

Effects of Perchlorate on the Molecules of Excitation–Contraction Coupling of Skeletal and Cardiac Muscle

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ABSTRACT To understand the nature of the transmission process of excitation–contraction (EC) coupling, the effects of the anion perchlorate were investigated on the voltage sensor (dihydropyridine receptor, DHPR) and the Ca release channel (ryanodine receptor, RyR) of the sarcoplasmic reticulum (SR). The molecules, from rabbit skeletal muscle, were either separated in membrane vesicular fractions or biochemically purified so that the normal EC coupling interaction was prevented. Additionally, the effect of ClO_4^- was investigated on L-type Ca^{2+} channel gating currents of guinea pig ventricular myocytes, as a native DHPR not in the physiological interaction of skeletal muscle. At 20 mM, ClO_4^- had minor effects on the activation of ionic currents through Ca channels from skeletal muscle transverse tubular (T) membranes fused with planar bilayers: a +7-mV shift in the midpoint voltage, \bar{V} , with no change in kinetics of activation or deactivation. This is in contrast with the larger, negative shift that ClO_4^- causes on the distribution of intramembrane charge movement of skeletal muscle. At up to 100 mM it did not affect the binding of the DHP [^3H]PN200-110 to triad-enriched membrane fractions (TR). At 8 mM it did not affect the kinetics or the voltage distribution of gating currents of Ca channels in heart myocytes. These negative results were in contrast to the effects of ClO_4^- on the release channel. At 20 mM it increased several-fold the open probability of channels from purified RyR incorporated in planar bilayers and conducting Ba^{2+} , an effect seen on channels first closed by chelation of Ca^{2+} or by the presence of Mg^{2+} . It significantly increased the initial rate of efflux of $^{45}\text{Ca}^{2+}$ from TR vesicles (by a factor of 1.75 at 20 mM and 4.5 at 100 mM). ClO_4^- also increased the binding of [^3H]ryanodine to TR fractions. The relative increase in binding was 50-fold at the lowest [Ca^{2+}] used (1 μM) and then decayed to much lower values as [Ca^{2+}] was increased. The increase was due entirely to an increase in the association rate constant of ryanodine binding. The chaotropic ions SCN^- and

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I⁻ increased the association rate constant to a similar extent. The binding of ryanodine to purified RyR protein reconstituted into liposomes had a greater affinity than to TR fractions but was similarly enhanced by ClO₄⁻. The reducing agent dithiothreitol (5 mM) did not reduce the effect of ClO₄⁻, and 5% polyethylene glycol, with an osmolarity equivalent to 20 mM ClO₄⁻, did not change ryanodine binding. The results are consistent with a primary effect of ClO₄⁻ on the release channel and suggest that the effect on charge movement may be secondary, mediated by a mechanical interaction between the voltage sensor and the release channel.

INTRODUCTION

The process of excitation–contraction (EC) coupling, or, more properly, of coupling between transverse tubular (T) membrane depolarization and gating of the sarcoplasmic reticulum (SR) Ca release channel, has in theory three functional stages: voltage sensing, the role of an identified T tubule membrane protein; transmission (a process whose nature remains unclear); and SR channel gating. The agonist effect of ClO₄⁻ on EC coupling has been traditionally rationalized as the consequence of a change in the first of the above, a shift in the voltage dependence of intramembranous charge movement, revealing an increased readiness of the voltage sensor to respond. Through this primary effect the release channel is gated at lower voltages, which is one of the main manifestations of the effect of the anion.

In the preceding paper (González and Ríos, 1993), it was shown that one of the effects of ClO₄⁻ on the voltage sensor, the slowing of charge movement, requires a fully functional system (the effect was not present if the fiber was inactivated by sustained depolarization even though the voltage sensor still generated charge movement in that situation). Additionally, it was shown that ClO₄⁻ has effects on the transmission process; that is, effects that cannot be reduced to primary shifts in the voltage dependence of the T membrane voltage sensor but specifically change the rules of transfer from voltage sensor to release channel.

Thus, ClO₄⁻ can no longer be considered a simple agonist or facilitator of voltage sensing, and must be considered a facilitator of transmission. The observation brings with it the tantalizing possibility that the effect on the voltage sensor is secondary to an effect downstream in the transmission process. If this were the case, and specifically if the effect on the voltage sensor were the consequence of a primary modification of the release channel, the fact that the effect can propagate backwards in the chain of transmission would be strong evidence that the transmission is allosteric, that is, by contact between the proteins.

With this possibility as motivation, we designed studies of the component molecules in isolation, devoid of the physiological interaction between sensors and channels. Four preparations were studied: (1) The dihydropyridine receptors (DHPRs) from T membrane fractions of skeletal muscle, fused with planar lipid bilayers were studied. In this preparation the voltage dependence was studied through gating of DHP-sensitive ion currents (which will be referred to as I_{Ca} , even when carried by Ba²⁺). (2) DHPRs from cardiac muscle (where they constitute L-type Ca channels) were studied in situ using whole cell patch clamp to measure their gating currents. (3) “Triad enriched” SR vesicle fractions were used to study binding of [³H]ryanodine, binding of the dihydropyridine [³H]PN200-110, and efflux of ⁴⁵Ca²⁺. (4) Finally, purified

ryanodine receptor protein, the release channel of the SR membrane, was reconstituted in planar bilayers for the study of single channel activity and into liposomes to evaluate [³H]ryanodine binding.

The rationale for using the cardiac preparation was to take advantage of a native DHPR protein not altered by fractionation, purification, or reconstitution, and known to interact with the corresponding release channel by Ca²⁺-mediated chemical transmission (Nabauer, Callewaert, Cleemann, and Morad, 1989) and not to control release channels as the skeletal muscle counterpart does (Tanabe, Mikami, Numa, and Beam, 1990). With many pharmacological and structural similarities, reviewed later, and this key functional difference, the cardiac DHPR constitutes an interesting term of comparison with the skeletal system. We also took advantage of the recent demonstration of methods to separate the L-type Ca channel component of intramembrane charge movement in cardiac myocytes of the guinea pig ventricle (Shirokov, Levis, Shirokova, and Ríos, 1992).

MATERIALS AND METHODS

Preparation of the Transverse Tubule Membranes from Skeletal Muscle

Transverse (T) tubule membranes were isolated from frozen rabbit skeletal muscle (Pel-Freez Biologicals, Rogers, AR) using the procedures described by Galizzi, Fosset, and Lazdunski (1984). Membrane vesicles were stored at a concentration of 5–7 mg protein/ml in 20 mM Tris-HCl (pH 7.4) in liquid nitrogen until use. This T tubule membrane preparation routinely contained 20–50 pmol DHPRs/mg protein, measured by the specific binding of (+)[³H]PN200-110.

