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EFFECTS OF PERCHLORATE ON EXCITATION–CONTRACTION COUPLING IN FROG AND CRAYFISH SKELETAL MUSCLE

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SUMMARY

1. The effects of perchlorate on various aspects of excitation–contraction coupling in frog and crayfish skeletal muscle have been examined in optical and electrophysiological experiments on voltage-clamped cut muscle fibres.

2. In the frog, perchlorate shifted the voltage dependence of charge movement and consequent sarcoplasmic reticulum (SR) Ca^{2+} release, but it had little effect on the slow inward calcium current.

3. In the crayfish, perchlorate had little effect on either calcium currents or the SR Ca^{2+} release that contributes to myoplasmic Ca^{2+} elevations.

4. Two alternative explanations for these results are discussed. There may be two functional types of dihydropyridine receptors, those (perchlorate sensitive) that communicate with the ryanodine receptor via charge movement and those (perchlorate insensitive) that function solely as Ca^{2+} entry points. Alternatively, the results would be consistent with two separate voltage sensors on each dihydropyridine receptor, only one of which is perchlorate sensitive.

INTRODUCTION

The process of excitation–contraction (E–C) coupling in crayfish striated muscle, as in cardiac muscle, appears to be mediated by voltage-dependent inward Ca^{2+} current triggering Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum (SR) (Györke & Palade, 1991, 1992). In vertebrate skeletal muscle the slow dihydropyridine (DHP)-sensitive Ca^{2+} current has no importance for E–C coupling (Armstrong, Bezanilla & Horowicz, 1972; Lüttgau & Spiecker, 1979). The dihydropyridine receptors instead appear to act as T-tubule voltage sensors which activate SR Ca^{2+} release channels by a mechanism related to intramembranous charge movement (Schneider & Chandler, 1973; Rios & Brum, 1987). It has been suggested that E–C coupling and I_{Ca} in frog and mammalian muscles are mediated by two different functional types of DHP receptors (Schwartz, McCleskey & Almers, 1985; De Jongh, Merrick & Catterall, 1989), but this issue is still unresolved (Lamb, 1991).

The chaotropic anion perchlorate has been reported to shift the voltage dependencies of charge movement (Lüttgau, Gottschalk, Kovacs & Fuxreiter 1983), contraction (Foulks & Perry, 1979) and intracellular Ca^{2+} transients (Csernoch,

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Kovacs & Szucs, 1987) in intact and cut frog muscle fibres. Effects of perchlorate appear to be specific for E–C coupling since other voltage-dependent processes are not affected (Gomolla, Gottschalk & Lüttgau, 1983).

The purpose of this study was to compare the effects of perchlorate on mechanisms of E–C coupling in frog and crayfish muscle under similar methodological conditions.

METHODS

Small diameter fibres were selected from the m. extensor carpopoditi of the crayfish *Procambarus clarkii* (Carolina Biological Supply, Burlington, NC, USA). The claws were surgically removed from the animals under tricaine anaesthesia. Fibre segments were mounted in a triple Vaseline-gap voltage clamp chamber (Hille & Campbell, 1976), with an external saline solution containing (mM): 115 $TEACH_3SO_3$, 115 $NaCH_3SO_3$, 10 $Ca(CH_3SO_3)_2$, 5 3-(*N*-morpholino) propane sulphonic acid (MOPS), pH 7·2) in the A pool, where the electrical and optical recordings were made. The other pools contained an internal solution containing (mM): 230 caesium aspartate, 5 sodium phosphocreatine, 3 MgATP, 0·2 EGTA and 5 MOPS, pH 7·2. After permeabilization of the fibre segment in the E pool with saponin (0·1 % for 1 min), 200–400 μM Rhod-2 (Molecular Probes Inc., Eugene, OR, USA) was added. After allowing 1 h to reach a steady state [Rhod-2] in the A pool portion of the fibre, Ca^{2+} transients were elicited upon depolarization from a holding potential of −90 mV. The fibre segment in the A pool was illuminated from above at 45 deg using a fibre optic transmitting the light from a 150 W tungsten–halogen source passed through a 550 nm broadband filter (40 nm half-bandwidth; Ditric Optics, Hudson, MA, USA). Fluorescence of the fibre fragment was collected through a long working distance 32 × objective (Leitz UMK-40), then passed through a long-pass barrier filter (OG 590, Omega Optical, Brattleboro, VT, USA) onto a photovoltaic cell (EG&G 440 UV) mounted on the microscope trinocular. Optical signals (ΔF) were normalized to the resting fluorescence (F).

