Stimulation-dependent Redistribution of Charge Movement Between Unavailable and Available States

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Abstract. A previous study (Stroffekova and Heiny 1997) demonstrated that changes in resting, intracellular free Ca^{2+} can modulate the amount of charge which is available to move upon depolarization and do excitation-contraction-coupling (E-C coupling) Charge movement reflects voltage-driven conformational changes of the dihydropyridine receptor which couple membrane excitation to Ca^{2+} release from the sarcoplasmic reticulum (SR) and contractile activation (c f review Melzer et al 1995) The present study demonstrates that dynamic changes in free Ca^{2+} that occur in the triadic gap during SR Ca^{2+} release can likewise produce a stimulation-dependent increase in the amount of available charge Thus this modulation occurs in the physiological range of Ca^{2+} changes that occur in the triad during normal muscle activity The modulation of charge movement by intracellular Ca^{2+} was rapid and maintained, it occurred within 2 3 suprathreshold depolarizations and remained for 5 10 minutes It could be prevented by intracellular BAPTA and by depleting the SR of Ca^{2+} but not by EGTA or agents known to alter ion channel phosphorylation These results are explained by a model in which a Ca^{2+} binding site on or near the voltage-sensor is normally populated by Ca^{2+} ions released into the triadic junction during activity and modulates the distribution of voltage sensors between available and unavailable states

Key words: Excitation-contraction coupling — Skeletal muscle — Charge movement

Introduction

In a previous report (Stroffekova and Heiny 1997) we proposed that a Ca^{2+} binding

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site on an internal domain of the skeletal muscle dihydropyridine receptor, termed the 'availability site', can modulate the amount of charge available to move upon depolarization. The charge that moves upon depolarization represents the charge available to do excitation-contraction coupling (Melzer et al. 1995). Charge movement reflects voltage-driven conformational changes of the dihydropyridine receptor which couple membrane excitation to Ca^{2+} release from the SR and contractile activation (cf. review Melzer et al. 1995). Based on the greater ability of BAPTA compared with EGTA to buffer Ca^{2+} at this site, we concluded that this site is normally populated by a local Ca^{2+} pool within the triad junction.

In cut fibers, this site is not normally populated at rest because intracellular Ca^{2+} is buffered to $pCa < 9$. However, when resting Ca^{2+} was raised to physiological resting levels of pCa 7 and above, more charge became available to move upon depolarization. The steepest increase in charge occurred over the range pCa 7 to pCa 6.5, near the threshold for contraction. This finding suggests that the postulated site functions in the range of physiological triadic Ca^{2+} changes during normal muscle activity.

In the present study we investigated whether *dynamic* Ca^{2+} changes in the triadic gap during Ca^{2+} release from the SR can modulate the maximum amount of charge by this mechanism. We found that repetitive suprathreshold depolarizations were able to increase the maximum amount of charge moved in a time and stimulation-dependent manner. This result is consistent with the idea that the availability site functions over the normal range of dynamic Ca^{2+} changes in the triad junction, and modulates the amount of charge available to do E-C coupling.

Materials and Methods

The experimental preparation, protocols, and recording apparatus were essentially the same as described previously (Heiny and Jong 1990; Jong et al. 1997; Stroffekova and Heiny 1997). Briefly, single cut skeletal muscle fibers from the semitendinosus muscle of *Rana catesbiana* were voltage-clamped using a vaseline-gap method. The cut fiber ends were permeabilized briefly (1–2 min) with saponin (0.01% in a Cs-Glutamate internal solution) and thereafter were perfused with a Cs-Glutamate internal solution. The holding potential was -90 mV. Pulses were applied to the fiber and data was acquired using a microcomputer-based pulse generation and data acquisition system. The command pulse to the voltage clamp was low-pass filtered at a corner frequency of 3 kHz with an 8-pole Bessel filter. Membrane currents were filtered at 1.2 kHz using an 8-pole Bessel filter before being digitized.

