# Effects of Perchlorate on Depolarization-Induced Conformational Changes in the Junctional Foot Protein and $Ca^{2+}$ Release from Sarcoplasmic Reticulum†

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ABSTRACT: Perchlorate is one of the most potent activators of skeletal muscle excitation−contraction (E−C) coupling reported in the literature, but the detailed mechanism of its action remains to be elucidated. In an attempt to further resolve the mode of perchlorate action, the effects of increasing concentrations of perchlorate on the voltage-dependent (T-tubule-mediated) and voltage-independent portions of $Ca^{2+}$ release were investigated using the isolated triad model. Low concentrations of perchlorate (≤10 mM) activated SR $Ca^{2+}$ release only when the T-tubule moiety was chemically depolarized. Higher concentrations of perchlorate (30−100 mM), on the other hand, produced significant activation of SR $Ca^{2+}$ release, regardless of whether or not the T-tubule was depolarized. In order to gain further insights, we monitored the conformational change in the junctional foot protein (JFP), which presumably is an important intermediate step in E−C coupling [Yano, M., El-Hayek, R., & Ikemoto, N. (1995) *J. Biol. Chem. 270,* 3017−3021], using the fluorescently labeled triad preparation. Again, low concentrations of perchlorate (≤10 mM) produced a preferential activation of voltage-dependent protein conformational change, while higher concentrations of perchlorate produced significant activation of voltage-independent protein conformational change. An increase in the ryanodine binding by perchlorate occurred only in the higher concentration range where the voltage-independent protein conformational change was activated. These results suggest that perchlorate activates E−C coupling by acting on at least two different steps: at lower concentrations, on the T-tubule-to-JFP signal transmission step; at higher concentrations, on the JFP directly.

Among several potentiators of skeletal muscle E−C coupling[1] function, a group of chaotropic anions such as perchlorate ($ClO_4^-$) and thiocyanate ($SCN^-$) have the most remarkable effects (Foulks et al., 1973; Foulks & Perry, 1979; Caputo, 1983; Gomolla et al., 1983; Lüttgau et al., 1983; Foulks & Morishita, 1985; Delay et al., 1990; Dulhunty et al., 1992). There are extensive studies on the mode of actions of these activating anions, especially of perchlorate. Several millimolar perchlorate shifts the midpoint potential for activation of charge movement and SR $Ca^{2+}$ release to more negative values (Csernoch et al., 1987; Györke & Palade, 1992), increases the steepness of voltage dependence of charge movement (Lüttgau et al., 1983), and, most importantly, reduces the threshold voltage for the amount of charge to be moved (Huang, 1986; Csernoch et al., 1987; Gonzalez & Rios, 1993). According to the widely accepted view, the net outcome of these effects would be an improvement of the efficiency of functional coupling between the T-tubule voltage sensor and the SR $Ca^{2+}$ release channel (Gomolla et al., 1983; Lüttgau et al., 1983; Csernoch et al., 1987; Stephenson, 1987, 1989; Fill & Best, 1990; Rios & Pizarro, 1991; Rios et al., 1992, 1993; Gonzalez & Rios, 1993; Gallant et al., 1993). Perchlorate also increases the hump ($Q\gamma$) component of charge movement (Huang, 1987) and prolongs the on and off time courses (Gonzalez & Rios, 1993). Rios and his colleagues (Rios et al., 1991; Ma et al., 1993) have recently reported that perchlorate activation of the T-tubule voltage sensor, as determined by the L-type $Ca^{2+}$ channel gating current, was virtually abolished under conditions in which the voltage sensor was isolated from the influence of the JFP/$Ca^{2+}$ channel protein. However, some functions intrinsic to the JFP (e.g., ryanodine binding, channel opening, and $Ca^{2+}$ efflux) are significantly activated by perchlorate even after separation of the protein from the voltage sensor if higher concentrations of perchlorate (20−100 mM) were used (Ma et al., 1993). These findings led them to the intriguing hypothesis that the primary target of perchlorate action is the RyR/SR $Ca^{2+}$ channel and the observed potentiation of charge movement by perchlorate may be a consequence of the activation of the JFP (Rios et al., 1993).