Planar Bilayer Recording of the Skeletal Muscle DHP-sensitive Ca Channels

Planar bilayers were formed across a ~200- μ m-diam aperture with a lipid mixture of phosphatidylethanolamine (PE)/phosphatidylserine (PS)/cholesterol in a ratio of 1:1:0.2. The lipids were dissolved in decane at a concentration of 50 mg/ml. The intracellular solution contained 200 mM KCl, 3 mM ATP-Mg, and 10 mM HEPES-Tris (pH 7.4). The *trans* (extracellular) solution contained 50 mM NaCl, 100 mM BaCl₂, and 10 mM HEPES-Tris (pH 7.4). Bay K 8644 (0.3 μ M) was always present in the intracellular solution because it permitted consistent detection of DHP-sensitive Ca²⁺ channel activity. All experiments were conducted at room temperature (22–24°C).

Single channel currents were recorded and voltage control was imposed with an Axopatch 1C unit (Axon Instruments, Inc., Foster City, CA). Pulse generation and data acquisition were carried out with an AT-compatible computer (model 386/25; CompuAdd Corp., Austin, TX) with a Labmaster DMA-100 converted board (Scientific Solutions Inc., Solon, OH). The current was filtered at 100 Hz using an 8-pole Bessel filter (902 LPF; Frequency Devices Inc., Haverhill, MA), digitized at 500 Hz, and stored.

The bilayer was initially held at –80 mV (holding potential [HP], *cis* – *trans*). When these methods of preparation were followed, the DHP-sensitive Ca channel activity was strictly dependent on membrane potential, being triggered by depolarizing pulses to –40 mV and beyond. As described by Ma, Mundiña-Weilenmann, Hosey, and Ríos (1991), the recorded currents were first corrected for linear capacitive and electrostrictive currents by subtraction of “controls,” averages of null sweeps. Thus, all the single channel currents shown in this article are differences between recorded currents and controls. To determine the effect of ClO₄[–] on channel properties, we first measured channel activity in reference solutions with pulse protocols to be described in Results, and then ClO₄[–] was added to both chambers to reach a

concentration of 20 mM and the series of pulses was repeated. The experiments usually lasted for 20–40 min without apparent channel run-down.

The procedures of data analysis have been described elsewhere (Ma et al., 1991). Most important in this article is the computation of the average open probability during a pulse (\bar{P}_o), which is calculated as the fraction of the total pulse duration occupied by open events (in turn detected using pCLAMP software). \bar{P}_o is an average of a time- and voltage-dependent P_o , which gives an overall measure of activation as a function of voltage (Ma et al., 1991).

Intramembrane Charge Movement in Ventricular Myocytes

The methods for measuring charge movement in guinea pig cardiomyocytes have been described in detail (González and Ríos, 1993). Briefly, cells were enzymatically isolated from the ventricles of guinea pig hearts (strain Duncan-Hartley, 250–400 g) by a method similar to those of Isenberg and Klockner (1982) and Bean and Ríos (1989) and kept at room temperature before the experiments, which were conducted at 19–22°C. Cells were superfused with a channel blocking solution containing (mM): 154 TEA Cl, 10 BaCl₂, 6 CdCl₂, 0.1 LaCl₃, and 10 HEPES, titrated to pH 7.25 with TEA hydroxide. The internal solution (in the patch pipette) contained (mM): 80 TEA aspartate, 20 creatine phosphate, 5 Mg ATP, 10 EGTA, and 10 glucose, titrated to pH 7.8 (Byerly, Meech, and Moody, 1984). Currents were measured in the whole cell mode of the patch voltage clamp technique (Hamill, Marty, Neher, Sakmann, and Sigworth, 1981) using borosilicate glass pipettes (Corning 7052; Garner Glass, Claremont, CA) heat polished to a resistance of 2–4 MΩ when filled with the internal solution.

Voltage clamping, pulse generation, and data acquisition were done with an Axopatch 1 system with Axolab interface (Axon Instruments, Inc.), controlled by an IBM AT-compatible computer (model 386/25; CompuAdd Corp.) running pCLAMP software. Currents were filtered at 1 kHz (4-pole Bessel filter) and sampled at 10–20 Hz.

The whole cell capacitance circuitry of the Axopatch 1 was modified to allow analogic compensation of linear capacity transients from cells of up to 300 pF by injection of an appropriate current through a capacitor directly into the headstage input.

After establishing the whole cell configuration, the access resistance was typically in the range of 5–10 MΩ and input capacitance was 100–250 pF. Series resistance was routinely compensated 70–90% and compensation was periodically adjusted.

The HP was usually in the range of –80 to –110 mV; control pulses for the conventional P/–4 procedure were taken at voltages more negative than –120 mV. A prepulse potential was maintained for 100 ms before the control pulse sequence and individual control pulses were separated by 100 ms at the prepulse potential. The sets of pulses were applied with a frequency of not more than 0.2 Hz to avoid contamination of controls with nonlinear charge movement (of the charge 2 type; Shirokov et al., 1992). All records illustrated in this article were obtained from single test and multiple control sweeps. The data were not analyzed if the linear capacity transient changed during the control pulses so that large artifacts due to residual linear current were apparent, or if records had substantial ionic current components (usually outward at positive potentials). The intramembrane charge moved by test voltage steps was measured by integrating the transient asymmetric current above a reference level computed usually as the average current for 5–10 ms before the test pulse. For computing the ON charge at very high positive test voltages, the reference level was taken from the steady-state baseline current between 40 and 50 ms in 50-ms pulses. No sloping baseline correction was made.

Preparation of SR Vesicle Fractions.