Charge movement currents were elicited in response to test pulses to 0 mV for 200 ms. The maximum charge movement, Q_{max}, was obtained by integrating the charge moved at 0 mV. Linear leak and capacity currents were subtracted off-line using a small scaled control pulse that was applied after each test pulse from a subtracting holding potential of −110 mV. For repetitive stimulation, ten identical test pulses were applied once every 6 seconds (0.17 Hz). The maximum charge measured at each test pulse, Q_n, was normalized to the charge measured at the final pulse, Q_{10}. The measurements were expressed as mean ± S.D. Significance was judged at the $P < 0.05$ level.

Table 1. Experimental solutions

Solution	CsGlu	EGTA	BAPTA	MOPS	Na_2CP	Na_2ATP	$MgSO_4$	$CaSO_4$	glucose	pCa
				Internal/end pools (mmol/l)						
A	76	10	0	10	5	5	5.80	0.023	5	9
B	66	0	10	10	5	5	6.38	0.019	5	9

Solution	TEA_2SO_4	$CsSO_4$	MOPS	$CaSO_4$	$MgSO_4$	$CdSO_4$	$LnCl_3$	TTX (μmol/l)
				External/central pool (mmol/l)				
C	85	5	5	3.25	0.5	0.5	0.1	1.56

EGTA and MOPS were added as the free acid. BAPTA was added as the tetracesium salt. The pH was adjusted using CsOH or TEA-OH.

The composition of the recording solutions is given in Table 1. The solutions were designed to eliminate all ionic currents. The osmolarity of internal and external solutions was adjusted to 235 and 255 mOsm (± 5 mOsm), respectively. The pH was 7.1 at 8 °C. The free Ca^{2+} concentration of the external solution was estimated as 1 mmol/l. The free Mg^{2+} concentration of the internal solutions was kept constant at 1 mmol/l. The internal solutions contained 10 mmol/l EGTA or 10 mmol/l BAPTA yielding an estimated pCa_i of 9. pCa_i was raised by adding $CaSO_4$ to solutions A or B. Paired fibers dissected from the same muscle were used for comparisons of charge movement measured in the EGTA and BAPTA containing internal solutions (Solutions A and B, Table 1). All experiments were performed at a temperature of 8 ± 1 °C.

Results

Effect of raising triadic Ca^{2+} concentration on the maximum charge movement

We first tested whether dynamic increases in intracellular Ca^{2+} during activity can increase charge movement, and measured the onset of the effect. During Ca^{2+} release, the Ca^{2+} concentration in the triadic space near the release sites is expected to rise rapidly and to greatly exceed resting myoplasmic levels, reaching the tens of micromolar range within a few milliseconds.

