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VOLTAGE-SENSOR CONTROL OF Ca^{2+} RELEASE IN SKELETAL MUSCLE: INSIGHTS FROM SKINNED FIBERS

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1. ABSTRACT

Important aspects of the excitation-contraction (EC) coupling process in skeletal muscle have been revealed using mechanically-skinned fibers in which the transverse-tubular system can be depolarized by ion substitution or electrical stimulation, activating the voltage-sensors which in turn open the Ca^{2+} release channels in the adjacent sarcoplasmic reticulum (SR). Twitch and tetanic force responses elicited in skinned fibers closely resemble those in intact fibers, showing that the coupling mechanism is entirely functional. It was found that ATP has to be bound to the Ca^{2+} release channels for them to be activated by the voltage-sensors and that the coupling mechanism likely involves the voltage-sensors removing the inhibitory effects of cytoplasmic Mg^{2+} on the release channels; such findings are relevant to the basis of muscle fatigue and to certain diseases such as malignant hyperthermia (MH). EC coupling is evidently not mediated by upmodulation of Ca^{2+}-induced Ca^{2+} release (CICR) or by an oxidation or phosphorylation reaction. The Ca^{2+} load in the SR of skinned fibers can be set at the endogenous level or otherwise. The normal coupling mechanism functions well in mammalian fast-twitch fibers even when the SR is only partially loaded, whereas CICR is highly dependent on SR luminal Ca^{2+} and caffeine is poorly effective at inducing release at the endogenous SR Ca^{2+} load level.

2. INTRODUCTION

2.1. Background

The mechanically-skinned fiber technique was first developed by Natori (1) and involves physically peeling or rolling back the sarcolemma, leaving the other structures more-or-less unaltered. This gives experimental access to the intracellular environment, which can then be manipulated in many ways to investigate particular aspects of muscle function, such as those involving the contractile machinery (2) or Ca^{2+} uptake and release by the sarcoplasmic reticulum (SR) (3). Importantly and quite remarkably, it is possible to study the normal excitation-contraction (EC) coupling process in such fibers. Upon skinning, the transverse-tubular system seals off (4,5), and if the skinned fiber segment is placed in a solution broadly mimicking the normal intracellular environment, in particular with high $[K^+]$, some Na^+, and physiological levels of ATP and free Mg^{2+} (~1 mM), the Na/K pump in the t-system keeps the $[Na^+]$ high and the $[K^+]$ low within the t-system, thereby re-establishing the normal transmembrane potential, with the lumen of the t-system positive with respect to the 'cytoplasmic' space (the 'intracellular' region that was opened to the bathing solution by the skinning procedure) (Figure 1). It was found that the t-system could then be depolarized by substituting the high $[K^+]$ bathing solution with a low $[K^+]$-high $[Cl^-]$ solution, eliciting Ca^{2+} release from the SR and contraction (6-8). However, increasing the 'cytoplasmic' $[Cl^-]$ directly stimulates the ryanodine receptor/Ca^{2+} release channels in the SR (8-11) and under some circumstances (such as when the SR is heavily loaded with Ca^{2+}), such Cl^- substitution can induce Ca^{2+} release in a manner unrelated to the normal voltage-sensor controlled mechanism (8). This problem was obviated by instead substituting the high $[K^+]$ solution with a high $[Na^+]$-low $[K^+]$ solution, which

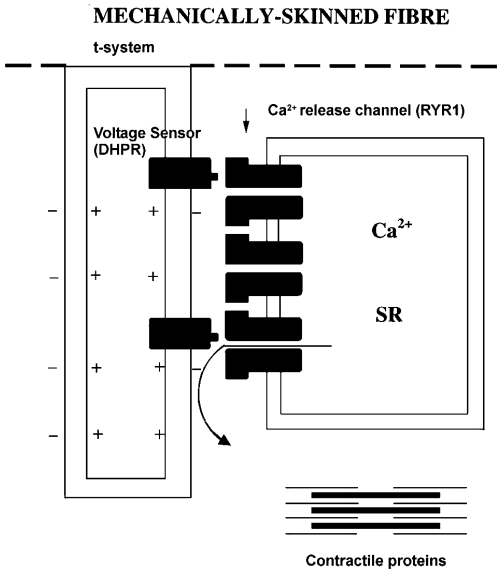

Figure 1. Schematic diagram of a mechanically-skinned muscle fiber. The sarcolemma is removed (dashed line) and t-system seals off and repolarizes (positive inside) if the segment is bathed in a high $[K^+]$ solution (see text). The voltage-sensors/dihydropyridine receptors (DHPR) in the t-system control the Ca^{2+} release channels (ryanodine receptor type 1, RYR1) in the sarcoplasmic reticulum (SR).