Rabbit leg and back muscle (40 g) were homogenized in a Waring blender (two times for 30 s on high setting) in 300 ml of buffer containing 0.1 M NaCl, 5 mM Tris maleate, pH 6.8, 2 mM

EDTA, 0.2 mM EGTA, and various protease inhibitors (0.2 mM PMSF, 100 nM aprotinin, 1 μ M leupeptin, 1 μ M pepstatin A, and 1 mM benzamidine). A 2,500–30,000-g crude microsomal pellet obtained from the homogenate was resuspended for 1 h in 50 ml of buffer A (100 mM NaCl, 10 mM K-PIPES, pH 6.8, 100 μ M EGTA, 75 μ M CaCl₂, 1 μ M leupeptin, and 0.2 mM PMSF). After repelleting the crude microsomal fraction by centrifugation at 100,000 *g* for 30 min, vesicle fractions were separated according to buoyant density by isopycnic centrifugation through 20–45% linear sucrose density gradients containing buffer A. SR membrane fractions used in this study were recovered from the 32–34% sucrose region of gradients. The collected fractions were diluted slowly in buffer B (0.1 M NaCl and 5 mM NaPIPES, pH 6.8) so that the final sucrose concentration was 0.3 M. After pelleting, the vesicles were taken up in buffer B, quick-frozen, and stored in aliquots at -75°C for subsequent determination of high affinity [³H]ryanodine and [³H]PN200-110 binding and ⁴⁵Ca²⁺ efflux.

Ryanodine Receptor Purification, Reconstitution, and Recording

The ryanodine receptor (RyR) was purified on sucrose gradients from a CHAPS-solubilized SR enriched microsomal fraction as previously reported (Lai, Erickson, Rousseau, Liu, and Meissner, 1988). The protein was stored in a buffer containing 0.5 M NaCl, 10 mM NaPIPES (pH 7.0), 2.5 mg/ml phosphatidylcholine, and 0.5% CHAPS at -75°C until use. The purified CHAPS-containing RyR-Ca²⁺ release channel complex was reconstituted into lipid bilayer vesicles by removal of detergent by dialysis. CHAPS-containing receptor gradient fractions were placed into a dialysis bag and dialyzed at 4°C for 44 h against a buffer containing 0.5 M NaCl, 100 μ M EGTA, 200 μ M Ca²⁺, 0.5 mM DIFP, 1 mM DTT, and 10 mM NaPIPES, pH 7 (Lee, H.B., and G. Meissner, unpublished observations). The dialyzed sample was diluted with 1 vol of deionized water, and proteoliposomes were sedimented by centrifugation in a Ti75 rotor (Beckman Instruments, Inc., Fullerton, CA) at 50,000 rpm for 2 h. The pellets were resuspended in 0.25 M NaCl, 50 μ M EGTA, 200 μ M Ca²⁺, and 10 mM NaPIPES, pH 7, quick-frozen in dry ice–acetone suspension, and stored at -75°C . Frozen samples were slowly thawed at room temperature and sonicated for 1 min at 23°C in a bath sonicator (Laboratory Supplies Co., Inc., Hicksville, NY) before use.

The single channel recording experiments were carried out at room temperature. Planar bilayers were formed with a lipid mixture of PE and PS in equal concentrations. 0.2–0.5 μ g of RyR was added to the *cis* solution, which contained 250 mM KCl, 3 mM ATP-Mg, 10 mM HEPES-Tris (pH 7.4), and 1 mM Ca-EGTA (free [Ca²⁺] = 10 μ M, measured with a Ca²⁺-sensitive electrode). Incorporation of the Ca release channel was facilitated by an initial *cis/trans* gradient of 250/50 KCl. After a successful channel incorporation, BaCl₂ was added to the *trans* solution to reach 100 mM. The final composition of the *trans* solution was 50 mM KCl, 100 mM BaCl₂, 10 mM HEPES-Tris, and 1 mM Ca-EGTA. The channels incorporated to the bilayer with their myoplasmic side facing the *cis* solution, as verified by their sensitivity to EGTA addition to the *cis* side.

The currents were filtered analogically at 2 kHz and digitized at 0.1 ms/point using the FETCHEX program of pCLAMP software and the same hardware described above for experiments on T membrane channels. Analysis of data, leading to “diary” (*P*_o vs. time) plots, was carried out with TIPS software (provided by Dr. A. Vandongen, Baylor College of Medicine, Houston, TX).

⁴⁵Ca Efflux from Triad-enriched SR Vesicles

Triad-enriched membrane fractions (0.1 mg protein/ml) were actively loaded at 25°C in a ⁴⁵Ca²⁺ uptake medium (5 mM NaPIPES, pH 7, 100 mM NaCl, 5 mM ATP, 8 mM MgCl₂, 0.25 mM EGTA, and 0.1 mM CaCl₂). After incubation for 6 min, aliquots were placed on 0.65- μ m

type DA Millipore filters and washed with either RyR channel inhibitory or activating release media for 0.2–180 s. Time points of 0.2–5 s were measured using a rapid filtration apparatus. Longer time points were measured by washing filters manually. Inhibitory media contained 5 mM NaPIPES, pH 7, 5 mM MgCl₂, 1 mM EGTA, and 20 μM ruthenium red. Release media contained 5 mM NaPIPES, pH 7, 5 mM nitrilotriacetic acid, and 175 μM CaCl₂ (free [Ca²⁺] of 5 μM). Inhibitory and inactivating media also contained either 100 mM NaCl (reference medium) or mixtures of NaClO₄ and NaCl such that total Na was always 100 mM (perchlorate media). ⁴⁵Ca²⁺ trapped by the vesicles on the filters was quantified by liquid scintillation counting.

[³H]Ryanodine Binding to SR Vesicles

Triad-enriched vesicles (0.08–0.7 mg protein/ml) were incubated in reference and perchlorate media which contained 100 mM NaCl or mixtures of NaClO₄ and NaCl such that total Na was always 100 mM. Media also contained 10 mM NaPIPES, pH 7.2, 5 mM AMP, 1–800 nM [³H]ryanodine, and [Ca²⁺] ranging from <0.1 μM to 5 mM. [Ca²⁺]’s of 5–100 μM were obtained with 5 mM nitrilotriacetic acid and calcium. All other [Ca²⁺]’s were obtained with 500 μM EGTA. All [Ca²⁺]’s were verified using a Ca²⁺ electrode or a pH titration method. Equilibrium binding was determined after 20 h at 12°C; longer incubation times failed to give additional binding. Free [³H]ryanodine was determined by centrifugation for 30 min at 90,000 g in an airfuge (Beckman Instruments, Inc.), the total bound was determined by a filtration assay, and nonspecific binding was determined in a 100-fold excess of unlabeled ryanodine. Radioactivity was quantified by liquid scintillation counting.

To determine [³H]ryanodine dissociation rates, vesicles (0.7 mg protein/ml) were labeled with 5 nM [³H]ryanodine as described above, followed by a 100-fold dilution into 25°C media containing no ryanodine. To determine [³H]ryanodine association rates, vesicles (0.3–0.7 mg protein/ml) were added to 25°C media containing 3–25 nM [³H]ryanodine.