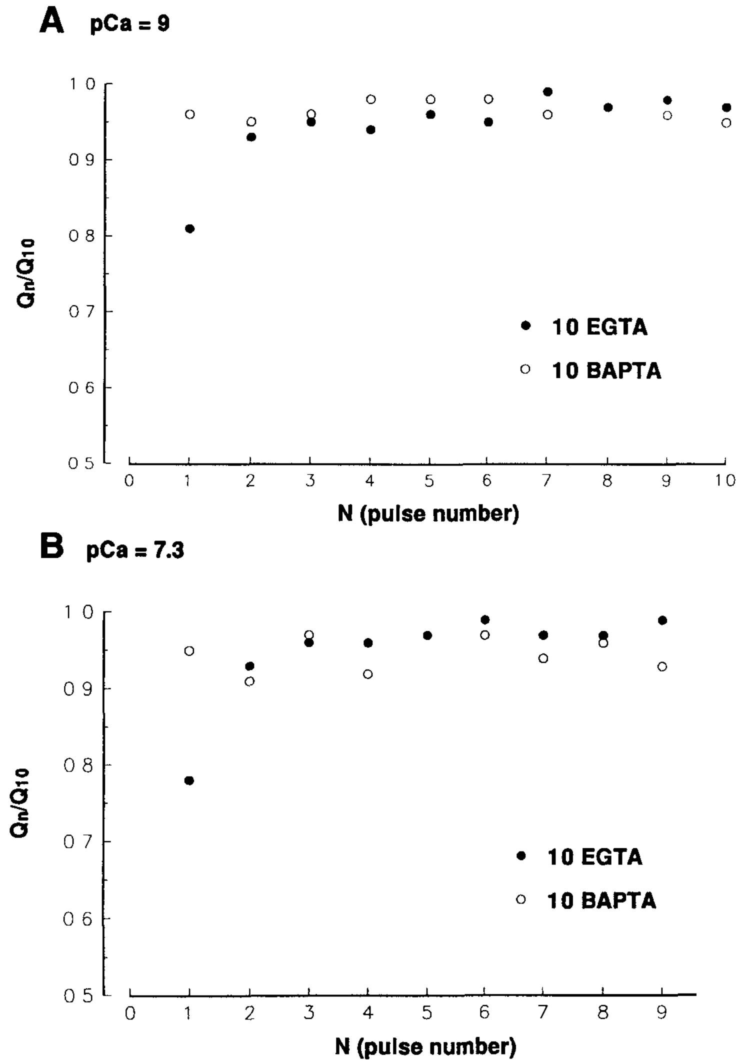

Figure 1. Effect of repetitive depolarization on the maximum charge measured at 0 mV in paired fibers perfused intracellularly with either the 10 mmol/l EGTA (filled circles) or 10 mmol/l BAPTA (open circles) internal solution (Table 1, Solutions A and B). A 200 ms pulse to 0 mV was applied every 6 seconds (0.16 Hz) for 10 cycles. The maximum charge measured during each pulse, Q_n, was normalized to the charge measured at pulse number ten, Q_{10}. Data points represent the mean of measurements from three pairs of fibers. Error bars were within the width of the symbols. *A*) pCa $\sim$ 9, *B*) After changing the internal solution to one containing the same buffer but with pCa raised to 7.3.

Fig. 1A examines the effect of repetitive suprathreshold stimulation on the maximum charge measured from paired fibers perfused with an internal solution containing either 10 mmol/l EGTA or 10 mmol/l BAPTA as the Ca^{2+} buffer, without added Ca^{2+} (pCa ≤ 9). In the EGTA perfused fiber, the maximum charge increased with increasing pulse number, reaching a steady value after 2–3 pulses. This was within 6–12 seconds after the first depolarization. The charge moved at the last pulse, Q_{10} was 20% greater than the initial charge, Q_1. After a rest of 5–10 minutes, the charge Q_1 returned to the initial value. In contrast, no increase in charge occurred in the paired BAPTA perfused fibers (filled symbols). Thus BAPTA was able to prevent the stimulation-dependent increase in charge movement.

Fig. 1B shows the results of similar measurements starting from a pCa of 7.3 (50 nmol/l). The initial charge is similar to the charge measured at a resting pCa of 9 (Stroffekova and Heiny 1997). Again, the charge increased during the first 2–3 pulses and Q_{10} measured after ten depolarizations was about 20% greater than the charge measured at the first pulse. After a rest of 5–10 minutes, the charge Q_1 returned to the initial value. The increase in charge did not occur in the BAPTA perfused fibers at either pCa value. The mean data from these measurements is summarized in Table 2. The charge measured at the first and last pulse was significantly different in the EGTA ($P = 0.01$ and $P = 0.05$ for pCa 9 and 7.3 respectively) but not in the BAPTA perfused fibers ($P > 0.8$ for pCa 9 and 7.3).

Table 2. Effect of repetitive suprathreshold stimulation on the maximum amount of charge available to move upon depolarization. Mean data ($n = 3$) from same fiber pairs described in Fig. 3. Q_1 is the mean charge measured at pulse #1 and Q_{10} is the mean charge measured at pulse #10.