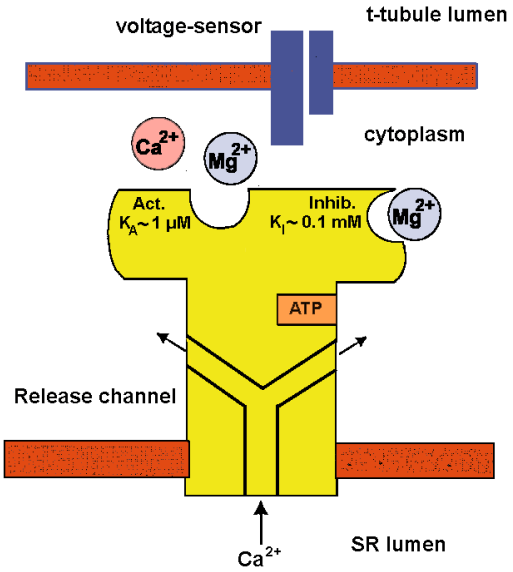

Figure 2. Regulation of the Ca^{2+} release channel. The channel is stimulated by ATP binding to a regulatory site (dissociation constant ~ 1 mM) and Ca^{2+} binding to a Ca^{2+}-activation site (act.) (dissociation constant (K_A) ~ 1 µM in the absence of Mg^{2+}). The channel is inhibited by Mg^{2+} binding at the Ca^{2+}-activation site (with >30 fold lower affinity than for Ca^{2+}) and by Ca^{2+} or Mg^{2+} binding at a non-specific inhibitory site (inhib.) (K_I ~ 0.1 mM for both ions). Mg^{2+}, normally present at 1 mM, lowers the apparent Ca^{2+} affinity of the activation site ~30 fold and causes almost full occupation of the Ca^{2+}/Mg^{2+} site. It is proposed that voltage-sensor activation opens the channel by lowering the Mg^{2+} affinity of these sites and thereby removing its inhibitory effects.

depolarizes the t-system without any appreciable direct effect on the Ca^{2+} release channels or the SR itself (8). Furthermore, skinning the muscle fibers under paraffin oil rather than in a low $[Ca^{2+}]$ 'relaxing' solution ensured that the dihydropyridine receptor (DHPR)/voltage-sensors in the t-system were kept in a functional state and that the endogenous level of Ca^{2+} in the SR was maintained, the latter being particularly important for distinguishing between normal and abnormal mechanisms of Ca^{2+} release (8,11).

2.2. Electrical stimulation and advantages of the preparation

The properties of this depolarization-induced Ca^{2+} release (in particular its dependence on the $[K^+]$ and t-system contents and integrity, and the effects of DHPs and D600 (8,12,13) showed that it was mediated by the DHPR/voltage-sensors in the t-system activating the SR Ca^{2+} release channels, in other words, the normal coupling mechanism (Figure 2). However, one draw-back with the ion substitution method of depolarization is that diffusional delays means that depolarization takes ~0.5 s and is thus considerably slower than in an intact fiber. Recently, this problem has been circumvented by stimulating the skinned fiber rapidly with a brief transverse electric field (~2 ms, 40 V/cm) applied via parallel platinum electrodes whilst the fiber is kept in the normal high $[K^+]$ solution (14). Such stimulation evidently induces an action potential (AP) in the sealed t-system all along the length of the skinned fiber, which elicits Ca^{2+} release and a twitch response, with stimulation at progressively higher frequencies inducing unfused and then fused tetanic force responses. As expected, these responses were dependent on the potential of the t-system and were blocked by tetrodotoxin in the t-system lumen (14). The close similarity between the twitch and tetanic responses in these skinned fibers (Figure 3) and those in intact fibers, proved unequivocally that the responses in the skinned fibers were mediated by the normal voltage-sensor dependent mechanism. An interesting incidental finding in these experiments was that action potentials also travel longitudinally between adjacent sarcomeres in mammalian muscles fibers via a longitudinal tubular network (14); this may well be an important fail-safe mechanism in muscle to ensure uniform activation of the whole fiber even under adverse conditions.

In summary, the key advantages of this mechanically-skinned fiber preparation are as follows. It allows ready access to the 'intracellular' environment, and manipulation of factors and structures therein, whilst still retaining the normal mechanism of voltage-sensor control of Ca^{2+} release. This mechanism can be stimulated by depolarizing the t-system by ion substitution or more rapidly by triggering an AP in the t-system, which of course is the process occurring in-vivo. Furthermore, the amount of Ca^{2+} in the SR can be maintained at endogenous level or varied as desired (8,15,16). Thus, with the skinned fiber preparation it is possible to examine how some manipulation affects the whole of the EC coupling sequence, as well individual elements such as the responsiveness of the contractile apparatus to applied Ca^{2+} or the release of Ca^{2+} from the SR to direct stimulation with Ca^{2+} or caffeine. In this way the skinned fiber preparation