The main aims of the present study are to examine whether perchlorate potentiates T-tubule-mediated SR $Ca^{2+}$ release in the isolated triad model in the same manner as in the intact cell and to identify the reaction steps of E−C coupling that are affected by perchlorate: (i) signal transmission from T-tubule to SR, (ii) conformational change in the JFP which is a mechanism necessary for channel opening (Yano et al.,

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[1] Abbreviations: BAPTA, 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid; E−C coupling, excitation−contraction coupling; JFP, junctional foot protein; MCA, methylcoumarin acetamide; 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; T-tubule, transverse tubular system; SR, sarcoplasmic reticulum.

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1995), and (iii) SR $Ca^{2+}$ release. As shown here, low concentrations of perchlorate (≤10 mM) potentiated specifically the voltage-dependent protein conformational change and SR $Ca^{2+}$ release, both of which were blocked by nimodipine, the blocker of step i above. Higher concentrations of perchlorate (15−100 mM) produced an additional activation of both JFP conformational change and SR $Ca^{2+}$ release, regardless of whether T-tubules were depolarized or not. This additional activation by high concentrations of perchlorate was not prevented by nimodipine, indicating that it was produced by direct stimulation of the JFP. Thus, the present results indicate that perchlorate activates E−C coupling by acting upon at least two clearly distinguishable steps: at lower concentrations, on the T-tubule-to-SR communication; at higher concentrations, on the JFP directly.

## EXPERIMENTAL PROCEDURES

*Preparations.* The triad-enriched microsomal fraction (triad) was prepared from rabbit leg and back muscles by differential centrifugation as described previously (Ikemoto et al., 1988, 1994). After the final centrifugation, the sedimented fraction was homogenized in a solution containing 0.3 M sucrose, 0.15 M potassium 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 preparation was quickly frozen in liquid nitrogen and stored at −70 °C.

*Chemical Depolarization-Induced $Ca^{2+}$ Release.* The $Na^+$-replacement protocol used originally for the skinned muscle fiber system (Lamb & Stephenson, 1990a,b) and recently for isolated triads (Ikemoto et al., 1994) or whole-cell homogenates (Anderson & Meissner, 1995) was employed as a principal method to produce graded depolarization of the T-tubule moiety of the triad. Triads that had been equilibrated in 150 mM potassium gluconate were mixed with 5 mM MgATP in the priming solution containing optimum concentrations of $Na^+$ for T-tubule polarization (Ikemoto et al., 1992) by mediation of the ($Na^+ + K^+$) pump and of $Ca^{2+}$ for active loading of the SR moiety with $Ca^{2+}$. The priming solution was placed in one syringe of a stopped-flow system (BioLogic SFM3) and incubated at 22 °C for 6 min or longer, which was sufficient for the completion of both T-tubule polarization and active loading of SR with $Ca^{2+}$. Then, 15 μL of priming solution was mixed with 135 μL of depolarizing solution (see below) or control solution (mixing but no depolarization) containing appropriate concentrations of a BAPTA−calcium buffer and the $Ca^{2+}$ indicator fluo-3. The time courses of SR $Ca^{2+}$ release were monitored with a stopped-flow fluorometer (BioLogic SFM-3 with MOS-200 optical system).

*(A) Priming solution:* 150 mM potassium gluconate, 1.6 mg/mL triad, 15 mM NaCl, 5 mM MgATP, an ATP-regenerating system (2.5 mM phosphoenolpyruvate, 10 units/mL pyruvate kinase), 50−150 μM $CaCl_2$, and 20 mM imidazole (pH 6.8).

*(B) Depolarization solution:* various concentrations of sodium gluconate, various concentrations of $NaClO_4$ (sodium gluconate + $NaClO_4$ = 150 mM), 15 mM NaCl, 0.1 mM BAPTA−calcium buffer ([$Ca^{2+}$] = 0.1 μM), 2.5 μM fluo-3, and 20 mM imidazole (pH 6.8).

*(C) Control solution:* various concentrations of potassium gluconate, various concentrations of $KClO_4$ (potassium gluconate + $KClO_4$ = 150 mM), 15 mM NaCl, 0.1 mM BAPTA−calcium buffer ([$Ca^{2+}$] = 0.1 μM), 2.5 μM fluo-3, and 20 mM imidazole (pH 6.8).