Frog skeletal muscle fibres were treated in a similar fashion except that slightly different solutions were employed. Single-fibre segments were dissected from m. semitendinosus of frogs (*Rana pipiens* or *catesbeiana*). The compositions of the external solutions were, for Ca^{2+} transient measurements (mM): 115 $NaCH_3SO_3$, 2 $Ca(CH_3SO_3)_2$, 5 $CoSO_4$, 10^{-7} TTX, 5 MOPS, pH 7·2; for I_{Ca} measurements (mM): 105 $NaCH_3SO_3$, 10 $Ca(CH_3SO_3)_2$, 10^{-7} TTX, 5 MOPS, p·H 7·2; for charge movement measurements (mM): 90 $NaCH_3SO_3$, 8 $Ca(CH_3SO_3)_2$, 10 $CoSO_4$, 10^{-7} TTX, 5 MOPS, pH 7·2. The basic composition of the internal solution in all experiments was (mM): 115 caesium aspartate, 5 sodium phosphocreatine, 3 MgATP, and 5 MOPS, pH 7·2. The solution for measuring intracellular Ca^{2+} transients contained in addition 200–400 μM Rhod-2. Solutions for measuring I_{Ca} and charge movement were supplemented instead with 10 mM EGTA to prevent fibre movement. $NaClO_4$ when added at 10 or 20 mM concentration to the external solutions, was substituted for an equal concentration of $NaCH_3SO_3$. *In vitro* calibration of Rhod-2 fluorescence as function of $[Ca^{2+}]$ is described elsewhere (Györke & Palade, 1992).

RESULTS

Effects of perchlorate on intracellular Ca^{2+} transients and charge movement in frog muscle fibres

Traces of intracellular Ca^{2+} transients recorded at different depolarizing potentials in a frog muscle fibre under control conditions and after addition of 10 mM sodium perchlorate to the external solution are shown in Fig. 1. The threshold for eliciting Ca^{2+} transients is shifted to more negative values. As shown in Fig. 1*B*, perchlorate shifts the voltage dependence of Ca^{2+} release by about 20 mV toward more negative potentials, without affecting maximal Ca^{2+} release at large depolarizations. Similar effects of perchlorate on intracellular Ca^{2+} transients were reported in frog muscle fibres using antipyrylazo III (Csernoch *et al.* 1987).

Examples of the effects of perchlorate on charge movements recorded from voltage

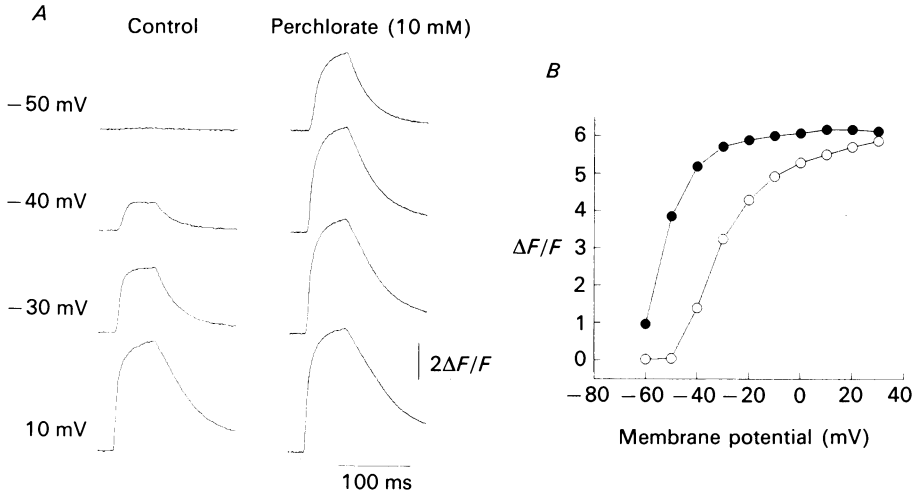

Fig. 1. Effect of 10 mM perchlorate on intracellular Ca^{2+} transients in a cut frog muscle fibre. *A*, records of intracellular Ca^{2+} transients during voltage steps to the potentials indicated before and after addition of 10 mM perchlorate to the external solution, at a holding potential of −90 mV. *B*, the results presented in *A* (peak amplitude attained during the pulse) and results obtained at other potentials are plotted as function of membrane potential under control condition (○) and in the presence of perchlorate (●). Curves are drawn by hand.

