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Reciprocal control of the conformational state of the sarcoplasmic reticulum calcium channel protein by polarization and depolarization in the transverse tubule

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Abstract We attached the conformational probe methylcoumarin acetate (MCA) specifically to the junctional foot protein (JFP) moiety of triads, and monitored conformational changes in the JFP during polarization and depolarization of the T-tubule moiety. The MCA fluorescence decreased upon T-tubule polarization, and the fluorescence changes were blocked by preventing T-tubule polarization or by a nimodipine block of the T-tubule-to-sarcoplasmic reticulum communication. Depolarization of the T-tubule reversed the MCA fluorescence decrease which had been produced by T-tubule polarization. These results suggest that the conformational and functional states of the JFP are regulated by T-tubule polarization and depolarization in a reciprocal fashion.

Key words: Excitation-contraction coupling; Triad; Junctional foot protein; Conformational change; Transverse tubule

1. Introduction

According to the electromechanical coupling hypothesis ([1], reviews: [2–8]), the depolarization signal elicited in the surface membrane is sensed by the dihydropyridine receptor (DHPR) in the T-tubule and is transmitted to the junctional foot protein (JFP) of the sarcoplasmic reticulum (SR). This would produce mechanical changes in the JFP, which in turn activates the Ca^{2+} release channel located in the JFP. Conversely, physical changes occurring in the JFP appear to be sensed by the DHPR [7–10]. Thus, it seems that protein-protein interactions between the two major components, viz. the voltage sensing DHPR of the T-tubule and the channel-containing JFP of the SR, play an important role in the skeletal muscle E-C coupling.

As shown in our recent study [11], chemical depolarization of the T-tubule moiety of the triad [12,13] produced a rapid increase in the fluorescence intensity of the conformational probe MCA attached specifically to the JFP moiety [14]. There was a close correlation between the chemical depolarization of the T-tubule and the MCA fluorescence change, and also between the MCA fluorescence change and the subsequent SR Ca^{2+} release [11]. Furthermore, the depolarization-induced MCA fluorescence changes in the JFP were blocked by the DHPR antagonist nimodipine [11]. These results suggested that T-tubule depolarization produces a conformational change in the JFP by mediation of the T-tubule voltage sensor, which in turn activates the Ca^{2+} release channel, as predicted by the electromechanical coupling hypothesis.

Here we report that in contrast to the depolarization-induced increase in the MCA fluorescence, polarization of the T-tubule moiety of the triad produces a decrease in the fluorescence intensity of the JFP-attached MCA probe. The fluorescence decrease was inhibited under non-polarizing conditions and in the presence of the DHPR antagonist nimodipine, suggesting that T-tubule polarization produces a new type of conformational change in the JFP by mediation of the T-tubule voltage sensor. As documented in this paper, the previously reported depolarization-induced conformational change [11,15] represents the reversal of the polarization-induced change described here. Thus, it appears that the conformational states of the JFP are regulated by the changes in the T-tubule membrane potential in a tightly coupled manner. The present results also suggest that the polarization-induced conformational change in the JFP represents a priming process required for the subsequent activation by T-tubule depolarization.

2. Materials and methods

2.1. Preparation

The triad-enriched microsomal fraction was prepared from rabbit leg and back muscles by differential centrifugation as described previously [12,16]. After the final centrifugation, the sedimented fraction was homogenized in a solution containing 0.3 M sucrose, 0.15 M K gluconate, proteolytic enzyme inhibitors (0.1 mM PMSF, 10 μg/ml aprotinin, 0.8 μg/ml antipain, 2 μg/ml trypsin inhibitor) and 20 mM MES, pH 6.8 (PI buffer) to a final protein concentration of 20–30 mg/ml. The preparations were quickly frozen in liquid nitrogen and stored at −70°.

2.2. Site-specific fluorescent labeling of the JFP moiety of the triad

Site-specific fluorescent labeling of the JFP moiety of the triad was performed using the cleavable hetero-bifunctional cross-linking reagent, sulfosuccinimidyl 3-((2-(7-azido-4-methylcoumarin-3-acetamido) ethyl) dithio)propionate (SAED; [14]) with the aid of neomycin as a carrier in the following way. First, neomycin-SAED conjugates were formed by incubating 0.4 mM neomycin with 0.2 mM SAED in 20 mM HEPES (pH 7.5) for 15 min at 22°C in the dark. The reaction was quenched by a 10-fold dilution with 10 mM lysine. Twenty ml of the neomycin-SAED conjugate (final neomycin concentration: 20 μM) was mixed in the dark with 300 mg triad protein, brought to 300 ml with PI buffer, and photolysed with UV light in a Pyrex tube