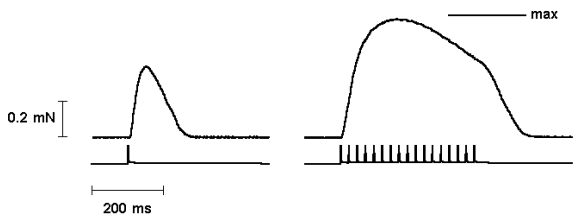

Figure 3. Twitch and tetanic force responses in a skinned fiber. Transverse electric field stimulation (40 V/cm, 2 ms) was applied to a skinned fiber segment from the extensor digitorum longus muscle of a rat, eliciting an action potential in the sealed t-system which resulted in SR Ca^{2+} release and contraction. A single stimulus induced a twitch response (left trace) and 50 Hz stimulation induced a fused tetanic force response. 'Max' indicates the maximum Ca^{2+}-activated force in the fiber.

can complement and extend the information obtained with other techniques, providing a powerful experimental tool that bridges the gap between studies on intact fibers and those on isolated SR or contractile or channel proteins.

3. KEY FINDINGS

A number of findings are immediately apparent from the fact that the EC coupling mechanism in skinned fibers is evidently entirely functional even though the normal cytoplasm has been replaced with a solution with only particular minimal requirements.

I. The coupling mechanism between the voltage sensor and the release channel does not require the presence of substances that readily dissociate and diffuse out of the fiber (other than those added experimentally, eg. K^+, Mg^{2+}, ATP), as it continues to function well for more than 30 minutes after skinning and cytoplasmic perfusion. (Even large proteins should diffuse out of the ~50 μm thick skinned fiber within a matter of minutes (17), and the bathing solution has a volume ~10^6 times that of the skinned fiber and is regularly changed.) This does not mean however that some important factors, such as calmodulin or FKPB12 (18), have not remained bound at key regulatory sites.

II. Cytoplasmic Mg^{2+} and ATP are both critical to the normal coupling mechanism (see later).

III. The coupling mechanism is not noticeably altered by the washout or addition of reducing agents glutathione (GSH) or dithiotreitol (DTT) and is evidently not mediated by an oxidation reaction (19).

IV. The stimulatory effect of caffeine and cytoplasmic Ca^{2+} on the Ca^{2+} release channels in mammalian muscle is greatly augmented by increasing the Ca^{2+} load level in the SR, probably via a stimulatory Ca^{2+} site within the SR lumen, but in contrast the normal coupling mechanism functions well even when the SR is relatively depleted of Ca^{2+} (11) (see later).

V. Concomitant with the last three observations, the normal coupling mechanism is not mediated by up-modulation of 'simple' Ca^{2+}-induced Ca^{2+} release (CICR) and is relatively insensitive to factors that favor CICR (such as oxidation) or inhibit CICR (such as low pH, raised [Mg^{2+}], lactate, DP4) (11,12,20-22). It is suggested that this is because voltage-sensor activation itself up-regulates and utilises the Ca^{2+} sensitivity of the release channels, meaning that there is 'voltage-sensor controlled-CICR', rather than 'simple CICR' that is independent of the voltage-sensors (23-25).

VI. Rapid Ca^{2+} release from the SR occurs in the absence of any Cl^- or other anion highly permeable to the SR, demonstrating that such Ca^{2+} efflux is not dependent on the flow of anion-mediated counter-current.

4. IMPORTANCE OF ATP AND Mg^{2+}

4.1. Voltage-sensor coupling requires ATP binding to the release channel

ATP is present in the cytoplasm of intact resting fibers at ~6 to 8 mM. Millimolar concentrations of cytoplasmic ATP stimulates Ca^{2+} release and augments CICR and caffeine-induced Ca^{2+}-release in skinned fibers (3,26,27), SR vesicles (28) and single release channels (29). However, this does not indicate whether ATP is in any way important in normal EC coupling, where the voltage-sensors activate the Ca^{2+} release channels apparently by some direct physical interaction (30). Experiments with skinned fibers show that despite such a direct interaction, ATP is absolutely required for normal coupling. In toad muscle depolarization-induced Ca^{2+} release by ion substitution was completely blocked in the absence of ATP (23) and the total amount of release was reduced approximately two-fold at 0.5 mM ATP (31). In mammalian (rat) muscle, the inhibitory effect of lowering the total [ATP] to 0.5 mM was only apparent in the ion substitution experiments when Ca^{2+} release had already been reduced by raising [Mg^{2+}] (32) (see next section), probably because of temporal limitations in those experiments, with recent experiments with electrical stimulation showing that low [ATP] does inhibit the release mechanism at the normal [Mg^{2+}] of 1 mM (33). The critical importance to EC coupling of ATP binding to a regulatory site on the Ca^{2+} release channel was further established using adenosine, an extremely weak agonist, as a competitor of ATP at that site. Adenosine competitively inhibited ATP-stimulation of voltage-sensor dependent Ca^{2+} release (34) in a manner quantitatively similar to its action on caffeine-stimulated release in skinned fibers (26,34) and on single Ca^{2+} release channels (35), showing that the inhibitory effect of low [ATP] was due to failure to stimulate a regulatory site on the release channel rather than being related to a decrease in phosphorylation or some other process dependent on ATP hydrolysis. 'Depolarization-induced' Ca^{2+} release in a triad (SR – t-tubular) preparation was also found to be inhibited in the absence of ATP (36), though it is not clear how much this effect was due to ATP favoring direct CICR in that preparation which was very heavily loaded with Ca^{2+} (11) (and see above). The fact that ATP binding to the release channel (Figure 2) is required for normal EC coupling

gives insight into how the voltage-sensor may regulate the release channel and is also relevant to the basis of muscle fatigue occurring in some conditions (see below).