Condition	Q_1 (nC/μF)	Q_{10} (nC/μF)	$Q_{10} - Q_1$ (nC/μF)	Q_{10}/Q_1
EGTA, pCa 9	15.51 ± 0.22	18.69 ± 0.63	3.18 ± 0.50	1.16 ± 0.03
EGTA, pCa 7.3	15.85 ± 0.21	19.83 ± 1.65	3.98 ± 0.42	1.27 ± 0.04
BAPTA, pCa 9	13.97 ± 0.79	13.75 ± 0.69	-0.22 ± 0.10	0.98 ± 0.01
BAPTA, pCa 7.3	12.43 ± 1.47	12.71 ± 1.02	0.28 ± 0.41	1.02 ± 0.03

This result is consistent with our previous finding that the maximum amount of charge that moves upon depolarization is not static but can be increased by increasing intracellular free Ca^{2+} (Stroffekova and Heiny 1997). Additionally, it demonstrates that charge movement can be increased rapidly by the dynamic local

Ca^{2+} increases that occur during activity. These are expected to transiently raise triadic Ca^{2+} levels from a resting pCa ~ 7 to above pCa 6. BAPTA was able to prevent the stimulation-dependent increase because it more effectively buffers Ca^{2+} at distances in the triadic space. Under these conditions, although EGTA effectively buffers resting Ca^{2+} uniformly throughout the myoplasm, it is not expected to buffer released Ca^{2+} effectively at distances less than about 100 nm from the release sites. With EGTA, contraction is prevented while the normal dynamic triadic Ca^{2+} changes continue essentially unperturbed.

Table 3. Effect of holding potential (HP) and calcium current blockers on the stimulation-dependent increase in Q_{max}. Mean data ($n = 5$)

Condition	Q_1 (nC/μF)	Q_{10} (nC/μF)	$Q_{10} - Q_1$ (nC/μF)	Q_{10}/Q_1
EGTA HP-100	23.65 ± 0.53	28.07 ± 1.36	4.83 ± 0.62	1.17 ± 0.02
EGTA HP-70	12.97 ± 1.15	15.80 ± 1.76	2.98 ± 0.51	1.22 ± 0.02
EGTA 0 La^{3+} 0 Cd^{2+} pCa 9	33.37 ± 1.98	39.42 ± 2.2	6.05 ± 0.22	1.18 ± 0.01

Table 3 examines whether changing the holding potential or blocking entry of extracellular Ca^{2+} can alter the activity-related increase in charge movement. When the holding potential was changed from −100 to −70 mV, the initial charge measured at 0 mV was less, but the stimulation-dependent increase remained. The charge measured at the first and last pulse was significantly different for both holding potentials ($P = 0.002$ and $P = 0.04$ for HP −100 and HP −70 mV, respectively). This suggests that the increase in charge movement is not due to recruitment of other gating currents. When La^{3+} and Cd^{2+}, which are normally present in our external solution, were omitted, the stimulation-dependent increase remained. The charge measured at the first and last pulses were significantly different ($P = 0.01$). This suggests that Ca^{2+} entry does not contribute significantly to the triadic Ca^{2+} pool that modulates charge movement during depolarization.

Mechanism of the effect

The next experiments examined possible mechanisms by which triadic Ca^{2+} could modulate charge movement. This modulation could occur either directly via Ca^{2+} binding to the voltage-sensor, or indirectly via activation of a closely associated Ca^{2+}-dependent enzyme for which the dihydropyridine receptor is a substrate. For example, a Ca^{2+}-dependent kinase located near or physically associated with the dihydropyridine receptor could rapidly phosphorylate it during activity to upregulate the voltage-sensor. The observed kinetics of the modulation are consistent with both types of mechanism. The onset was fast, with a maximal increase occurring within 6–12 seconds of the first suprathreshold depolarization. The effect was maintained and charge was reset to the initial value over the next 5–10 minutes.