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Abbreviations: BAPTA, 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; DHPR, dihydropyridine receptor; E-C coupling, excitation-contraction coupling; HEPES, *N*-2-hydroxyethyl piperazine-*N'*-2-ethane sulfonic acid; JFP, junctional foot protein; MCA, methylcoumarin acetate; MES, 2-(*N*-morpholino)ethanesulfonic acid; PI buffer, buffer solution containing proteolytic enzyme inhibitors; PMSF, phenylmethanesulfonyl fluoride; SAED, sulfosuccinimidyl 3-((2-(7-azido-4-methylcoumarin-3-acetamido) ethyl) dithio)propionate; SR, sarcoplasmic reticulum; T-tubule, transverse tubular system

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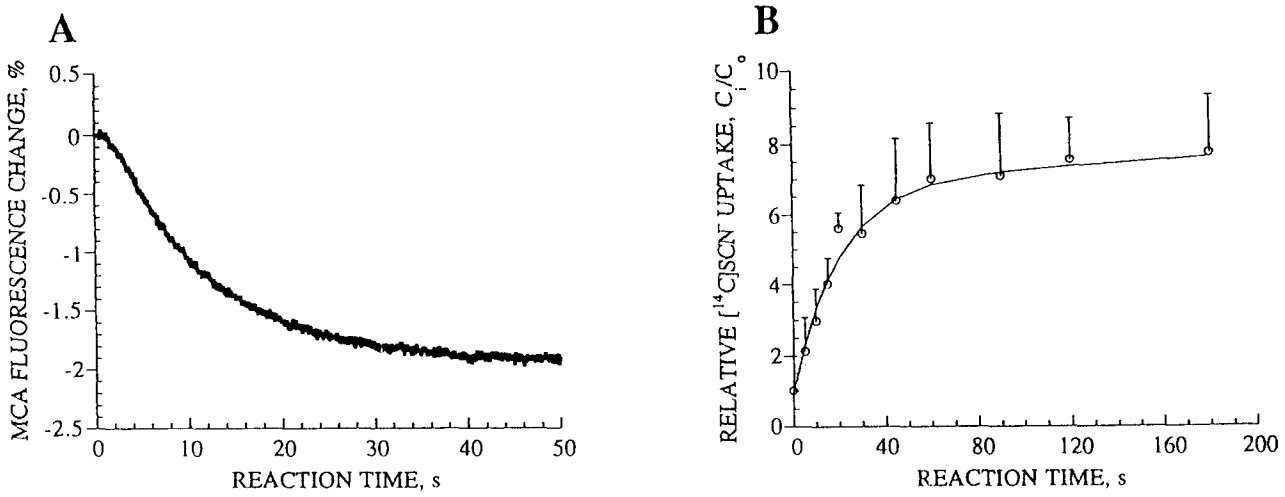


Fig. 1. Incubation of triads in the polarization solution induces a decrease of the fluorescence intensity of the MCA probe attached to the JFP moiety of the triad (A), which approximately parallels the time course of T-tubule polarization assessed by the $[^{14}C]SCN^-$ uptake (B). Triads (0.192 mg/ml for A; 3.0 mg/ml for B) were incubated in the polarization solution, and the time courses of MCA fluorescence changes (A) and relative $[^{14}C]SCN^-$ uptake, viz. C_i/C_o (B) were determined by the stopped-flow fluorometry and the $[^{14}C]SCN^-$ partitioning method, respectively (for details, see Section 2). MCA fluorescence change (%) = $(\Delta F/F_0) \times 100$, where ΔF is a change in the fluorescence intensity, and F_0 is the fluorescence at $t = 0$. Each datum point in B represents the mean plus standard deviation ($n = 4$–12).

at 4°C for 10 min. β-Mercaptoethanol was added (100 mM final concentration) to cleave the disulfide bond of SAED. After incubation on ice for 1 h, the mixture was centrifuged for 15 min at 100 000 × g, and the sedimented triads were resuspended in PI buffer to a final protein concentration of ~20 mg/ml. In agreement with our previous result [14], the neomycin-mediated incorporation resulted in the specific incorporation of the MCA into the JFP moiety of the triad as determined by fluorometry of electrophoretically separated protein bands.