In order to examine possible osmotic effects by this protocol, the same experiments described above were carried out in the absence of added fluo-3. There was no appreciable change in the light scattering of triads, suggesting that this protocol produced little osmotic effect.

*Monitoring of Conformational Changes in the Junctional Foot Protein.* The JFP moiety of the triad was labeled with the cleavable heterobifunctional cross-linking reagent sulfosuccinimidyl 3-[[2-(7-azido-4-methylcoumarin-3-acetamido)ethyl]dithio]propionate (SAED; Kang et al., 1992) by using neomycin as a site-specific carrier as follows. First, to form neomycin−SAED conjugates, 0.4 mM neomycin was incubated 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. Fifty microliters of neomycin−SAED conjugate (final neomycin concentration was 2 μM) was mixed in the dark with 1 mg of triad protein, brought to 1 mL with PI buffer, and centrifuged for 15 min at 100000*g*. The sedimented fraction was resuspended in 1 mL of PI buffer and photolyzed with UV light in a Pyrex tube at 4 °C for 10 min. In order to cleave the disulfide bond of SAED, β-mercaptoethanol was added (100 mM final), and then the labeled triad solutions were incubated on ice for 1 h. After a final centrifugation for 15 min at 100000*g*, the sedimented triads were resuspended in 50 μL of PI buffer to a final protein concentration of 20 mg/mL. 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 (data not shown).

*Fluorescence Assays of the Protein Conformational Change.* The time course of depolarization-induced changes in the fluorescence intensity of the JFP-bound MCA probe was monitored as follows. After the fluorescently labeled triads (2.0 mg/mL) were incubated in the priming solution for at least 6 min to complete both T-tubule polarization and $Ca^{2+}$ loading of the SR, 15 μL of the primed triads was mixed with 135 μL of depolarization solution devoid of fluo-3. The time courses of depolarization-induced change in the fluorescence intensity of the JFP-bound MCA (excitation at 368 nm, emission at 440 nm using an interference filter with 70 nm bandwidth) were monitored with the same stopped-flow fluorometer as described above (Yano et al., 1995). The time course of the change in MCA fluorescence was expressed as $\Delta F/F_0$ (in percent), where $\Delta F$ is the change in MCA fluorescence and $F_0$ is the baseline fluorescence intensity of the MCA probe. Approximately 50 traces of the MCA signal were averaged for each experiment.

*[$^3$H]Ryanodine Binding Assay.* Triads (0.4 mg/mL) were incubated for 120 min at 36 °C in 100 μL of a reaction solution containing 8 nM [$^3$H]ryanodine, 150 mM potassium gluconate, 20 mM imidazole (pH 6.8), various concentrations of $NaClO_4$, various concentrations of NaCl ([$NaClO_4$] + [NaCl] = 115 mM), and 0.1 mM BAPTA−calcium buffer ([$Ca^{2+}$] = 0.1 μM). The reaction mixture was filtered through Whatman GF/A filters and washed twice with 5 mL of distilled water. The specific binding was calculated as the difference between the binding in the absence (total binding) of and in the presence (nonspecific binding) of 10 μM unlabeled ryanodine (El-Hayek et al., 1993). The