Table 4. Effects of phosphorylation modifying agents on the stimulation-dependent increase in maximum charge movement. Protein kinase inhibitor (PKI), okadaic acid (OkA), calmodulin inhibitor peptide (CAM_{inh}), $ATP_{\gamma s}$ and ATP. Each fiber was first perfused with the standard EGTA internal solution and allowed to equilibrate for 45 minutes. Charge movement was then recorded in response to a series of repetitive pulses to 0 mV applied at 0.16 Hz. $Q_{1\ control}$ is the charge recorded from pulse #1 of this train and $Q_{10} - Q_{1\ control}$ is the difference in charge between pulses #10 and #1. At that time the internal solution was exchanged for a test solution having the same composition but with the phosphorylation modifying agent added. In the case of nominally 0 ATP, the 0 ATP solution was used from the beginning because of the difficulty of washing out ATP. After another 20 minutes equilibration, the pulse train was repeated. $Q_{1\ test}$ is the charge recorded from pulse #1 of this series and $Q_{10} - Q_{1\ test}$ is the difference in charge between pulses #10 and #1.

TEST AGENT	$Q_{1\ control}$ (nC/μF)	$Q_{1\ test}$ (nC/μF)	$Q_{10} - Q_{1\ control}$ (nC/μF)	$Q_{10} - Q_{1\ test}$ (nC/μF)
PKI ($n = 4$)	35.34 ± 1.62	36.44 ± 1.19	9.36 ± 1.50	9.40 ± 0.92
OkA ($n = 2$)	30.12 ± 2.92	30.59 ± 3.60	4.64 ± 0.81	4.86 ± 0.64
CAM_{inh} ($n = 4$)	36.34 ± 2.01	35.20 ± 2.20	8.25 ± 1.04	9.49 ± 2.61
$ATP_{\gamma s}$ ($n = 4$)	33.72 ± 1.07	31.63 ± 1.45	5.91 ± 0.87	7.89 ± 1.89
0 ATP ($n = 5$)		30.26 ± 3.07		6.99 ± 0.33

To examine whether an indirect mechanism might be involved, we examined the effects of agents known to promote or block phosphorylation of ion channels. These results are summarized in Table 4. As shown, the maximum charge measured from rested fibers at the start of pulsing (Q_1) was similar in control and test conditions. Q_1 was not significantly different in control and test conditions for any agent tested ($P = 0.4, 0.9, 0.5, 0.1$ for PKI, OkA, CAM_{inh} and $ATP_{\gamma s}$, respectively). Repetitive stimulation increased the maximum charge movement under control conditions, by an amount similar to that shown in Table 2. None of these agents was able to eliminate or prevent the stimulation-dependent increase which was the same in control and test conditions ($P = 1.0, 0.8, 0.5$, and 0.2 for PKI, OkA, CAM_{inh} and $ATP_{\gamma s}$, respectively). 20 μmol/l PKI, a specific blocker of cAMP dependent phosphorylation, or 10–20 μmol/l calmodulin inhibitor, a specific blocker of Ca^{2+}-

calmodulin dependent phosphorylation did not alter resting Q_{max} or prevent the stimulation-dependent increase in charge. Likewise these were not changed by 10 μmol/l okadaic acid in combination with 4 mmol/l ATP$_\gamma$s and 1 mmol/l ATP suggesting that phosphorylation via PKA or PKC pathways was not involved. The stimulation-dependent increase remained when ATP was removed from the internal solution. Although these results cannot exclude all possible pathways of indirect modulation they make it unlikely that a phosphorylation mechanism is involved.