4.2. Role of Ca^{2+} activation and Mg^{2+} inhibition

Cytoplasmic Ca^{2+} stimulates the opening of the Ca^{2+} release channel by binding to a high affinity site(s) (dissociation constant, K_a ~0.2-1 μM in mammalian (3,10,28), but possibly ~10 μM in frog muscle (27,37)). It is likely that this Ca^{2+}-activation site is required for normal coupling because recent experiments expressing mutated release channels in myotubes found that greatly lowering the affinity of the activation site for Ca^{2+} largely or completely blocked coupling (38). Mg^{2+} is known to inhibit the opening of the Ca^{2+} release channels by acting both at this 'Ca^{2+}-activation site' and at a low affinity Ca^{2+}/Mg^{2+} inhibitory site **(**Figure 2) (3,28,39). Mg^{2+} competes with Ca^{2+} for the activation site but with a >30-80 fold lower apparent affinity (K_I ~ 20-50μM) (10,27,28,39,40) and it apparently not only prevents Ca^{2+} stimulation of the channel but also directly inhibits channel opening when bound at this site (28). Mg^{2+} also competes with Ca^{2+} for the low affinity site, but the site is quite non-specific, with both ions binding with similar affinity and both causing similar inhibition of channel opening (10,27,28,39,40). It seems most appropriate to regard this latter site as a 'Mg^{2+}-inhibitory site' (41) because there is ~1 mM Mg^{2+} normally present in the cytoplasm, and consequently the site would be primarily occupied by Mg^{2+} rather than Ca^{2+} under physiological circumstances (Figure 2). Furthermore, as the dissociation constant for this Ca^{2+}/Mg^{2+} inhibitory site is ~0.1-0.2 mM at physiological ionic strength (10,28,42), it could be expected to be substantially occupied by Mg^{2+} in-vivo. Thus, Mg^{2+} will inhibit simple CICR in skeletal muscle by acting at both sites, but it is because of its occupancy of the low affinity Ca^{2+}/Mg^{2+} inhibitory site that such release will be considerably depressed no matter how high the $[Ca^{2+}]$ may rise in the vicinity of the release channels (3,12,23,25) (Figure 4).

The inhibitory action of Mg^{2+} on Ca^{2+} release is clearly present in skinned fibers even when EC coupling is entirely functional and the release channels are under the control of the voltage-sensors, because lowering the free $[Mg^{2+}]$ induces full release of SR Ca^{2+} in both mammalian and amphibian muscle independently of any changes in free or total [ATP] or [MgATP] (21,23,43). Of course, E-C coupling does work in-vivo in the presence of ~1 mM cytoplasmic $[Mg^{2+}]$, and further it appears that most or all the release channels must open almost fully (albeit transiently) if the observed peak rates of Ca^{2+} efflux are to be achieved (44). Consequently, it appears that the stimulatory effect of voltage-sensor activation must in some way bypass or overcome the inhibitory effect of Mg^{2+} on the release channel, in particular at the low affinity Ca^{2+}/Mg^{2+} site where raised $[Ca^{2+}]$ will only compound, not relieve, the inhibitory action of Mg^{2+}.

Importantly too, experiments in skinned fibers showed that depolarization-induced Ca^{2+} release is inhibited almost completely in mammalian and amphibian fibers if the $[Mg^{2+}]$ is raised to 10 mM (21,23) including when stimulating rapidly by electrical stimulation (unpublished observations); inhibition of Ca^{2+} release is also seen in intact fibers with raised $[Mg^{2+}]$ (45). This effect of raised $[Mg^{2+}]$ is not due to inactivation of the voltage-sensors, nor to an inability of Ca^{2+} to flow out through the release channels – instead it appears simply that the channels do not open with voltage-sensor stimulation in the presence of such a high cytoplasmic $[Mg^{2+}]$ (23). These observations led to the following proposal.

4.3. Voltage-sensor control of Mg^{2+} inhibition and CICR

As voltage-sensor activation overcomes the Mg^{2+} inhibitory effect at 1 mM Mg^{2+} but not at 10 mM Mg^{2+}, we have proposed that the voltage-sensor activates the release channel by lowering its affinity for Mg^{2+} at both the Ca^{2+}-activation site (23,24) and the low affinity Ca^{2+}/Mg^{2+} site (21,25,41) (Figure 4). The proposal of a Mg^{2+}-affinity change is also supported by recent findings in voltage-clamped cut fibers from mammalian muscle (46).