2.3. Assays of protein conformational changes in the JFP moiety of the triad induced by T-tubule polarization and depolarization

2.3.1. Solutions. Polarization solution: 150 mM K gluconate, 15 mM NaCl, a 0.1 μM Ca^{2+} buffer (0.5 mM BAPTA/0.2 mM $CaCl_2$), 20 mM imidazole (pH 6.8).

Depolarization solution: 150 mM Na gluconate, 15 mM NaCl, a 0.1 μM Ca^{2+} buffer (0.5 mM BAPTA/0.2 mM $CaCl_2$), 20 mM imidazole (pH 6.8).

2.3.2. T-tubule polarization. The MCA-labeled triads (0.24 mg/ml) were first equilibrated in the polarization solution. Then, the (Na^+/K^+) ATPase reaction (for T-tubule polarization) was initiated by mixing 120 μl of the triad suspension with 30 μl of the polarization solution with added 12.5 mM Mg·ATP and an ATP-regenerating system (2.5 mM PEP, and 10 units/ml pyruvate kinase).

2.3.3. T-tubule depolarization. This was done following the Na^+ replacement protocol described previously ([12,15]; cf. [17–19]). The MCA-labeled triads were first polarized by incubating the triads (1.9 mg/ml) in the polarization solution with added 5.0 mM Mg·ATP and an ATP-regenerating system (see above) for 10–15 min. To depolarize the T-tubule, 15 μl of the polarization solution containing the polarized triads was mixed with 135 μl of the depolarization solution using a stopped-flow apparatus (BioLogic SFM3).

The time courses of fluorescence change of the protein-bound MCA (excitation at 368 nm, emission at 440 nm using an interference filter with 70 nm bandwidth) induced by polarization and depolarization were monitored with the stopped-flow fluorometer (BioLogic SFM-3 with MOS-200 optical system). Approximately 20 traces (polarization-induced changes) and 30 traces (depolarization-induced changes) of the MCA fluorescence signal were averaged for each experiment.

2.4. Assays of T-tubule polarization with the membrane potential probe

The time course of T-tubule polarization was monitored using $[^{14}C]SCN^-$ as a membrane potential probe as described previously [13]. The assay was based upon the following principle. When a potential difference ($\Delta\psi$) is generated across a membrane by polarization, $[^{14}C]SCN^-$ is taken up by T-tubules until electrochemical equilibrium is established. At equilibrium, $\Delta\psi = -(RT/zF)\ln(C_i/C_o)$, where C_i and C_o are the intravesicular (extracellular) and extravesicular (cytoplasmic) concentrations of $[^{14}C]SCN^-$ (review: [20]). Under polarizing conditions, there were significant amounts of $[^{14}C]SCN^-$ uptake by purified T-tubules and triads, but there was very little uptake by the SR vesicles (cf. [13]). Hence, the determination of C_i/C_o at different times of polarization permitted us to follow the approximate time course of T-tubule polarization in the triads. The triads (3.0 mg/ml) were incubated in the polarization solution (see above) with added 2.5 mM Mg·ATP, an ATP regenerating system, and 13.7 μM $[^{14}C]SCN^-$ (55 mCi/mmol; Amersham batch #B40, 1993)[1]. At various times ranging from 4 s to 7 min, a 110 μl portion of the reaction solution was mixed with 990 μl of the polarization solution, immediately followed by filtration through Whatman glass microfiber filter (Whatman type GF/F). The filter was washed with 5 ml of the polarization solution, air-dried, and the radioactivity retained on the filter was counted. Voltage-dependent and independent portions of $[^{14}C]SCN^-$ uptake were determined and the C_i/C_o values were calculated as described in [13].

3. Results

We followed the fluorescence intensity of the MCA probe attached to the JFP moiety of the triad during the incubation of the triads in the polarization solution (Fig. 1A) and compared it with the time course of T-tubule polarization assessed by the $[^{14}C]SCN^-$ uptake (Fig. 1B). As seen, the fluorescence intensity of the JFP-bound MCA decreased approximately in parallel to the time course of T-tubule polarization. This suggests a new concept that polarization in the T-tubule moiety of the triad induces a conformational change in the JFP moiety.

This concept has been further supported by the fact that the MCA fluorescence decrease was inhibited when T-tubule polarization was prevented (Fig. 2A–C). Thus, the omission of Mg·ATP (the substrate for the (Na^+, K^+) pump) during the polarization attempt prevented the fluorescence decrease (Fig.

