abolition of the delayed charge movement. We observed similar effects in seven other experiments.

The most straightforward interpretation of the results above is that the interventions applied (pulsing at low frequency in 15 mM EGTA, exposure to high EGTA, 0 Ca) deplete calcium in the SR, and that the suppression of I_γ after the same interventions is secondary to the depletion effect. This in turn supports the conclusion of the previous paper, that Q_γ is a consequence of Ca^{2+} release. Charge movement currents with clear, stable humps can usually be recorded in 15 mM EGTA, provided that the pulsing frequency is not greater than about one per minute. For the above conclusion to hold, this pulsing should not deplete the SR of calcium.

We recorded Ca^{2+} transients from fibers exposed to 15 mM EGTA internal solution for 40–60 min (cf. Fig. 1) and found that the size of the transients was not affected by previous activity, provided that the stimulation frequencies were below one per minute.

DISCUSSION

Pulsing in EGTA Causes Depletion

García et al. (1989) demontrated that repetitive pulsing of a fiber with 1 mM EGTA internally results in a condition in which the SR does not contribute to the Ca^{2+} transient. In such situations the Ca^{2+} transient becomes proportional to an integral of the Ca^{2+} inward current.

A straightforward interpretation of those experiments is that the SR has been depleted of Ca^{2+} by the procedure. In fibers exposed to much lower EGTA, Schneider, Simon, and Szücs (1987) have shown that a single depolarization of 150 ms may reduce as much as 40% of the SR Ca^{2+} ready to be released, even after 900 ms of recovery. In these fibers the missing Ca^{2+} remains in the myoplasm, bound to parvalbumin and other Ca^{2+}-binding molecules, and release recovers with a time constant of ~ 20 s, reflecting replenishment of the store.

In the presence of much higher [EGTA], as used here, the free $[Ca^{2+}]_i$ is made smaller at all times. Kovacs, Klein, Simon, and Schneider (1989) have shown that Ca^{2+} uptake by the SR depends on $[Ca^{2+}]_i$ in a steep fashion around 60 nM, roughly halving its rate when $[Ca^{2+}]_i$ is reduced by one-tenth. Therefore, in the presence of 15 mM EGTA the uptake of released Ca^{2+} should be much slower, and repetitive pulsing should lead to depletion.

Pulsing in High EGTA Causes Elimination of Release and Q_γ

To test the hypothesis that I_γ is a consequence of Ca^{2+} release we applied repetitive pulsing in either 15 or 62.5 mM EGTA. The observations were: (a) Stimulation at frequencies below 2 min^{-1} permits stable Ca^{2+} transients and I_γ in 15 mM EGTA. (b) Stimulation every 0.5 min causes broadening of the Q_γ "hump," then its virtual elimination, in ~ 5 min (10 pulses). This effect is fully reversible after 5 min of rest (see Fig. 2). (c) In 62.5 mM EGTA even stimulation at 10-min intervals causes elimination of Ca^{2+} release and concomitant elimination of the hump (See Fig. 3). This effect can be partially reversed if the cut ends of the fiber segment are then exposed to a solution with 200 nM $[Ca^{2+}]$ and the same high [EGTA] (see Fig. 4).

The fact that I_γ can be eliminated reversibly by these interventions, and that the elimination of I_γ is parallel to the elimination of Ca^{2+} transients and intrinsic optical signals, is consistent with the hypothesis tested.

In the first paper of this series a specific mechanism was proposed by which the release of Ca^{2+} could cause a delayed component of charge movement. Fig. 1 demonstrates that a Ca^{2+} transient is recorded by the dye, even in the presence of 15 mM EGTA. This implies that the buffer cannot prevent the increase in $[Ca^{2+}]_i$. It is likely that this increase, in the presence of EGTA, is restricted to the vicinity of the release sites. The Ca^{2+} transients shown are averages of increases in $[Ca^{2+}]_i$ that are very inhomogeneous spatially; the actual local concentrations near the triad should therefore be much greater than what the dye reports. In any case, Ca^{2+} transients are

presented here as an indication that the SR still contains calcium and is able to release it upon depolarization. Further support for the idea that Ca^{2+} plays a direct role in the generation of I_γ is given in the next paper (Szűcs, Csernoch, Magyar, and Kovács, 1991).

Two Alternative Interpretations of Q_γ

One curent interpretation of Q_γ is that it is a charge originating at a subset of voltage sensors (the γ sensors) different from those that originate Q_β (e.g., Hui, 1983). In this paper we found that interventions shown to reduce Ca^{2+} release by depleting the SR of Ca^{2+} suppress Q_γ currents. To make this result consistent with a separate carrier for Q_γ, it would be necessary to postulate that either EGTA or SR depletion causes blockade of the γ voltage sensor as a primary effect.

Healthy Q_γ currents can be recorded for long periods in high EGTA; therefore, it is the concurrence of EGTA and a pattern of stimulation (frequencies above 1 min^{-1}) that abolishes I_γ. Also, it seems unlikely that depletion per se can specifically block a T membrane sensor.

Finally, the kinetic properties of the current suppressed do not support the interpretation that the effect is a blockade of a specific sensor. The fact that this current has delayed onset, undershoot after the positive peak, a much greater ON, and a variable OFF is incompatible with the simple expectations of the blockade of an independent subset of voltage sensors.

The alternative interpretation, that I_γ involves the same ensemble of sensors, moving as a consequence of Ca^{2+} release, is strongly supported by the present results.

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