The above proposal seems the simplest way to account for information to date. The fact that high cytoplasmic $[Mg^{2+}]$ inhibits coupling does not fit with a proposal in which the inhibitory effect of Mg^{2+} is bypassed altogether (eg. that the voltage-sensor simply directly opens the release channel irrespective of Mg^{2+} binding) but is consistent with a Mg^{2+} affinity change. Furthermore, it is known that the Mg^{2+} affinity of the sites on the release channel can indeed be reduced by certain treatments (eg. high $[Cl^-]$ (10)) or agents (eg. bastadin (47)).

It is apparent from many experiments that it would not be sufficient for the voltage-sensors to only increase the Ca^{2+} affinity and/or decrease the Mg^{2+} affinity of the Ca^{2+} activation site without changing the inhibitory effect of Mg^{2+} at the Ca^{2+}/Mg^{2+} site. For example, 'depolarization-induced' Ca^{2+} release and CICR in triads are strongly inhibited under conditions (0.3 mM Ca^{2+}, 1.2 mM Mg^{2+}, 5 mM AMPPCP) in which the Ca^{2+}-activation site would be occupied almost exclusively by Ca^{2+} but where the low affinity Ca^{2+}/Mg^{2+} site would be appreciably occupied (36). Comparable results are also seen in ryanodine binding studies (with 1 mM Ca^{2+}, 5 mM AMP (10)). Also, increasing the Ca^{2+}-affinity of the activation site with 30 mM caffeine elicits little Ca^{2+} release in mammalian skinned fibers in the presence of 1 mM Mg^{2+} even though the voltage-sensor activation works extremely well under the same conditions (11). (Here it is presumed that caffeine increases only the Ca^{2+} affinity and not the Mg^{2+} affinity of the Ca^{2+}-activation site, in agreement with results in amphibian muscle fibers (27) – recent results in mammalian muscle SR (40) however have suggested that the Mg^{2+} affinity is increased as well, but this was with no ATP present and only 5 mM caffeine, and occurred only with SR from normal muscle and not in muscle from malignant hyperthermia-susceptible animals). Thus, it appears that the voltage-sensors must at least lower the affinity of the Ca^{2+}/Mg^{2+} site to get physiological Ca^{2+} release rates (41).