[1] The product is not commercially available any more, and future experiments will have to rely upon custom labeling.

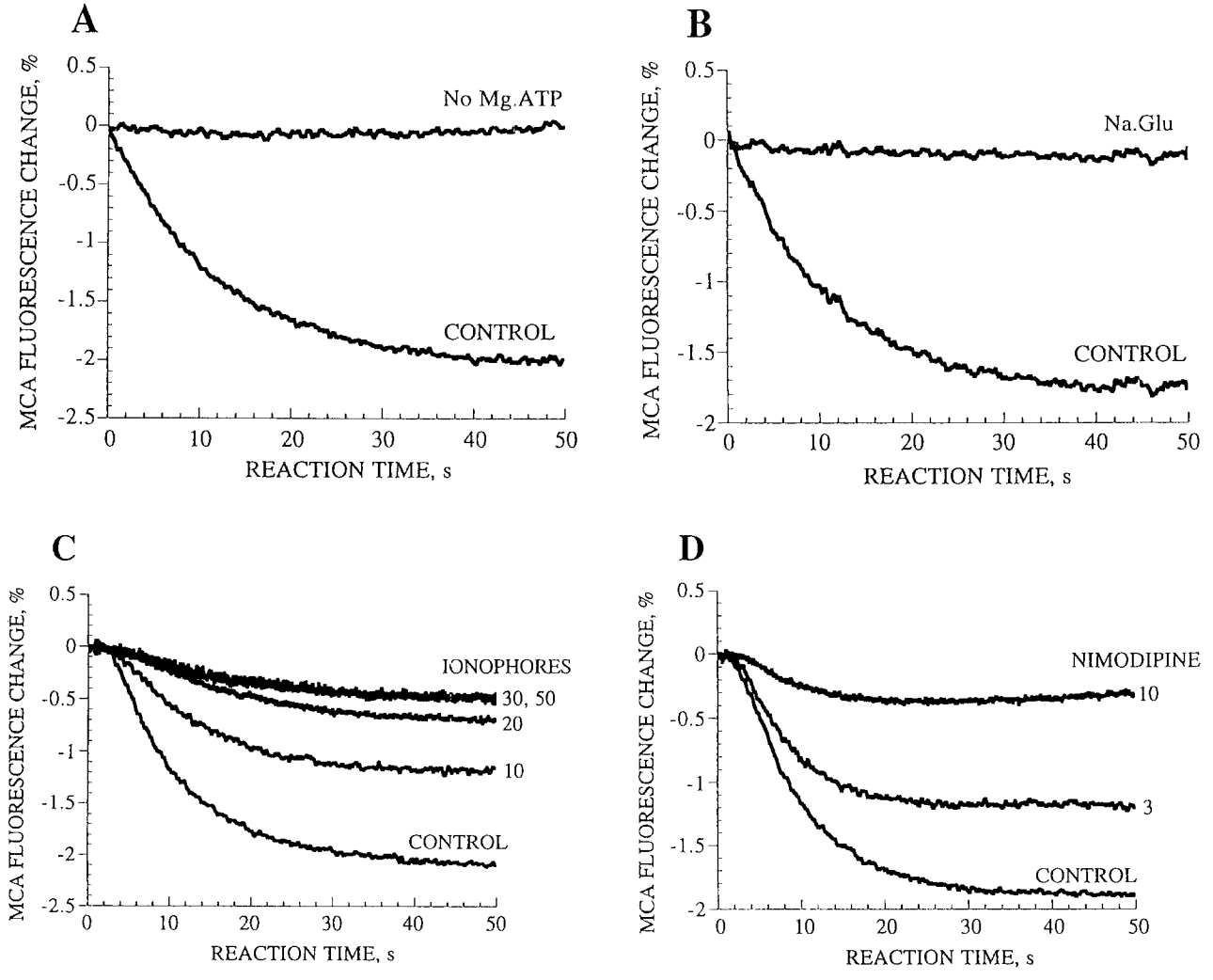


Fig. 2. Several procedures that prevent T-tubule polarization block the decrease of MCA fluorescence (A–C), and the DHPR antagonist nimodipine blocks the MCA fluorescence decrease even in the polarizing conditions (D). Triads were incubated in solutions with various modifications as follows, and the time course of the changes in the MCA fluorescence was determined. A: No added Mg·ATP in a polarization solution; B, with Mg·ATP in a depolarization solution; C: with Mg·ATP in the polarization solution containing an equimolar mixture of monensin/valinomycin (μM); D, with Mg·ATP in a polarization solution containing nimodipine (μM).