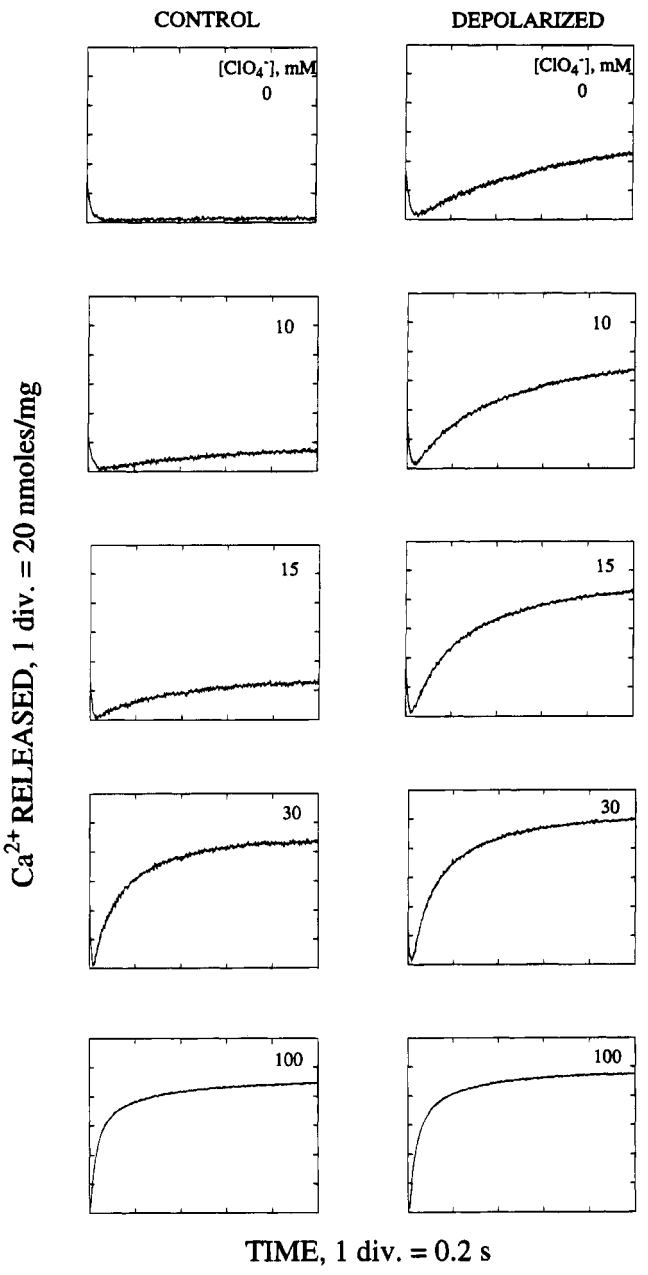



FIGURE 1: Time courses of $Ca^{2+}$ release from SR upon mixing the primed triads with depolarizing (right panel) or control (nondepolarizing) (left panel) solution in the presence of various concentrations of perchlorate as indicated (the numbers within the figure frame). Upward excursion represents $Ca^{2+}$ release. The initial rapid descending phase is a mixing artifact. Each trace was obtained by signal-averaging a total of 90−150 traces originating from three to five different experiments.

experiments were carried out in duplicate; each datum point is the average.

## RESULTS

*Increasing Concentrations of Perchlorate First Activate Voltage-Dependent $Ca^{2+}$ Release from SR and Then Activate Voltage-Independent $Ca^{2+}$ Release.* In the experiments shown in Figure 1, the primed triads were mixed with the depolarizing solution (right panel) or nondepolarizing control solution (left panel) containing various concentrations of perchlorate as indicated, and $Ca^{2+}$ release induced under these conditions was investigated. As seen, an increase in the concentration of perchlorate added to the dilution solution

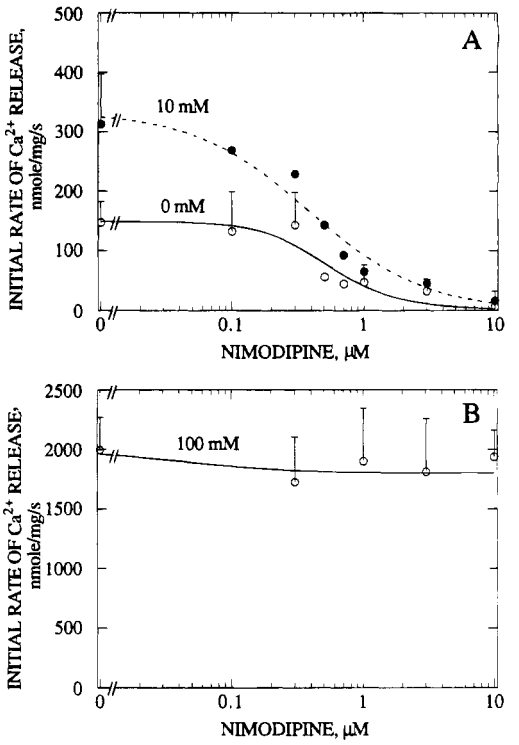



FIGURE 2: Nimodipine dose dependence of the inhibition of SR $Ca^{2+}$ release induced by mixing with depolarizing solution containing various concentrations of perchlorate (A, 0 and 10 mM; B, 100 mM). Each datum point represents the mean ± standard deviation (with error bar, $n = 3$; without error bar, $n = 2$).