Effect of SR depletion

We next examined the effect of removing SR Ca^{2+}. This was accomplished by applying the same protocol of repetitive depolarizations to fibers before and after the SR had been depleted of Ca^{2+}. Depletion of the SR was accomplished by applying suprathreshold pulses once per second for one hour to fibers perfused with high concentrations of an EGTA-containing internal solution (Table 1 Solution A), while monitoring Ca^{2+} release optically (Jong et al. 1995, 1997). The results are shown in Fig. 2. Under control conditions, the charge moved after ten suprathreshold depolarizations was greater than the charge elicited by the first depolarization from

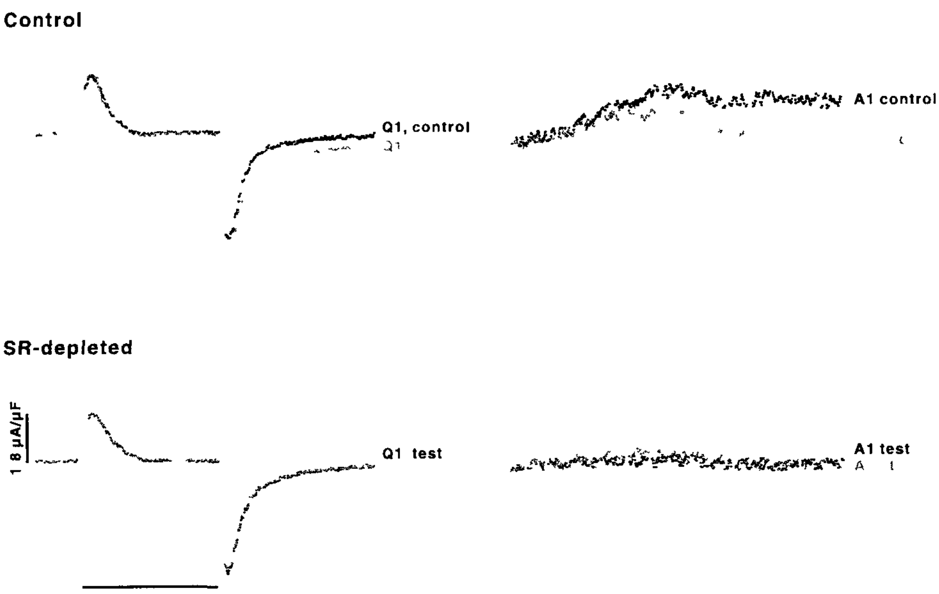

Figure 2. Effect of SR depletion on the stimulation-dependent increase in charge movement. Charge movement currents (left) and intrinsic optical signals (right) recorded in response to repetitive stimulation at 0.16 Hz in control conditions and after SR depletion. Charge movement currents and intrinsic optical signals elicited by pulse #1 (thicker trace) and pulse #10 (thinner trace) are shown superimposed. The average Q_{10}/Q_1 ratio in two fibers subjected to this protocol was 1.14 ± 0.001 in control conditions, and 1.02 ± 0.02 after depletion.

a rested fiber (top left traces) The SR released Ca^{2+} during both stimulations as evident from the presence of an intrinsic optical signal (top right traces) After the SR was depleted of Ca^{2+}, charge movements elicited by the first and tenth depolarizations were identical (lower left traces) There was no Ca^{2+} release during either stimulation, as evident from the absence of an intrinsic optical signal (lower right traces) This result indicates that the SR is the normal source of Ca^{2+} for the stimulation dependent effect

Discussion

We previously demonstrated that the amount of charge which can move upon depolarization is not static but can be increased by raising resting intracellular free Ca^{2+} We proposed the existence of a Ca^{2+} binding site on or near the voltage-sensors which can modulate the distribution of voltage sensors between available and unavailable states The present study demonstrates that charge can be increased by dynamic Ca^{2+} increases in the triadic gap elicited by repetitive depolarizations to voltages above threshold for releasing Ca^{2+} from the SR Thus this modulation occurs in the physiological range of Ca^{2+} changes that occur in the triad during normal muscle activity The effect is rapid and maintained Charge is increased within 6 12 seconds and takes 5 10 minutes to reset to the initial value after which it can be increased again by subsequent depolarizations The stimulation dependent increase in charge could be prevented by intracellular BAPTA and by depleting the SR of Ca^{2+} but not by agents known to alter ion channel phosphorylation This suggests that the modulation most likely occurs by a direct binding of Ca^{2+} to a site on or closely associated with the voltage sensor This effect is seen in EGTA but not in BAPTA perfused fibers because triadic Ca^{2+} changes continue in the former but not the latter The different results with BAPTA and EGTA support the idea that the postulated site is normally populated by a local Ca^{2+} pool-that is by Ca^{2+} released from the SR into the triadic space