2A). Incubation of triads with Mg·ATP under depolarizing conditions (in the presence of 165 mM Na^+ and 0 mM added K^+) produced no fluorescence decrease (Na·Glu, Fig. 2B). Permeabilizing the T-tubule membrane to both Na^+ and K^+ by adding increasing concentrations of an equimolar mixture of monensin and valinomycin resulted in an increased extent of inhibition of the MCA fluorescence decrease (Fig. 2C).

As previously described, depolarization-induced MCA fluorescence increase was blocked by several μM nimodipine, indicating that the depolarization-induced conformational change in the JFP is mediated via the T-tubule voltage-sensor ([11], cf. [21]). In the experiments shown in Fig. 2D, triads were polarized in the presence of increasing concentrations of nimodipine. As seen, 10 μM nimodipine produced almost complete inhibition of the polarization-induced MCA fluorescence decrease. This suggests that conformational changes in the JFP induced by polarization, as well as those induced by depolarization, are mediated via the T-tubule voltage sensor.

In order to analyze the relationship between the polarization-induced MCA fluorescence decrease described here and the previously reported depolarization-induced fluorescence increase [11,15], we carried out both experiments with the same sample (Fig. 3). As seen from the comparison between the polarization-induced changes (curve a, left panel) and the depolarization-induced changes (curve a, right panel), the magnitude of depolarization-induced fluorescence increase is approximately identical to that of polarization-induced fluorescence decrease, although the rate constants of the changes ($k_{pol} = 0.093\ s^{-1}$; $k_{dep} = 17.9\ s^{-1}$) are significantly different. Fig. 3 also illustrates that the depolarization attempt on the non-polarized sample produced no MCA fluorescence change (Fig. 3, curve b). These results suggest that the polarization-induced conformational change is a prerequisite for the depolarization-induced conformational change and channel activation.

4. Discussion

Our previous studies [11,15] on the depolarization-induced conformational change in the JFP and Ca^{2+} release from the SR suggested that upon T-tubule depolarization the JFP undergoes a rapid conformational change from a low MCA fluorescence state R to a high MCA fluorescence state (*R)

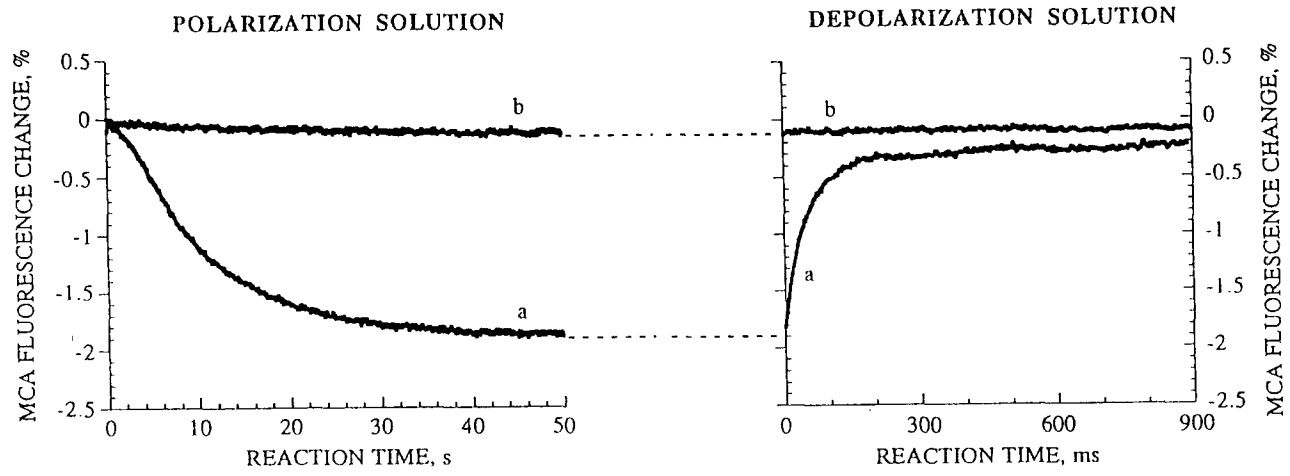
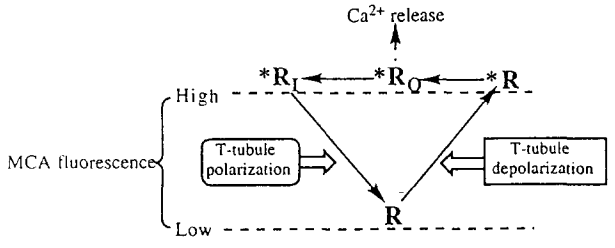