produced significant activation of the voltage-dependent $Ca^{2+}$ release at lower concentrations and activation of the voltage-independent release at higher concentrations. Thus, in the low concentration range (0−10 mM), perchlorate activated $Ca^{2+}$ release induced by mixing with the depolarization solution (right panel), with much smaller effect on the time course obtained by dilution with control (nondepolarizing) solution (left panel). When the concentration was increased further to 30 mM, however, perchlorate began to produce significant activation of $Ca^{2+}$ release induced under nondepolarizing conditions (left panel) and plateaued thereafter. In contrast, increasing concentrations of perchlorate from 10 to 30 mM under depolarizing conditions produced a relatively small activation of $Ca^{2+}$ release. At the higher concentrations (30−100 mM), the amount of $Ca^{2+}$ released in the depolarizing solution became comparable to that induced under nondepolarizing conditions. Thus, it appears that $Ca^{2+}$ release in the depolarizing solution containing lower concentrations of perchlorate is induced by T-tubule depolarization but that in the presence of higher concentrations it is induced by a direct effect of perchlorate on the SR.

In the absence of perchlorate, 10 $\mu$M nimodipine [cf. Rios and Brum (1987)] produced almost complete inhibition of the voltage-dependent portion of $Ca^{2+}$ release. However, it had no appreciable effect on voltage-independent $Ca^{2+}$ release produced by higher concentrations of perchlorate. Figure 2 shows the nimodipine concentration dependence of inhibition of $Ca^{2+}$ release induced by the depolarizing solution in the presence of three concentrations of perchlorate: 0, 10, and 100 mM. As shown in Figure 2A, 10 mM perchlorate produced no appreciable effect on the $IC_{50}$ for nimodipine inhibition (~0.5 $\mu$M), confirming that the portion of SR $Ca^{2+}$ release activated by 10 mM perchlorate is also

under the control of T-tubule. In the presence of 100 mM perchlorate (Figure 2B), however, there was virtually no inhibition in the nimodipine concentration range investigated. As seen in the following section, this is due to the fact that at these high concentrations perchlorate works as a direct trigger of the JFP.

*Further Analysis of the Concentration-Dependent Dual Effects of Perchlorate by Fluorometric Monitoring of Conformational Changes in the Junctional Foot Protein (JFP) Moiety.* As shown in our recent studies, upon stimulation of SR either *via* T-tubule depolarization (Yano et al., 1995) or by direct stimulation with polylysine (El-Hayek et al., 1995), the JFP undergoes protein conformational changes before the $Ca^{2+}$ release channel opens. Thus, the protein conformational change appears to be a mechanism that is localized in the JFP moiety and is a prerequisite for channel activation. To investigate perchlorate effects on protein conformational change under both depolarizing and nondepolarizing conditions, we utilized triad preparations whose JFP moiety had been labeled in a site-specific manner with the fluorescent conformational probe MCA [cf. Kang et al. (1992) and Experimental Procedures].

As seen in Figure 3, the effects of increasing concentrations of perchlorate on the JFP conformational change in the depolarizing (right panel) and control (nondepolarizing) (left panel) conditions follow essentially the same pattern as in the $Ca^{2+}$ release experiments (cf. Figure 1). Thus, in the <15 mM range perchlorate activated primarily the depolarization-induced protein conformational change (right panel). At the higher concentrations, however, perchlorate induced substantial protein conformational changes in both depolarizing (right panel) and nondepolarizing (left panel) conditions. As seen in Figure 4A, under depolarizizng conditions, perchlorate activated protein conformational change in a monotonic fashion in the range of 0−30 mM. Under nondepolarizing conditions, however, perchlorate activation occurred in two clearly distinguishable phases: no effect in the range of 0−10 mM and a sharp increase in the activation in the range of 10−30 mM. Nimodipine (10 $\mu$M) produced almost complete inhibition of depolarization-induced protein conformational change in the presence of low concentrations of perchlorate (<15 mM), but it had virtually no effect on protein conformational change produced by high concentrations of perchlorate (data not shown).