[³H]PN200-110 Binding to SR Vesicles

Effects of ClO₄⁻ on [³H]PN200-110 equilibrium binding were determined by incubating triad-enriched vesicles (0.05 mg protein/ml) for 1 h at 23°C (longer incubations failed to result in additional binding) in reference and ClO₄⁻-containing media (as defined above for ryanodine binding) containing 25 mM Tris HCl, pH 7.5, 50 μM EGTA, 50 μM Ca, and 0.1 nM [³H]PN200-110. [³H]PN200-110 association rates were determined in the presence and absence of ClO₄⁻ by adding vesicles (0.05 mg protein/ml) to the above described media at 25°C. To determine B_{max} values, equilibrium binding was measured as described above except that saturating [³H]PN200-110 (15 nM), instead of 0.1 nM, was included in the incubation media (Dunn, 1989). Total bound [³H]PN200-110 was determined by a filtration assay and nonspecific binding was defined as that occurring in the presence of 500 nM unlabeled nifedipine. Radioactivity was quantified by liquid scintillation counting.

RESULTS

Effects of Perchlorate on DHP-sensitive Ca Channels in Bilayers

Previous studies demonstrated that Ca channels from T membrane of skeletal muscle, reconstituted in bilayers held polarized at -80 mV, are strictly voltage dependent (when studied with the methods and solutions described; Ma et al., 1991). When Ba²⁺, present in the extracellular chamber only, is the carrier of the ionic current, the channels open when depolarized to or beyond -40 mV. Single channel

currents during successive pulses to the voltages indicated are shown in Fig. 1 (as described in Materials and Methods, the currents have been corrected for linear capacity and electrostriction components). In the records shown, which were obtained contiguously but represent a much longer series of sweeps, there is no activity at -50 mV but there is activity at -30 and -10 mV both with and without ClO_4^- . There are indications in the records shown that the activity at -30 mV could be somewhat more frequent in reference, whereas at -10 mV it seems somewhat more intense in ClO_4^- .

Fig. 2 is the quantitative confirmation of these impressions using pooled data from many experiments. The activity was quantified as described before by an average open probability during the 2-s pulse, \bar{P}_o . \bar{P}_o was evaluated in sets of ~ 30 sweeps from five different bilayers successively in reference and 20 mM ClO_4^- . Fig. 2

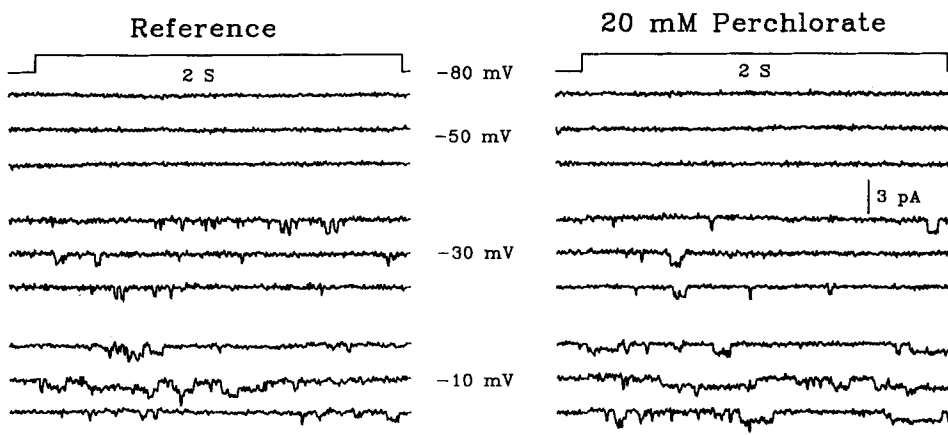


FIGURE 1. Effects of ClO_4^- on current through T membrane Ca channels. (Left) Ba currents through dihydropyridine-sensitive channels in a reference solution (described in Materials and Methods). (Right) Currents in the same experiment in the presence of 20 mM ClO_4^- on both sides of the membrane. The membrane was held at -80 mV and pulsed to the potentials indicated. Averaged nulls recorded at the same voltage were subtracted from each record. Channel opening is downward, corresponding to *trans* \rightarrow *cis* Ba current.

represents the averages of \bar{P}_o over the five experiments in reference (open symbols) and ClO_4^- (filled symbols). The bars represent one standard error of the mean.

There is no major difference induced by ClO_4^- except at the two highest voltages (-10 and 0 mV). At those potentials the differences were appreciable, but not significant ($P > 0.1$) in two-sided t tests at both voltages). However, the significance increases if the results at -10 and 0 mV are combined. The variable t of the combined difference is 2.09, which corresponds to a P of ~ 0.05 . Thus the average open probability is unchanged at the voltages where ClO_4^- has its greatest physiological effects, but may increase at high voltages.

To further compare this effect with those on muscle fibers, the activation of \bar{P}_o with increasing voltage was described with a Boltzmann (two-state canonical distribution)

function:

$$P = \bar{P}_{\max} / [1 + \exp(\bar{V} - V)/K] \quad (1)$$

The fits are shown as curves in Fig. 2. The best fit parameters in reference were: $\bar{P}_{\max} = 0.092$ (0.005), \bar{V} (transition voltage) = -23 (0.2) mV, and K (steepness factor) = 5.9 (0.2) mV. In ClO_4^- the parameters were: $\bar{P}_{\max} = 0.139$ (0.01), $\bar{V} = -16$ (0.4) mV, and $K = 5.9$ (0.4) mV. Thus, ClO_4^- changed the transition potential slightly, but in the *positive* direction, contrary to the effects in the muscle fiber.