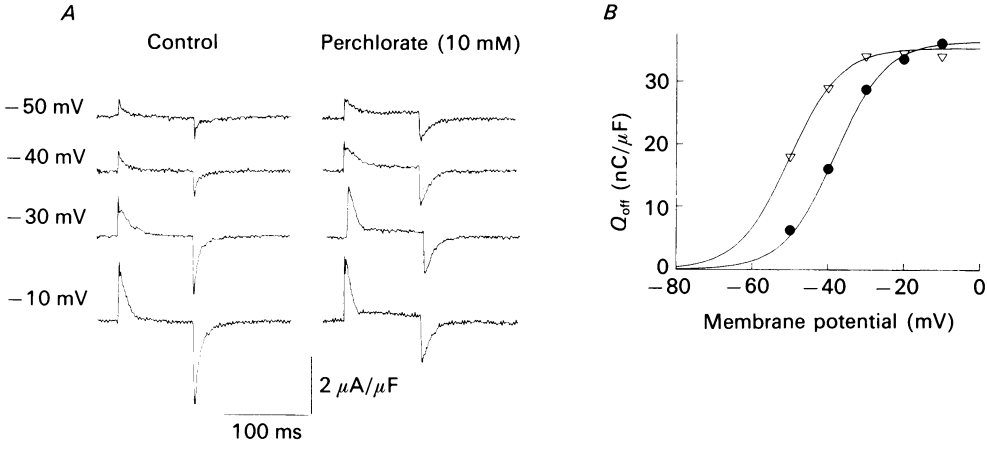

Fig. 2. Effect of 10 mM perchlorate on the intramembrane charge movements in a cut frog muscle fibre. *A*, charge movement records obtained at depolarizations to the indicated membrane potentials before and after addition of 10 mM perchlorate to the external solution; holding potential −90 mV. *B*, voltage dependence of the amounts of charge displaced by 'off' transients (Q_{off}) in *A*; ● correspond to control solution and ▽ to the presence of 10 mM perchlorate. Curves are the best fits to a two-state Boltzmann distribution $Q = Q_{max}/1+\exp-(V-\bar{V})/k)$, where Q_{max} is the maximum amount of charge, $\bar{V}$ is the potential at which half of Q_{max} has moved, and $1/k$ is a steepness factor, with Q_{max} = 35·92 nC/μF, V = −38·27 mV and rate constant k = 6·66 mV for ●, and Q_{max} = 34·80 nC/μF, $\bar{V}$ = −49·00 mV and k = 6·700 mV for ▽.

clamped frog fibres are shown in Fig. 2*A*. In agreement with earlier reports (Lüttgau *et al.* 1983; Csernoch *et al.* 1987), charge movement was activated at more negative potentials in the presence of 10 mM perchlorate, and 'off' tails exhibited a more prolonged relaxation. In addition to these effects, a small maintained outward current consistently appeared in the perchlorate records, as if a non-linear ionic current were introduced by the perchlorate.

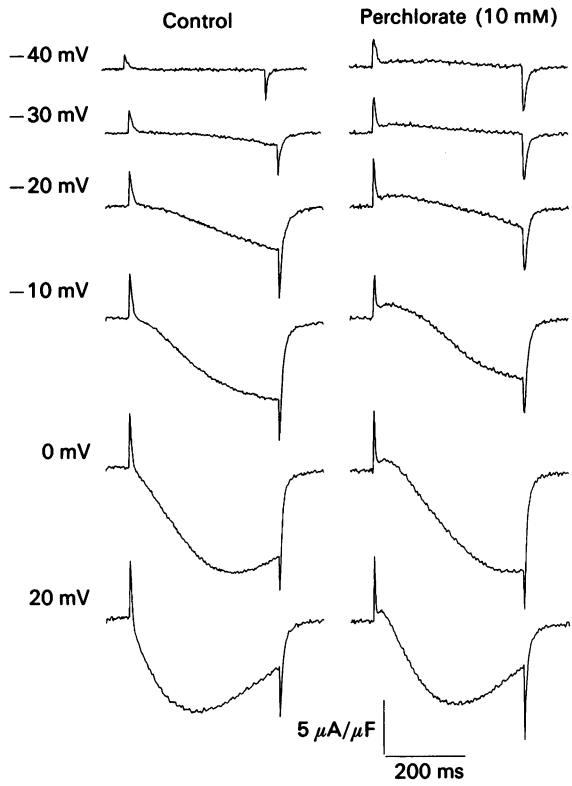

Fig. 3. Effect of perchlorate on membrane currents in a cut frog muscle fibre. The records show currents during voltage steps to the potentials indicated under control conditions and in the presence of 10 mM extracellular perchlorate, from a holding potential of −90 mV.