*Activation of Ryanodine Binding Occurs Only at High Concentrations of Perchlorate.* The ryanodine binding property is widely used as a tool to assess protein conformation corresponding to the open state of the ryanodine receptor/$Ca^{2+}$ channel [for review, see Lai and Meissner (1989)]. It was shown that 100 mM perchlorate produced a severalfold increase of the ryanodine binding to the triad-enriched membrane fraction (Ma et al., 1993), but the perchlorate concentration dependence of the activation of ryanodine binding remains to be investigated. We carried out ryanodine binding assays under nondepolarizing conditions comparable to those used for the studies of perchlorate activation of SR $Ca^{2+}$ release and JFP conformational change. Similar to the MCA fluorescence data, at low concentrations (0−15 mM) perchlorate had virtually no effect on ryanodine binding. Significant activation occurred only in the higher concentration range of perchlorate (15−100 mM).

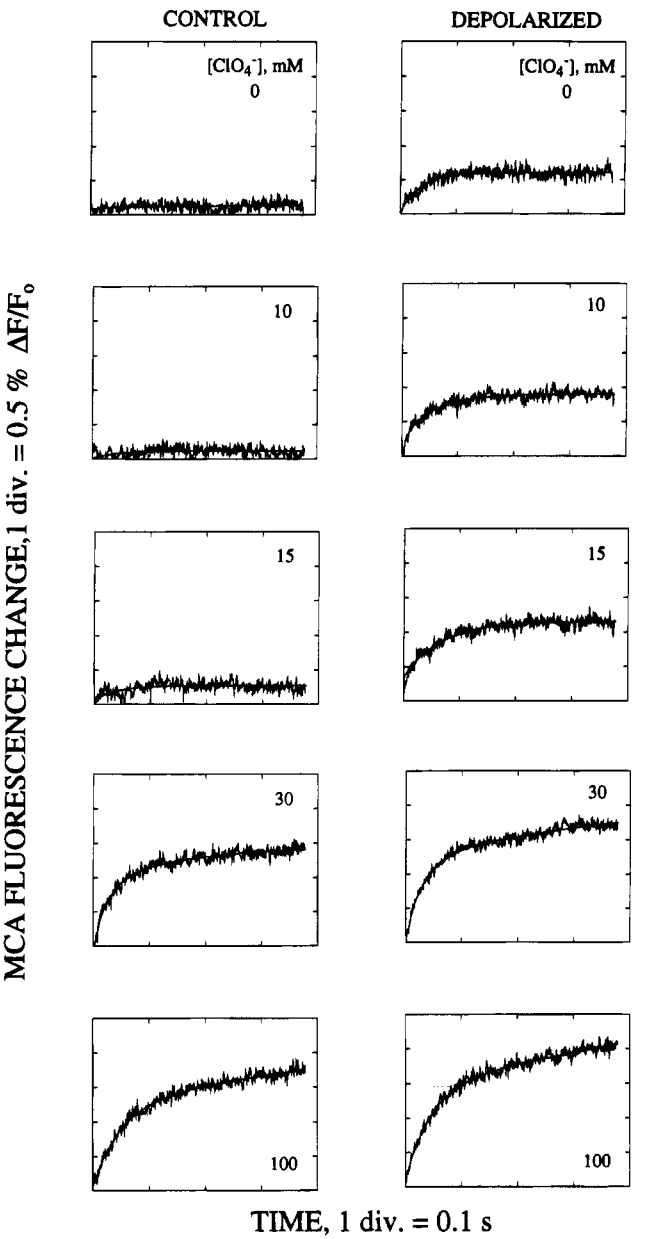
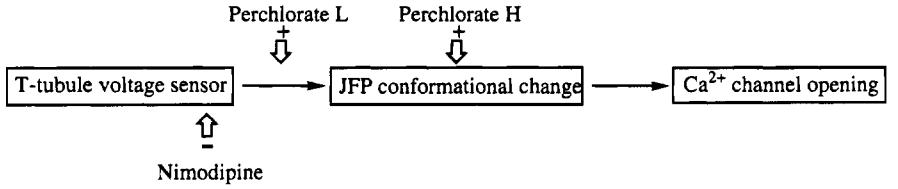