One of the most characteristic effects of ClO_4^- in the fibers is the slowing, sometimes by several-fold, of the intramembrane charge movement current at the OFF of depolarizing pulses. Therefore it was of interest to seek effects on the kinetics

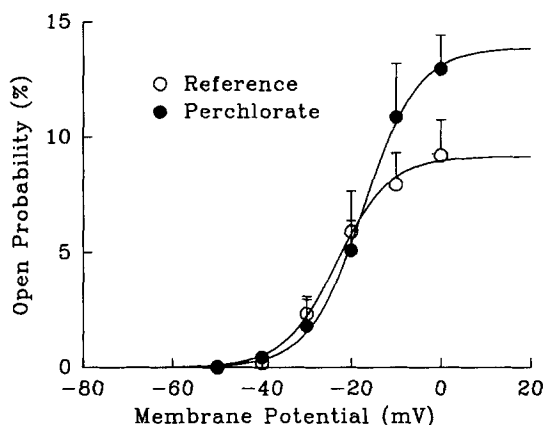


FIGURE 2. Voltage dependence of open channel probability. Average open probability (\bar{P}_o) in reference (*open symbols*) and 20 mM ClO_4^- . \bar{P}_o was calculated in each sweep as the fractional open time. Open time was evaluated according to a half-amplitude criterion (Ma et al., 1991). Double, and occasionally triple, amplitude events were observed and were interpreted as the superimposed opening of two or three channels. When two open

events superimposed their currents, their durations were independently added for calculation of \bar{P}_o (therefore, the \bar{P}_o evaluated here is affected by the number of channels active in the bilayer). \bar{P}_o was obtained by averaging ~ 30 sweeps at each voltage in individual experiments. Individual values averaged over five experiments are plotted ± 1 SEM. The lines were generated with Eq. 1 and parameter values given in the text. A discussion of the relationship between \bar{P}_o and single channel properties, as well as the errors involved in this calculation, can be found in Ma et al. (1991).

of Ca channel closing (deactivation) at the end of depolarizing pulses. A large change in the kinetics of return of charge movement should result in a comparable change in the kinetics of voltage-dependent channel closing (in a number of different models of transfer from charge movement to channel gating). The kinetics of deactivation in the bilayer was studied in ensemble averages of many sweeps. Such averages are shown in Fig. 3 for a collection of many sweeps from three experiments in reference and two bilayers in ClO_4^- . The pulses were shorter in this case (400 ms) to repolarize and close the channel at about the time of maximum activation (maximum of the time-dependent open probability).

No major changes are associated with the presence of ClO_4^- . The activity remaining after the end of the pulse averages to a tail current that was fitted by a single exponential decay (smooth lines in Fig. 3). The time constant of this exponential was

7.2 (0.29) ms in reference and 9.0 (0.22) ms in ClO_4^- . The difference (1.8 ms) is significant but very small.

In summary, when tested on the Ca channel activity induced by voltage in bilayers, the anion caused neither the voltage shift nor the kinetic changes that it induces at comparable concentrations in the charge movement of muscle fibers. It caused an increase in activation at high voltage and a marginal slowing of channel closing.

Effect of Perchlorate on the Gating Currents of Cardiac Ca Channels

Asymmetric currents were measured in four cardiac myocytes in reference external solution and in a solution with 8 mM ClO_4^- substituted for Cl^- . A family of asymmetric currents from one experiment is shown in Fig. 4. Very little kinetic change, if any, was induced by ClO_4^- .

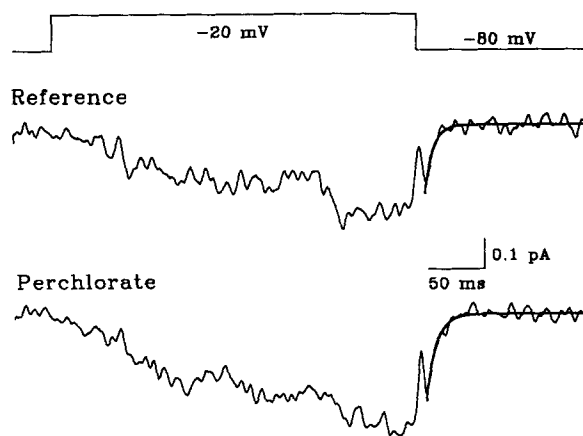


FIGURE 3. Kinetics of DHPR channel closing. Averaged currents during 144 sweeps in three bilayers in reference solution (*top*) and during 130 sweeps in two bilayers in 20 mM ClO_4^- (*bottom*). The pulse was 400 ms long. The currents were corrected by subtraction of averages of null sweeps. The smooth lines at the OFF in both records are single exponential fits ($I = I(0) \exp(-t/\tau)$), with $\tau = 7.2$ ms (standard error of estimate was 0.29) in reference and $\tau = 9.0$ ms (0.22) in 20 mM ClO_4^- . The first 8 ms of the OFF were omitted in the fit.

The charge transferred at each voltage was computed from the asymmetric currents as their time integral after subtraction of a steady level during the ON only (as described by Shirokov et al. [1992], the OFF charge is less than the ON, especially at high voltages, due to inactivation). The values of charge transfer measured in each cell were first fitted with a Boltzmann function (Eq. 1, with charge Q instead of P) and then normalized to the best fit Q_{max} . Values from four cells, normalized in this manner, were averaged and are represented in Fig. 5 as a function of pulse voltage for reference (open symbols) and ClO_4^- (filled symbols). The Boltzmann parameters fitted to the individual cells are listed in Table I together with their averages. The continuous curves of Fig. 5 were generated with Boltzmann functions fitted to the averages, which have parameters not very different from the averages in Table I.

Neither the maximum charge nor the transition voltage were changed by ClO_4^- . ClO_4^- increased the steepness factor somewhat. The difference, 4.3 mV, is significant to $P < 0.01$. Here, as when the effects were studied on skeletal muscle Ca channels in

bilayers, the effects of ClO_4^- were minimal and not consistent with the effects on EC coupling in muscle fibers (ClO_4^- decreases the steepness factor in skeletal muscle charge movement; cf. González and Ríos, 1993).

Effects of Perchlorate on SR Ca Channels in Bilayers

We found apparent effects of perchlorate on the Ca release channel in a number of different $[\text{Ca}^{2+}]$; however, differences could be demonstrated best by first lowering