The voltage dependence of the charge moved in 'off' transitions is plotted in Fig. 2*B*. As with Ca^{2+} transients, the voltage dependence of the charge moved is shifted to more negative potentials with application of perchlorate, without significant change in the maximal amount of the charge moved.

Effect of perchlorate on the slow inward Ca^{2+} current in frog muscle fibres

In contrast to charge movement, the slow inward calcium current was not affected by perchlorate. Examples of records of calcium current at different membrane potentials in control conditions and after addition of 10 mM sodium perchlorate to the external solution are shown in Fig. 3. Although the amounts of charge moved at depolarizing pulses to −50 and −70 mV became clearly increased after introduction

of perchlorate, calcium currents in the presence of perchlorate appear and reach their maximum at the same membrane potentials as they do under control conditions. At the same time, the onset of the current measured in the presence of perchlorate appears to be slower than under control conditions. The slowing down of the rise of

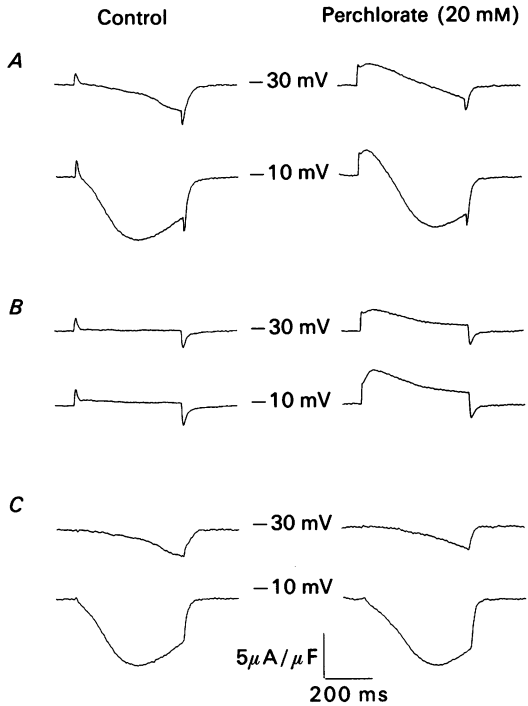

Fig. 4. Lack of effect of perchlorate on the net membrane Ca^{2+} currents in frog muscle fibres. *A*, membrane currents recorded during depolarizing pulses to −30 and −10 mV under control conditions and in the presence of 20 mM extracellular perchlorate. *B*, membrane currents under the same conditions as in *A* with addition of 1 mM Cd^{2+} to the external solution. *C*, membrane Ca^{2+} currents (I_m) estimated as difference: I_m in reference solution − I_m with Cd^{2+}. Left (before) and right (after) addition of 20 mM perchlorate to the external solution. Holding potential −90 mV.

the inward current could be a result of the increase in outward current induced by perchlorate.

To test this hypothesis, we applied 1 mM Cd^{2+} to block I_{Ca} and then subtracted the traces obtained from corresponding records obtained in the absence of Cd^{2+} (Fig. 4). The two upper panels show traces recorded at −30 and −10 mV in the presence and absence of 20 mM perchlorate with no Cd^{2+} in the external bath solution. Cd^{2+} at a concentration of 1 mM completely blocks I_{Ca} and reveals a substantial outward current in the presence of perchlorate (Fig. 4*B*). The outward currents increased with increasing perchlorate concentration and depolarization. We speculate that these currents may represent perchlorate entry through anion channels.

After substraction of the outward current, I_{Ca} in the presence and absence of perchlorate exhibits similar time courses at both potentials. Similar results were obtained on six other fibres using 10 or 20 mM perchlorate.