FIGURE 3: Time courses of the changes in the fluorescence intensity of the JFP-bound MCA probe (protein conformational change) upon mixing the primed triads with depolarizing (right panel) or control (nondepolarizing) (left panel) solution in the presence of various concentrations of perchlorate as indicated (the numbers within the figure frame). Upward excursion represents an increase in the fluorescence intensity of the JFP-bound MCA probe. Each trace was obtained by signal-averaging a total of 120−300 traces originating from two to five different experiments.

## DISCUSSION

As outlined in the introduction, a considerable body of information is available concerning the potentiating effects of perchlorate on E−C coupling in skeletal muscle cells and on the isolated JFP. It appears that perchlorate in the millimolar range acts upon the initial steps of E−C coupling, such as voltage-sensing and signal transmission from T-tubule to SR. There are several reports suggesting direct activation of the SR $Ca^{2+}$ release channel protein by perchlorate between 10 and 100 mM concentrations (Ma et al., 1993; Fruen et al., 1994). Thus, the accumulated information in the literature suggests that the action of perchlorate is targeted to several different reaction steps of E−C coupling.

Scheme 1



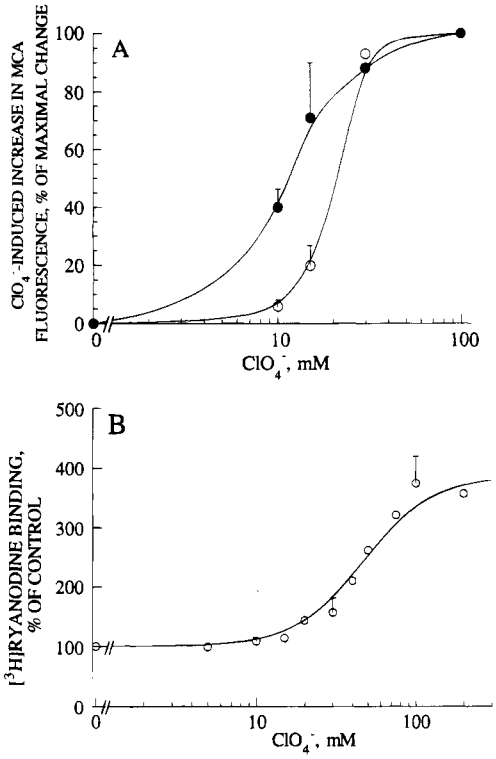




FIGURE 4: (A) Perchlorate-induced increase (percent of maximal change) of the fast phase of the MCA fluorescence change (JFP conformational change) upon mixing the primed triads with depolarizing (●) or control (nondepolarizing) (○) solution in the presence of various concentrations of perchlorate. The magnitude of the change in MCA fluorescence in the fast phase ($A_1$) was calculated by fitting a double-exponential function [$y = y_0 + A_1(1 - e^{-k_1 t}) + A_2(1 - e^{-k_2 t})$] to the time course of the change in MCA fluorescence. Then, the relative value of the perchlorate-induced increase was calculated by taking the $A_1$ values at 0 and 100 mM perchlorate as 0% and 100%, respectively. Actual values of $A_1$ ($\Delta F/F_0$ in percent) at different concentrations of perchlorate are listed in the table below. Each datum point represents the mean ± standard deviation (with error bar, $n = 3-5$; without error bar, $n = 2$). (B) Percent activation of [$^3$H]ryanodine binding under conditions comparable to those of the control protein conformational assays at different concentrations of perchlorate added. [$^3$H]-Ryanodine binding in the absence of perchlorate (control) was 0.12 ± 0.03 pmol/mg. Nonspecific binding was ≤10% of total binding. Each datum point represents the mean ± standard deviation (with error bar, $n = 3$; without error bar, $n = 2$).