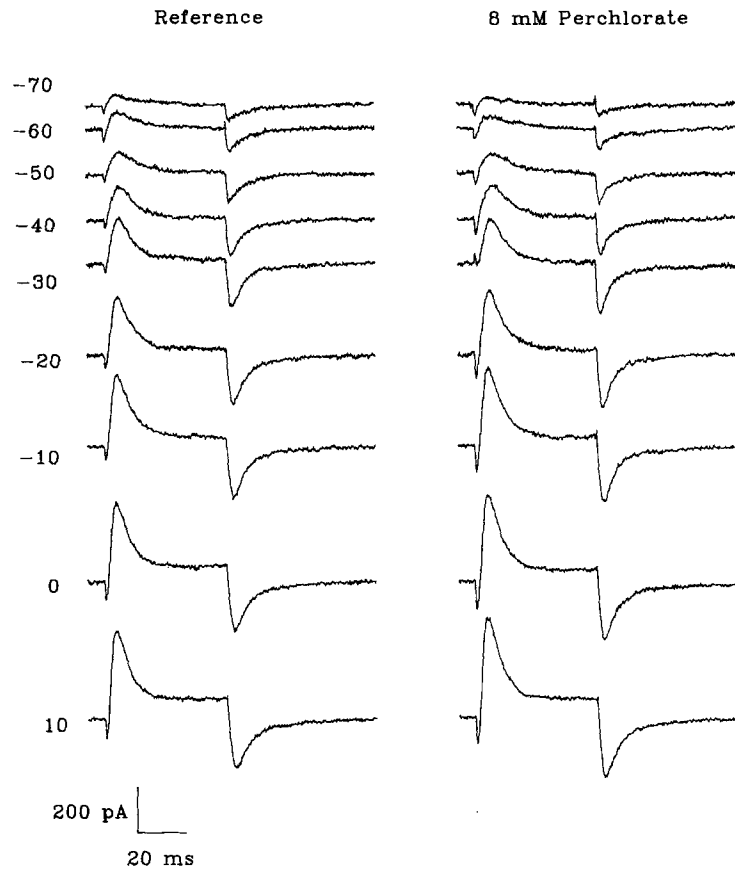


FIGURE 4. Effect of ClO_4^- on gating currents in heart myocytes. Asymmetric currents were obtained by subtraction of currents during test pulses to the voltages indicated (from a HP of -100 mV) and a scaled control current during $P/-4$ pulses, applied from -120 mV in the hyperpolarizing direction. The records are single sweeps, obtained first in reference, then in 8 mM ClO_4^- . Experiment 90004c02. Series resistance of electrode plus cell, 10 m Ω , not compensated.

$[\text{Ca}^{2+}]$ or increasing $[\text{Mg}^{2+}]$; this decreased the P_o and its standard deviation to essentially zero, giving a baseline from which the effects of ClO_4^- were clearer. The following procedure was used: (1) channel currents carried by K^+ were monitored to detect incorporation of a RyR channel; (2) Ba^{2+} was added to the *trans* solution; the

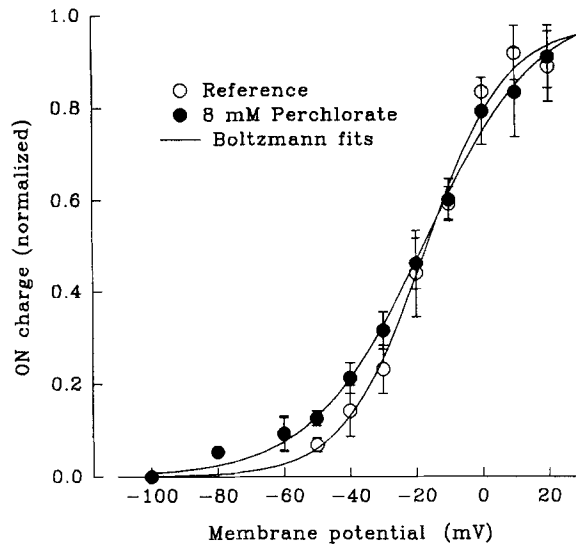


FIGURE 5. Effect of ClO_4^- on the voltage distribution of Ca channel gating charge. Averages of charge transferred during ON transitions from -100 mV to the potential in the abscissa. Asymmetric currents as in Fig. 4 were corrected by subtraction of a constant level (the average during the last 5 ms of the pulse). A Boltzmann distribution function (Eq. 1 with Q instead of P) was fitted to the individual ON values. The values at each voltage were then normalized to the parameter Q_{\max} and averaged among different experiments. Vertical lines span ± 1 SEM. The con-

tinuous lines were obtained with Boltzmann functions fitted to the averages (reference, *open symbols*, $\bar{V} = -17.1$ mV, $K = 12.2$ mV; 8 mM ClO_4^- , *filled symbols*, $\bar{V} = -17.1$ mV, $K = 17.1$ mV). A total of four cells were studied. Their individual Boltzmann parameters are listed in Table I.

activity of the channel was monitored for at least 3 min in this situation and the experiment continued when no rundown or appearance of substates was observed; (3) channel activity was then brought down close to zero by *cis* addition of either EGTA (reducing $[\text{Ca}^{2+}]_{\text{cis}}$ nominally to 40 nM) or 10 mM Mg^{2+} ; and (4) perchlorate was then added to both sides of the membrane to a final concentration of 20 mM.

Currents from a representative experiment are shown in Fig. 6; the downward deflections represent inward current carried by Ba^{2+} at a holding potential of -40 mV.

The complete, continuous record of current was digitized in 250-ms episodes and the open probability was computed for each successive episode. The successive values of P_o for one experiment are plotted in Fig. 7. After 4 min in reference myoplasmic

TABLE I
Effects of Perchlorate on the Voltage Distribution of Charge Movement in Ventricular Myocytes

Cell No.	Reference			8 mM Perchlorate		
	\bar{V}	K	Q_{\max}	\bar{V}	K	Q_{\max}
	mV	mV	fC	mV	mV	fC
90918c05	-19.4	9.8	3,033	-18.7	17.7	3,683
90918c21	-9.8	10.4	3,893	-22.7	12.4	3,199
90004c02	-28.2	12.9	2,664	-19.6	15.1	2,961
90004c10	-16.6	11.1	3,282	-19.8	16.8	2,854
Average	-18.5	11.2	3,218	-20.2	15.5	3,174
SEM	3.8	0.7	258	0.9	1.2	184

The values of ON charge as a function of test pulse voltage were fitted with Eq. 1.

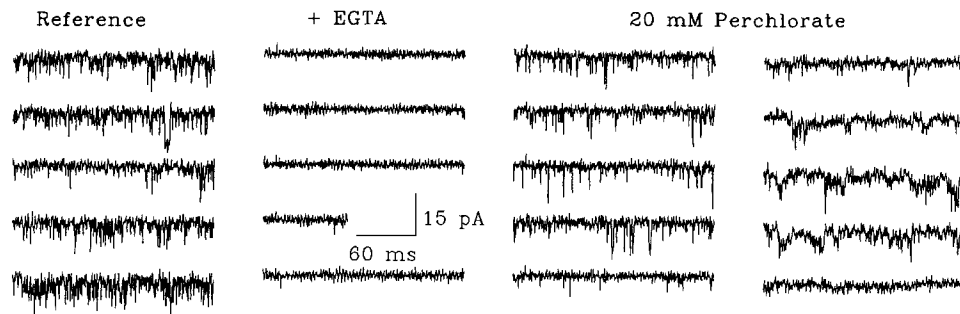


FIGURE 6. Effect of ClO_4^- on single channel currents of purified RyR channels. Currents were carried by Ba^{2+} in the *trans* \rightarrow *cis* direction (downward). The membrane was held at -40 mV. The first panel represents current in reference. The second shows records after *cis* addition of EGTA at a final concentration of 6.25 mM. The last two panels plot episodes recorded 1 and 5 min after addition of 20 mM ClO_4^- to both sides.

solution, during which the P_o was variable but measurable, 6.25 mM EGTA was added to the myoplasmic side. This caused the channels to close. Recording in this situation was continued for 4 min, during which the channels remained closed (as they did in all experiments with EGTA). After this period, 20 mM ClO_4^- was added to both sides of the membrane. After a delay of ~ 20 s, a sizable activity was elicited.