Effects of perchlorate on Ca^{2+} transients and I_{Ca} in crayfish muscle fibres

Ca^{2+} currents and associated myoplasmic Ca^{2+} transients elicited by depolarizing pulses to different membrane potentials in the voltage clamped crayfish muscle fibre under control conditions are shown in Fig. 5. Both I_{Ca} and Ca^{2+} transients begin to

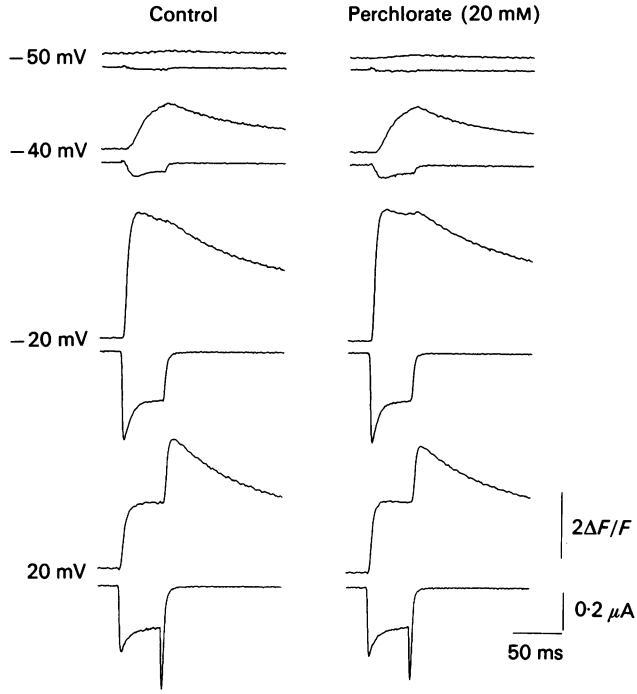

Fig. 5. Effect of perchlorate on I_{Ca} and intracellular Ca^{2+} transients in a cut crayfish muscle fibre. I_{Ca} and Ca^{2+} transients (the lower and upper of each pair of traces, respectively) were measured in response to voltage clamp pulses to the potentials indicated before and after addition of 20 mM perchlorate to the external solution. Holding potential −90 mV.

be observed at a threshold of approximately −40 mV. The Ca^{2+} transients, like I_{Ca}, grow larger as the membrane is depolarized further, reaching a maximum at about −10 mV. Over this range of potentials the Ca^{2+} transients resemble those recorded from frog fibres, though Ca^{2+} transients in crayfish were generally lower in amplitude ($6{\cdot}3 \pm 1{\cdot}2$ $\Delta F/F$ ($n = 6$) in frog *vs.* $3{\cdot}2 \pm 0{\cdot}9$ $\Delta F/F$ ($n = 7$) in crayfish). In contrast to frog muscle fibres, where amplitude of Ca^{2+} transients saturates at high depolarizations, Ca^{2+} transients in crayfish fibres show a gradual decrease in the amplitude with depolarizations to increasingly positive potentials. The decrease in Ca^{2+} transients is associated with the decrease in I_{Ca} as membrane potential (+10 mV trace) approaches the Ca^{2+} equilibrium potential. In addition, after pulses to high potentials the Rhod-2 signals exhibit a secondary large increase following the 'tail' I_{Ca} upon repolarization. As demonstrated previously, these features of Ca^{2+} transients in crayfish muscle fibres can be explained by the mechanism of Ca^{2+}-

induced Ca^{2+} release from the SR (which constitutes at least 90 % of the intracellular Ca^{2+} transient) triggered by I_{Ca} (Györke & Palade, 1991).

As seen in Fig. 5*B*, addition of 20 mM perchlorate to the external solution does not produce any significant effect on either I_{Ca} or Ca^{2+} transients at any potentials. Our

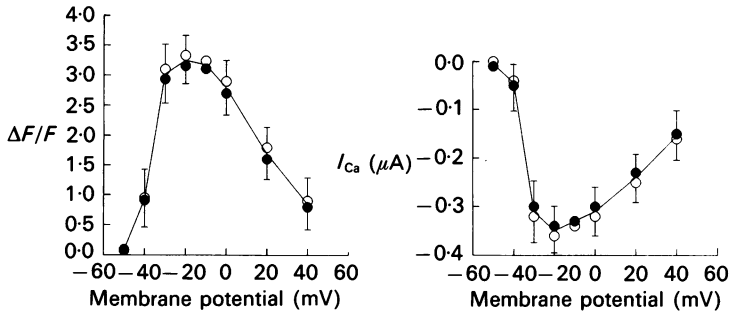

Fig. 6. Membrane potential dependence of peak I_{Ca} (below) and intracellular Ca^{2+} transients measured 20 ms after depolarization (above) of crayfish muscle fibres in control solution (●) and in the presence of 20 mM extracellular perchlorate (○). The symbols give the means (±S.E.M.) of the values obtained from four individual fibres before and after addition of perchlorate. Curves are drawn by hand.

experiments on crayfish muscle with and without perchlorate are summarized in Fig. 6. As seen, there is no significant change in voltage dependence or amplitude of either I_{Ca} or Ca^{2+} transients in the presence of perchlorate.