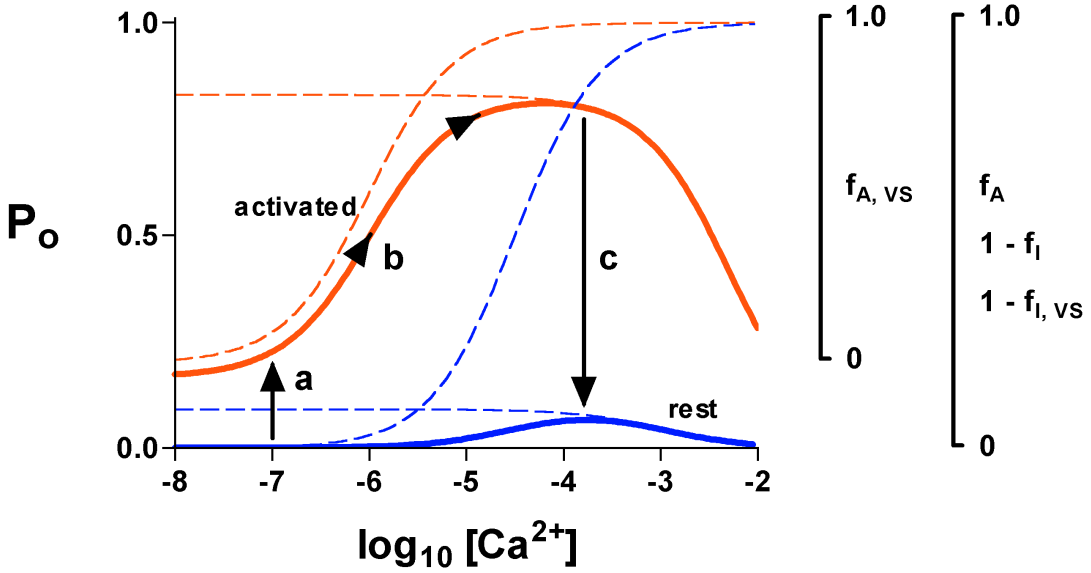

Figure 4. Effect of voltage-sensor activation on the Ca^{2+} dependence of release channel opening (P_O). In a resting fiber (ie. with the voltage-sensor not activated and physiological cytoplasmic [Mg^{2+}] of ~1 mM), Mg^{2+} will compete strongly with Ca^{2+} for the Ca^{2+}-activation site, lowering its apparent Ca^{2+} affinity ~30 fold (K_A increased to ~30 μM; see ascending dashed blue curve) and the Ca^{2+}/Mg^{2+} inhibitory site (K_I ~0.1 mM) will be almost fully occupied by Mg^{2+} (see lower dashed blue line) resulting in a low channel open probability (P_O <0.1) even at optimum cytoplasmic [Ca^{2+}] (see blue bell curve). It is proposed that voltage-sensor activation substantially reduces the affinity for Mg^{2+} at both sites so that i) the limitation on peak P_O caused by Mg^{2+} occupation of the Ca^{2+}/Mg^{2+} site is largely removed (red descending dashed curve, K_I ~5 mM) and ii) the apparent Ca^{2+}-affinity of the Ca^{2+}-activation site returns towards ~1 μM and the stimulatory effect of ATP will open the channel even in the absence of [Ca^{2+}] (eg. P_O ~0.2) (a), with the released Ca^{2+} further stimulating channel opening (b). This is 'voltage-sensor controlled CICR'. When the voltage-sensor is deactivated, the Mg^{2+} inhibition at both sites is restored and P_O rapidly declines **(c)** even in the presence of high local [Ca^{2+}]. Ascending blue and red dashed curves show the fraction of Ca^{2+}-activation sites occupied by Ca^{2+} at rest (f_A) and with the voltage-sensor activated ($f_{A,VS}$) respectively. Descending blue and red dashed curves show the fraction of Ca^{2+}/Mg^{2+} inhibitory sites that are unoccupied in the two cases respectively ($1 - f_I$ and $1 - f_{I,VS}$). P_O is calculated as (f_A x ($1-f_I$)) and (($f_{A,VS}$ + 0.2) x ($1-f_{I,VS}$)).

The question then arises as to whether it is sufficient to remove the inhibitory effect of Mg^{2+} only at the low affinity Ca^{2+}/Mg^{2+} site. This would require that the channels in vivo (with ATP present etc.) open to some extent even when Mg^{2+} is bound to the Ca^{2+}-activation site. If they do, the released Ca^{2+} could be expected to bind to the activation site, very rapidly inducing maximal channel opening. However, if the channels are not appreciably open under such conditions, it must be proposed that the voltage-sensors also remove or reduce the inhibitory effect of Mg^{2+} at the activation site, by reducing its affinity for Mg^{2+} and possibly also increasing its affinity for Ca^{2+} (23). Given that it was found in skinned mammalian fibers with functional coupling that decreasing the free [Mg^{2+}] to 50 μM induced considerably less Ca^{2+} release than did decreasing [Mg^{2+}] to 15 μM (21), it appears that reducing the level of occupancy of the low affinity Ca^{2+}/Mg^{2+} site to ~25% with 50 μM Mg^{2+} is not in itself sufficient to give substantial Ca^{2+} release at resting [Ca^{2+}] (~0.1μM), suggesting that for potent channel activation in functional fibers it is indeed necessary to reduce the Mg^{2+} inhibition at the activation site. As the voltage-sensors can induce Ca^{2+} release even when the cytoplasmic [Ca^{2+}] is buffered to nM levels (16), it seems that increasing the Ca^{2+}-sensitivity of the activation site without lowering its affinity for Mg^{2+} would not be sufficient to induce release.

Thus, it seems most parsimonious to propose that the voltage-sensor activates the release channel by reducing Mg^{2+}-inhibition at both sites (23,25). This would affect the Ca^{2+} dependence of release channel opening dramatically (Figure 4). When the voltage-sensors are not activated, Mg^{2+} would severely limit Ca^{2+} binding to the Ca^{2+}-activation sites (ascending dashed blue line in Figure 4) and also nearly saturate the Ca^{2+}/Mg^{2+} inhibitory sites (descending dashed blue line) so that the channel would open to only a small extent even at optimal [Ca^{2+}] (note the low peak of blue bell curve). Activating the voltage-sensors would move the 'activation' function to the left and 'inhibition' function to the right (to the red ascending and descending dashed curves respectively), with the latter effect greatly increasing the peak of the resulting bell curve (ie. giving a high probability of channel opening). Thus, the stimulatory effect of ATP will induce some degree of channel activation even when the cytoplasmic [Ca^{2+}] is at or below the normal resting level (<0.1μM) ('a' in Figure 4), with the Ca^{2+} efflux then able to maximally stimulate the channel by acting on the Ca^{2+}-activation site ('b' in

Figure 4). This is 'voltage-sensor controlled CICR', not simple uncontrolled CICR, and importantly Ca^{2+} release can be rapidly stopped, irrespective of any high local [Ca^{2+}], by deactivating the voltage-sensor and restoring the Mg^{2+} inhibition (23) ('c' in Figure 4) (see also section on termination of Ca^{2+} release). The proposed effect of voltage-sensor activation on the bell curves in Figure 4 is similar to that observed when adding high cytoplasmic [Cl^-] (10), possibly suggesting that high [Cl^-] stabilises the same 'activated' state favored by voltage-sensor activation. Increasing the level of Ca^{2+} loading in the SR also may have a similar effect (presumably mediated via a SR luminal Ca^{2+} site (48-50)), because it not only increases the apparent Ca^{2+}-sensitivity of the activation site (3, 28) but also seemingly reduces the extent of inhibition occurring via the low affinity Ca^{2+}/Mg^{2+} site, given that caffeine and Ca^{2+} can induce rapid and considerable Ca^{2+} release even in the presence of ~1 mM Mg^{2+} when the SR is very loaded (11,36,51).