The plot of Fig. 7, which is representative of most of the 10 experiments carried out using EGTA, and of five experiments in which Mg^{2+} was used, shows two features that made the experiments more difficult. One is the variability of the activity in the presence of calcium (early portion of plot), which prompted us to first inhibit the activity before adding ClO_4^- . Another feature of the ClO_4^- effect is the variable latency of the increase in activity, which in our experiments ranged from a few seconds to minutes.

An additional observation, illustrated in the right-most panel of Fig. 6, was the appearance of channel events of reduced conductance and prolonged duration. This only occurred in a few experiments and after prolonged exposure to ClO_4^- . In the quantitative evaluation described below, we did not use the segments of recording obtained after such events started.

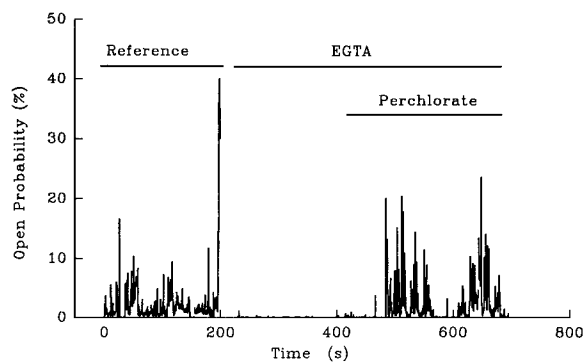


FIGURE 7. Effect of ClO_4^- on RyR open probability. Diary plot of the experiment of Fig. 6. Plotted are values of \bar{P}_o , evaluated as fractional open time during successive 2-s sweeps. The horizontal bars represent in time the presence of ClO_4^- and EGTA.

To quantify the effect of ClO_4^- and assess its statistical significance, an average open probability was calculated for the whole period in reference, then for the interval spent in the presence of EGTA or Mg^{2+} , and then for the time in ClO_4^- . These values are listed in Table II for all experiments. 15 experiments concluded successfully with the addition of ClO_4^- , while four others were interrupted before adding the anion. Fig. 8 is a bar diagram comparing averages over these experiments. The first bar collects the average probability of the bilayers in the presence of Ca^{2+} , the initial portion of the experiments, lasting between 2 and 6 min. The second and third bars represent the probabilities in EGTA or Mg^{2+} , in which the bilayers

TABLE II
Effect of Perchlorate on Single Ca Release Channels in Bilayers

1 Experiment No.	2 Reference	3 + EGTA	4 + Mg^{2+}	5 + ClO_4^-	6 5 - 3	7 5 - 4
1	2.56	0.03		2.20	2.17	
3	1.01	0.27		1.74	1.47	
4	10.8	0.41		9.75	9.34	
6	8.20	0.83		1.91	1.08	
7	10.8	0.12		0.21	0.09	
8	27.0	7.53		44.3	36.8	
10	23.7	0.62		3.09	2.47	
12	10.2	0.11		2.18	2.07	
15	8.63	0.58		1.21	0.63	
16	9.55	0.20		0.86	0.66	
2	17.7		0.31	2.45		2.14
5	18.0		3.26	22.8		19.5
17	3.00		0.15	7.25		7.10
18	24.2		4.44	33.8		29.4
19	3.69		0.50	17.7		17.2
9	3.53					
11	33.3					
13	72.4	0.54				
14	12.3					
Avg.	15.8	1.09	1.73		5.68	15.1
SEM	3.78	0.72	0.89		3.56	4.80
n	19	11	5		10	5

All values of probability are in percent.

stayed for ~ 4 min. These bars have to be compared with the fourth and fifth bars, representing the averaged probability after addition of ClO_4^- , over ~ 6 min. Standard errors of the mean are represented in all cases.

Due to the large variability of the activities in reference, the differences of average open probabilities shown in the figure are only moderately significant. The paired differences induced by ClO_4^- are more significant. The last columns of Table II list the paired differences, showing that ClO_4^- increased the open probability by 5.7% in the presence of EGTA and by 15.1% in the presence of Mg^{2+} . The effect is a several-fold increase; the significance is high in the presence of Mg^{2+} ($P < 0.02$) and marginal in EGTA ($0.05 < P < 0.08$).

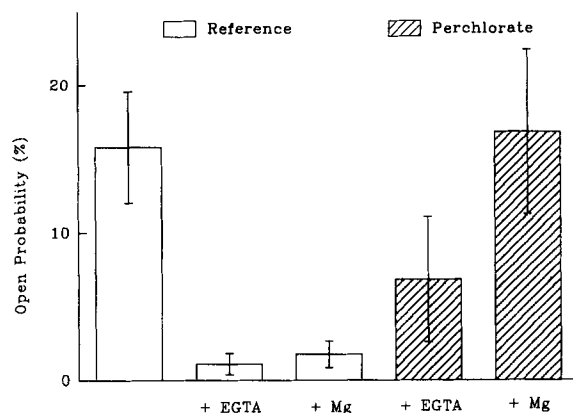


FIGURE 8. Averaged open probability of the RyR channel in various conditions. A value of \bar{P}_o was obtained for each experiment during the time spent in reference (4–6 min), in an inhibitory solution (in the presence of either EGTA or 10 mM Mg^{2+} , *cis*, 4 min), and after adding ClO_4^- to the inhibitory solution (4–6 min). The vertical bars represent the average values of \bar{P}_o over all experiments, and the vertical lines span ± 1 SEM. 10 experiments with

EGTA and 5 with Mg^{2+} are represented. There were 19 experiments in reference, including two that were interrupted shortly after exposure to the inhibitory solution and two that went directly to ClO_4^- without an inhibitory intervention. These were not included in the other averages. Individual experiments are listed in Table II.