DISCUSSION

In accordance with earlier studies in voltage-clamped frog muscle fibres (Lüttgau *et al.* 1983; Csernoch *et al.* 1987) perchlorate shifted the activation curve for intramembranous charge movement and associated SR Ca^{2+} release toward more negative potentials. At the same time perchlorate did not have a significant effect on the inward Ca^{2+} current. This confirms the preliminary results of Feldmeyer & Lüttgau (1988) on the lack of effect of perchlorate on I_{Ca} in intact frog fibres measured using a microelectrode technique. This observation is also consistent with the lack of a perchlorate effect on single DHP-sensitive Ca^{2+} channels incorporated in bilayers (Rios, Ma & Gonzalez, 1991).

Both charge movement and I_{Ca} in vertebrate skeletal muscle are believed to be generated by the T-tubule DHP receptors (Rios & Brum, 1987; Tanabe, Beam, Powell & Numa, 1988). Whether the two functions are mediated by the same individual molecule is not clear (Schwartz *et al.* 1985; Lamb, 1991). The different effect of perchlorate on I_{Ca} and E–C coupling would be consistent with the possibility that these two phenomena are mediated by two different functional types of DHP receptors. Perchlorate interacts specifically with DHP receptors associated with charge movement generation, not affecting those that mediate I_{Ca}. The functional diversity of DHP receptors could be accounted for by post-translational modification (De Jongh *et al.* 1989), reversible modification via phosphorylation (Nunoki, Florio

& Catterall, 1989) or different subunit composition of the heteropolymeric DHP receptor (Lory, Varadi & Schwartz, 1991). In addition, the selective perchlorate sensitivity of certain DHP receptors could be a result of interactions with other parts of the E–C coupling machinery (Rios *et al.* 1991). An alternative explanation for the differential effect of perchlorate on I_{Ca} and charge movement may reflect two processes mediated by two different charges in a bifunctional molecule (e.g. Feldmeyer, Melzer, Pohl & Zollner, 1990; Ma, Hosey, & Rios, 1992).

A completely new finding reported here is that perchlorate does not have any effect on I_{Ca} or SR Ca^{2+} release in crayfish muscle fibres. As reported earlier (Györke & Palade, 1992), E–C coupling in crayfish skeletal muscle is mediated by Ca^{2+} influx through sarcolemmal/T-tubule Ca^{2+} channels (i.e. Ca^{2+}-induced Ca^{2+} release) rather than through a mechanical interaction between DHP receptors and the SR Ca^{2+} release channel, as is believed to occur in vertebrate skeletal muscle (Rios *et al.* 1991). Despite the functional differences, it appears that the E–C coupling mechanism in crayfish muscle is served by homologous ryanodine receptors (Formelova, Hursnak, Novotova & Zachar, 1990), dihydropyridine receptors (Krizanova, Novotova & Zachar, 1990), and surface/T-tubule membrane Ca^{2+} channels that are sensitive to both dihydropyridines (Hurnak, Proks, Krizanova & Zachar, 1990) and verapamil (Zahradnik & Zachar, 1983). Similarly no significant effects of perchlorate on the voltage dependence of DHP-sensitive intramembrane charge movement have been reported in cardiomyocytes (Rios *et al.* 1991), a system that closely resembles crayfish muscle with respect to its dependence on Ca^{2+}-induced Ca^{2+} release. The lack of perchlorate effect on intracellular Ca^{2+} transients in crayfish muscle is consistent with the notion that alteration of SR Ca^{2+} release in frog muscle is a consequence of its effects on charge movement rather than a result of its direct interaction with SR Ca^{2+} release (Csernoch *et al.* 1987; Fill & Best, 1990). These results indicate that perchlorate might be a selective probe for the charge-related 'mechanical' interaction between DHP receptors and SR Ca^{2+} release channels.

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