| | [$ClO_4^-$], mM | | | | |
|---|---|---|---|---|---|
| | 0 | 10 | 15 | 30 | 100 |
| control | 0.05 ± 0.06 | 0.10 ± 0.07 | 0.22 ± 0.11 | 0.83 | 0.89 ± 0.24 |
| depolarized | 0.37 ± 0.07 | 0.64 ± 0.10 | 0.84 ± 0.19 | 0.95 | 1.03 ± 0.20 |

As shown here, perchlorate exerted clearly two distinguishable types of effects on both JFP conformational change and $Ca^{2+}$ release. At low concentrations (≤10 mM), it activated primarily the voltage-dependent protein conformational change and subsequent $Ca^{2+}$ release, which were induced only by T-tubule depolarization and were inhibited by the voltage-sensor antagonist nimodipine. At higher concentrations, however, perchlorate produced a voltage-independent protein conformational change and $Ca^{2+}$ release, which were induced by the nondepolarizing protocol and were not inhibited by nimodipine.

One of the most important new aspects of this study is that we have investigated perchlorate effects not only on $Ca^{2+}$ release but also on the JFP conformational change which presumably represents a mechanism necessary for the opening of the $Ca^{2+}$ release channel (Yano et al., 1995). This has permitted us to localize the sites of perchlorate action in the updated reaction model including the newly resolved protein conformational change (Scheme 1). In the presence of low concentrations of perchlorate (perchlorate L), blocking of the voltage-sensor function by nimodipine resulted in the inhibition of both protein conformational change induced by T-tubule depolarization and the subsequent $Ca^{2+}$ release. The present observation that ≤10 mM perchlorate produced no appreciable change in the $IC_{50}$ for nimodipine inhibition suggests that perchlorate activation occurred after voltage-sensing but before the conformational change in the JFP. However, we cannot exclude an alternative possibility that depolarization-induced conformational change might have increased the affinity of the JFP for perchlorate, which allowed perchlorate to act upon the JFP even at low concentrations. Higher concentrations of perchlorate (perchlorate H) obviously acted directly on the JFP, leading to JFP conformational changes which seem to be a basic mechanism prerequisite for activation of opening the $Ca^{2+}$ channel regardless of the types of release trigger (T-tubule depolarization or polylysine). As a matter of fact, higher concentrations of perchlorate activate all SR $Ca^{2+}$ release, protein conformational change, and ryanodine binding under nondepolarizing conditions. In light of this reaction model, this suggests that at higher concentrations perchlorate works by itself as the $Ca^{2+}$ release trigger and induces $Ca^{2+}$ release by mediation of the protein conformational change mechanism. Thus, the activation by high concentrations of perchlorate represents in fact perchlorate-induced $Ca^{2+}$ release.

According to an allosteric feedback model proposed by Rios et al. (1993), the potentiation of charge movement by perchlorate is a consequence of the activation of the JFP (cf. introduction). In light of the present results, this model predicts that the activation of the T-tubule voltage sensor by perchorate would occur in a biphasic fashion: a relatively small activation by low concentrations of perchlorate *via* voltage-dependent JFP conformational change and a larger activation by high concentrations of perchlorate *via* voltage-independent direct stimulation of the JFP. It would be interesting to test this hypothesis in the intact fiber system. In order to investigate this hypothesis in the triad system, it is necessary to develop a method that permits one to monitor a protein conformational change in the T-tubule voltage

sensor in parallel to the JFP conformational change.

In conclusion, perchlorate, at low concentrations (≤10 mM), produces a specific potentiation of depolarization-induced JFP protein conformational change and SR $Ca^{2+}$ release in the isolated triad preparation. This resembles the effect of several millimolar perchlorate on charge movement and on the intracellular $Ca^{2+}$ transient *in vivo* and is probably governed by the mechanism by which perchlorate promotes tighter coupling between the T-tubule voltage sensor and the SR $Ca^{2+}$ release channel. Higher concentrations of perchlorate directly stimulate the JFP leading to SR $Ca^{2+}$ release (perchlorate-induced $Ca^{2+}$ release), as evidenced by a significant increase in conformational change in the JFP and in ryanodine binding under nondepolarizing conditions.

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