Taken together these data can be explained by the model shown in Fig 3 In a fully rested fiber the putative availability site(s) is not expected to be fully populated A suprathreshold depolarization will move this charge to the active state causing Ca^{2+} release and a transient rise in the triadic Ca^{2+} concentration As this Ca^{2+} binds to the 'availability site on the voltage sensors, more sensors move to the normal resting or available state and are able to move upon subsequent depolarization The maximum amount of charge moves when all sites are saturated-i e all voltage sensors are in the available state Normally one or two pulses above threshold for Ca^{2+} release are all that is needed to populate the site Presumably binding of Ca^{2+} to this site has fast ON rate and a slow OFF rate According to this model, a resting fiber which has not been stimulated to twitch for some time would have less than the maximum charge available to do E-C coupling After a

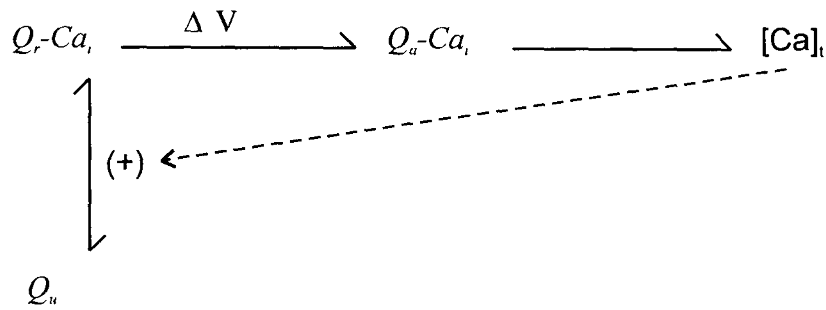

Figure 3. Proposed model of modulation of the voltage-sensor by triadic Ca^{2+}. Q_r and Q_a *represent the normal resting and active states of the voltage-sensor respectively.* Only charge which is initially in the resting state can move upon depolarization and do E-C coupling—i.e. charge which has Ca^{2+} bound to the proposed 'internal availability site'. Q_u represents an unavailable state. Binding of Ca^{2+} to the unavailable state promotes the transition of charge to the resting state. This Ca^{2+} comes from the increase in triadic Ca concentration, [Ca]t during Ca^{2+} release. Thus, SR Ca^{2+} release feeds back positively on the voltage sensor to recruit more charge into the available state from which it can move upon subsequent depolarization.

few depolarization however the remaining charge would fully 'prime' and become available to do E-C coupling. It is possible that this modulation represents a cellular mechanism for conserving energy by making voltage-sensors fully functional only when needed during periods of muscle activity.

More generally this model although different in the details supports data from other studies which suggest that the state of the dihydropyridine receptor and/or the number of activable voltage-sensors can be influenced by Ca^{2+} ions released into the triad junction during SR Ca^{2+} release (Pizarro et al. 1991; Csernoch et al. 1992; Rios et al. 1993; Jong et al. 1995, 1996; Pape et al. 1996). Such cross-talk between the Ca^{2+} release and voltage-sensing processes has been used to support the idea of a close physical association and/or an allosteric interaction between the SR Ca^{2+} release channels/ryanodine receptors and the voltage-sensors/dihydropyridine receptors during excitation-contraction coupling.

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