The fact that ATP is a strong stimulant to channel opening and is absolutely required for voltage-sensor activation of the channels (see above) suggests that the release channels have to be in a 'primed' state to be opened by the voltage-sensors, and consequently is consistent with the idea that the release channels can be activated simply by removing a resting inhibition, that is, the Mg^{2+}-inhibition. Experiments with SR vesicles from both mammalian (28,36) and amphibian muscle (52) indicate that the Ca^{2+} release rate would be indeed high enough to explain release in-vivo if the vesicles are stimulated by cytoplasmic ATP (mM) and Ca^{2+} (>1μM) in the absence of inhibition by Mg^{2+}. A recent study with amphibian skinned muscle fibers however concluded that removing Mg^{2+} inhibition would not by itself increase channel opening sufficiently to give physiological Ca^{2+} release rates (27), though the fibers in that study did not have functional EC coupling and the rate of Ca^{2+} release may have been limited by diffusional delays when applying ATP. The question of whether removing Mg^{2+}-inhibition is sufficient in itself to give physiological Ca^{2+} release may be best resolved in the future by experiments in which the [Mg^{2+}] is very rapidly lowered in fibers with functional EC coupling. It is possible that in functioning fibers the combined stimulatory effects on the release channels of cytoplasmic ATP (and calmodulin) and luminal Ca^{2+} loading are still not enough to stimulate maximal Ca^{2+} release in the absence of Mg^{2+} inhibition – in that case it would have to be concluded that the voltage-sensors, in addition to removing the inhibitory effect of Mg^{2+}, must also have a direct stimulatory action on the release channels.

4.4. Malignant hyperthermia

The above mechanism also gives further insight into basis of malignant hypethermia (MH). MH is an inherited disorder of skeletal muscle, in most cases caused by mutations in the Ca^{2+} release channel, where volatile anesthetics and stress can trigger uncontrolled contractures, leading to hyperthermia and often to death (53). Ca^{2+} release in muscle fibers from MH-susceptible individuals is not inhibited as strongly by cytoplasmic Mg^{2+} as it is in normal muscle (54). This is due both to a two to three fold reduction in the Mg^{2+} affinity of the Ca^{2+}/Mg^{2+} inhibitory site (42) and a two fold decrease in the Mg^{2+} affinity and a two fold increase in the Ca^{2+} affinity of the Ca^{2+}-activation site (40). In view of the discussion above, this readily explains why the bell-curve describing the Ca^{2+} dependence of channel opening of release channels from MH muscle at rest (ie. with no voltage-sensor activation') is somewhat higher and wider than that for normal channels shown by the blue bell curve in Figure 4. So long as the Mg^{2+} inhibition is not reduced so much that the MH channel is appreciably open at the resting [Ca^{2+}] (ie. the point on the bell curve at ~0.1 μM Ca^{2+} remains close to a P_O of zero), this would mean that muscle function would be little if at all affected, with the MH channel remaining closed in the resting muscle and opening normally upon voltage-sensor activation when its characteristics would be the same as the normal channel (ie. red bell curve in Figure 4). However, if some extraneous factor such as a volatile anaesthetic or stress stimulated the release channel in any way (or raised the cytoplasmic [Ca^{2+}]), this could well be enough to induce a self-reinforcing cycle of CICR in the MH channel, which would produce the observed uncontrolled muscle contraction. In comparison, the normal release channel would be much less susceptible to any such stimuli.

Recent results indicate that the mutations in the release channel responsible for MH cause hyper-activation because they interfere with the normal interactions between different domains of the protein (55). It was found that the properties of the normal Ca^{2+} release channel altered to those of MH channels when a synthetic peptide (DP4), corresponding a particular region of the channel, was added (55). The exogenous peptide evidently competed with the corresponding region on the channel for its binding site on a different region of the channel, thereby preventing the channel from adopting its normal stable closed state. Addition of DP4 to skinned fibers caused similar sensitising effects, giving them the characteristics of MH-susceptible fibers (22). These experiments suggest the view that voltage-sensor activation opens the release channel by 'destabilising' the resting state that the channel protein normally adopts in the presence of Mg^{2+}.