Fig. 3. Composite time courses of MCA fluorescence changes (see curve a), induced first by T-tubule polarization (left panel) and then by depolarization (right panel), suggest that the polarization-induced JFP conformational change is reversed upon depolarization to activate the release channel. Treatment with a depolarization solution produces no fluorescence change in the non-polarized triad (see curve b). Curve a: In the first reaction (left panel), the MCA-labeled triads (0.19 mg/ml) were incubated in a polarization solution containing 0.15 M K·gluconate, 15 mM NaCl, 0.5 mM BAPTA/Ca buffer ($[Ca^{2+}]=0.1$ μM), 2.5 mM Mg·ATP, and an ATP-regenerating system. In the second reaction (right panel), one volume of the polarized triads (1.9 mg/ml protein, 5 mM Mg·ATP) was diluted with nine volumes of a depolarization solution (0.15 M Na·gluconate, 15 mM NaCl, 0.5 mM BAPTA/Ca buffer, $[Ca^{2+}]=0.1$ μM). Curve b: The MCA-labeled triads were first incubated in a polarization solution devoid of Mg·ATP (left panel), then diluted with a depolarization solution (right panel).

followed by channel opening ($*R_O$) and inactivation ($*R_I$), as illustrated in Scheme 1. Thus, the fluorescence intensity of the JFP-attached MCA probe increases rapidly upon depolarizing the T-tubule moiety of the primed triads and remains at a high MCA fluorescence level (*) even after SR Ca^{2+} release is inactivated.

The most important new finding in the present study is that the MCA fluorescence decreases during incubation of the non-polarized/depolarized triads in the polarizing solution. As determined with the membrane potential probe, the MCA fluorescence decreased in parallel to the (Na^+, K^+) pump-mediated T-tubule polarization [13]. Furthermore, various inhibitors of T-tubule polarization inhibited the MCA fluorescence change. The DHPR antagonist nimodipine also blocked the polarization-induced MCA fluorescence changes. These results suggest that polarization of the T-tubule membrane is sensed by the DHPR/voltage sensor; the message is then transmitted to the JFP, producing a conformational change in the JFP ($*R_I \rightarrow R$, Scheme 1).

The magnitude of MCA fluorescence decrease induced by T-tubule polarization is about identical to, or slightly larger than, that of the fluorescence increase induced by the highest level of T-tubule depolarization (cf. [12]) as shown here (cf. Fig. 3). Importantly, the fluorescence labeling method used in this study permitted specific incorporation of the MCA into the JFP moiety of the triad (cf. [14]), suggesting that the fluorescence signals produced by both T-tubule polarization and depolarization were derived primarily from the JFP moiety. Therefore, the previously reported depolarization-induced MCA fluorescence increase ($R \rightarrow *R$, see Scheme 1) is, as a matter of fact, the reversal of the polarization-induced decrease of the MCA fluorescence ($*R_I \rightarrow R$, Scheme 1) which has occurred prior to depolarization. This also suggests that the polarization-induced conformational change represents a mechanism required for the subsequent activation by T-tubule depolarization. In support of this view, the depolarization attempt on the non-polarized triads failed to produce an MCA fluorescence increase as seen in Fig. 3.



Scheme 1. A scheme explaining reciprocal conformational changes produced by polarization and depolarization in the T-tubule. This model implies that upon T-tubule polarization, the $*R_I$ state, which presumably prevails in the depolarized or non-polarized triads, changes to the R state resulting in the decrease in the MCA fluorescence. Upon T-tubule depolarization, the JFP returns to the high fluorescence state, and the Ca^{2+} channel opens ($*R_o$). This is followed by isomerization to the $*R_I$ state, which will be reprimed to R again if the T-tubule is re-polarized.

In conclusion, the present results suggest the new concept that the conformational state of the JFP is regulated by polarization and depolarization of the T-tubule in a reciprocal fashion. The polarization-dependent conformational change seems to represent a priming mechanism required for the subsequent depolarization-induced channel activation. Identification of the regions and proteins involved in the voltage-dependent control of the conformational states and channel functions will be one of the key issues to better understand the molecular mechanism of E-C coupling.

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