5. TERMINATION OF Ca^{2+} RELEASE

Deactivation of the voltage-sensor rapidly stops Ca^{2+} release in skeletal muscle (56,57). As mentioned above, this can be explained by there being marked inhibition of CICR by Mg^{2+} when the voltage-sensors are not activated – simply deactivating the voltage-sensors would restore this strong level of Mg^{2+} inhibition, which would then greatly decrease Ca^{2+} release even if the presence of a high local [Ca^{2+}] (Figure 4). Furthermore, the fact that the sensitivity of CICR is greatly inhibited by partially depleting the SR of Ca^{2+} (3,11) (with depolarization-induced release being little affected (11,58)) means that any tendency for the Ca^{2+} release to be self-sustaining would be further reduced following the initial channel activation and Ca^{2+} efflux. (This can also account for the phenomenon of 'repolarization-induced stop of Ca^{2+} release (11,59)). Termination of Ca^{2+} release will be further aided if release

channels also display 'inactivation', in which the open probability of the release channels greatly decreases from an initial high level to a relatively low level, due either to a type of Ca^{2+}-specific (56,57,60) or use-dependent inactivation (61-63). Finally, depleting the SR Ca^{2+} would also reduce the absolute rate of Ca^{2+} release because of the reduced Ca^{2+} gradient, and this could be exacerbated by local depletion of Ca^{2+} inside the lumen of the SR near the release channels caused by diffusion limitations on Ca^{2+} as it binds and unbinds to calsequestrin within the SR. Together these phenomena explain why the amount of Ca^{2+} released during a single twitch response (ie. to stimulation by a single action potential) remains virtually unchanged when fibers are given treatments that up-modulate the sensitivity to simple CICR, such as addition of DP4 (22) or oxidation of the release channels (G. S. Posterino & G. D. Lamb, unpublished observations).

6. INVESTIGATIONS INTO MUSCLE FATIGUE

Finally, the skinned fiber preparation has also provided important information about the basis of muscle fatigue. Muscle fatigue, which is a decline in muscle performance with repeated activity, has many facets and causes (64,65). It can be caused by direct effects on the contractile apparatus and, of particular interest here, by a reduction in Ca^{2+} release from the SR (64). The reduction in Ca^{2+} release is not caused by a decline in intracellular pH (20,21,66-68), even though this depresses CICR in skinned fibers (20,21) and Ca^{2+}- and ATP-activation of isolated release channels (69), once again showing that voltage-sensor controlled Ca^{2+} release is not mediated by simple CICR. The reduction is Ca^{2+} release is also not due to the inhibitory effect of lactate ions on the release channels, as the twitch response to electrical stimulation in skinned fibers is quite unaffected by the presence of even 30 mM lactate (70).

Two factors that are likely to be important in muscle fatigue in some circumstances are a) a decrease in cytoplasmic [ATP] and b) the accompanying increase in free [Mg^{2+}] that occurs with a fall in [ATP] owing to the fact that most cellular ATP is in the form MgATP and the hydrolysis products of ATP bind Mg^{2+} with much lower affinity than does ATP. Following vigorous stimulation of human fast-twitch fibers, the average [ATP] in the cytoplasm falls to <1 mM (71), and the concentration may well be lower near the Ca^{2+} release channels in the triad junction. The ability of the voltage-sensors to induce Ca^{2+} release is inhibited when the [ATP] falls into this range (31-33), and this would be exacerbated if the local [Mg^{2+}] reached ~3 mM (23,32,45,46), with the reasons for this explained above. In other circumstances where [ATP] does not fall, reduced Ca^{2+} release may occur due to inorganic phosphate, which rises to high concentrations in the cytoplasm (64), moving into the SR and precipitating with Ca^{2+}, thereby reducing the amount of readily releasable Ca^{2+} (72,73). Glycogen depletion, which occurs with sustained activity, can also by some unknown means cause a reduction in depolarization-induced Ca^{2+} release (17,74). Lastly, it appears that if the cytoplasmic [Ca^{2+}] is raised for prolonged periods, voltage-sensor control of Ca^{2+} release is hindered (5,75), apparently due to physical disruption of the triad junction (5), leading to 'low-frequency fatigue' which may last for a day or more.

7. CONCLUSIONS / PERSPECTIVES

The skinned fiber preparation with functional EC coupling has given important insight into the properties and basis of the coupling mechanism in skeletal muscle. Because the 'intracellular' environment can be readily manipulated in these fibers, it has been possible to investigate which factors influence normal coupling and which do not. This has given information not only about how the voltage-sensors control the Ca^{2+} release channels, but also about the possible basis of muscle fatigue and particular diseases. These experiments have highlighted how Ca^{2+} release controlled by the voltage-sensor differs from direct stimulation or modulation of the channels by Ca^{2+}, H^{+}, lactate and caffeine etc. It appears that voltage-sensor regulation of the release channels may be achieved by varying the affinity of certain sites for Mg^{2+} - a mechanism in which channel activation is initiated by removal of resting inhibition rather than by a direct stimulatory effect. It may be that many other cellular processes are also regulated by varying the affinity of key sites for Mg^{2+}, an ion present in most cells at a relatively constant, high (millimolar) level, properties which, perhaps contrary to general thinking, make it useful for dynamic regulation of molecular processes.

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