Effects of Perchlorate on Triad-enriched Membrane Fractions

In the 32–34% region of the gradients, membranes exhibited B_{max} values of 15.0 pmol/mg protein (SEM 4.3, $n = 3$) and 8.2 (1.2) pmol/mg protein for high affinity [3H]PN200-110 and [3H]ryanodine binding, respectively, giving a B_{max} ratio of 1.8 (0.3). This comigration of [3H]ryanodine and [3H]PN200-110 binding to the middle of the gradients indicated that T tubule/SR junctional complexes (triads) were present in this fraction since free T tubule membranes, detached from SR, are known

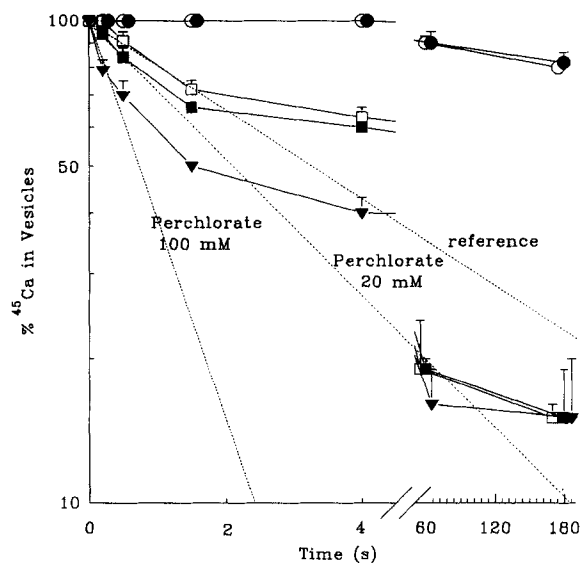


FIGURE 9. ClO_4^- increases $^{45}Ca^{2+}$ efflux from SR. $^{45}Ca^{2+}$ efflux from triad-enriched vesicular fractions was measured as described in Materials and Methods. Loaded vesicles were washed with different media as follows: reference inhibitory (open circles), 100 mM ClO_4^- inhibitory medium (filled circles), reference release activating medium containing 20 mM ClO_4^- (filled squares), or release activating medium containing 100 mM ClO_4^- (triangles). Values (\pm SEM) are averages from three experiments. Inhibitory media contained 5 mM NaPIPES, pH 7, 5 mM $MgCl_2$,

1 mM EGTA, and 20 μ M ruthenium red. Release media contained 5 mM NaPIPES, pH 7, 5 mM NTA, and 175 μ M $CaCl_2$ (nominal $[Ca^{2+}]$ of 5 μ M).

to exhibit buoyant densities corresponding to 20–30% sucrose (Caswell, Lau, and Brunschwig, 1976; Sabbadini and Dahms, 1989; Kim, Caswell, Brunschwig, and Brandt, 1990). Also, others have reported that populations of skeletal muscle microsomes exhibiting buoyant densities corresponding to 30–35% sucrose are enriched in morphologically intact triads (Caswell et al., 1976; Mitchell, Palade, and Fleischer, 1983; Kim et al., 1990).

The initial rates of efflux of $^{45}\text{Ca}^{2+}$ from the triad-enriched membrane fractions were measured either in $5\ \mu\text{M}$ $[\text{Ca}^{2+}]$ media containing 100 mM NaCl (reference), or in ClO_4^- -containing media. The results are summarized in Fig. 9, where open squares

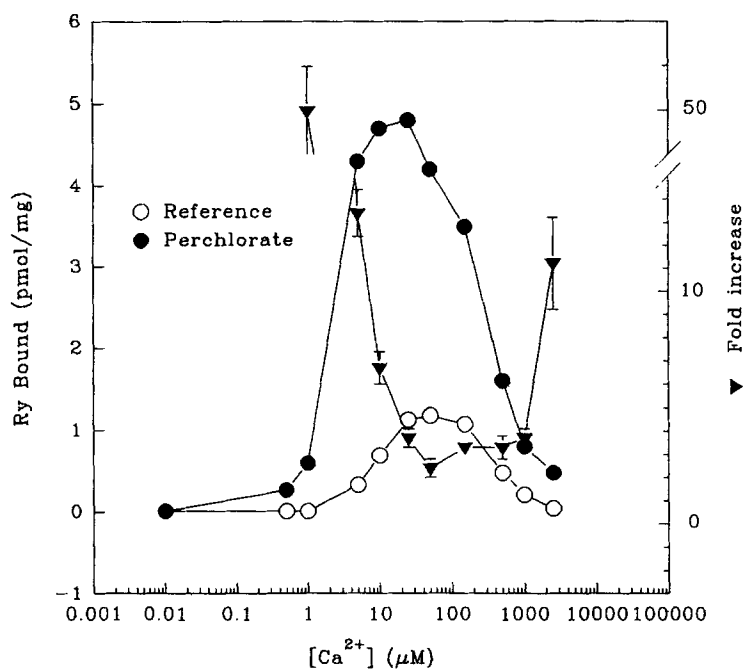


FIGURE 10. ClO_4^- increases ryanodine binding to triad-enriched fractions in a Ca^{2+} -dependent manner. Equilibrium binding to a triad-enriched vesicle fraction measured at varying $[\text{Ca}^{2+}]$ in media containing 5 nM $[\text{^3H}]\text{ryanodine}$, 10 mM NaPIPES (pH 7.2), 5 mM AMP, 0.08–0.7 mg protein/ml, and either 100 mM NaCl (*open circles*) or 100 mM ClO_4^- (*filled circles*). Shown is one of three similar experiments. The triangles represent the average ratio of bound ryanodine in ClO_4^- to bound ryanodine in reference medium ($\pm\text{SD}$, $n = 3$).

represent the efflux caused by washing vesicles suspended in reference solution with a release-activating medium and filled squares and triangles represent the corresponding results again using a reference suspension medium, but with a release-activating medium containing either 20 or 100 mM ClO_4^- , respectively. The kinetics of efflux are complex, probably reflecting heterogeneity of vesicle size and channel density. $^{45}\text{Ca}^{2+}$ efflux occurring in the first 0.2–1.5 s was used to derive initial efflux rates (depicted by dashed lines in the semilog plot). The rate constants were ~ 0.2 , 0.35 , and $0.9\ \text{s}^{-1}$ in reference and 20 and 100 mM ClO_4^- , respectively. This represents 1.75- and 4.5-fold increases, which were found to be significant ($P < 0.02$) in paired

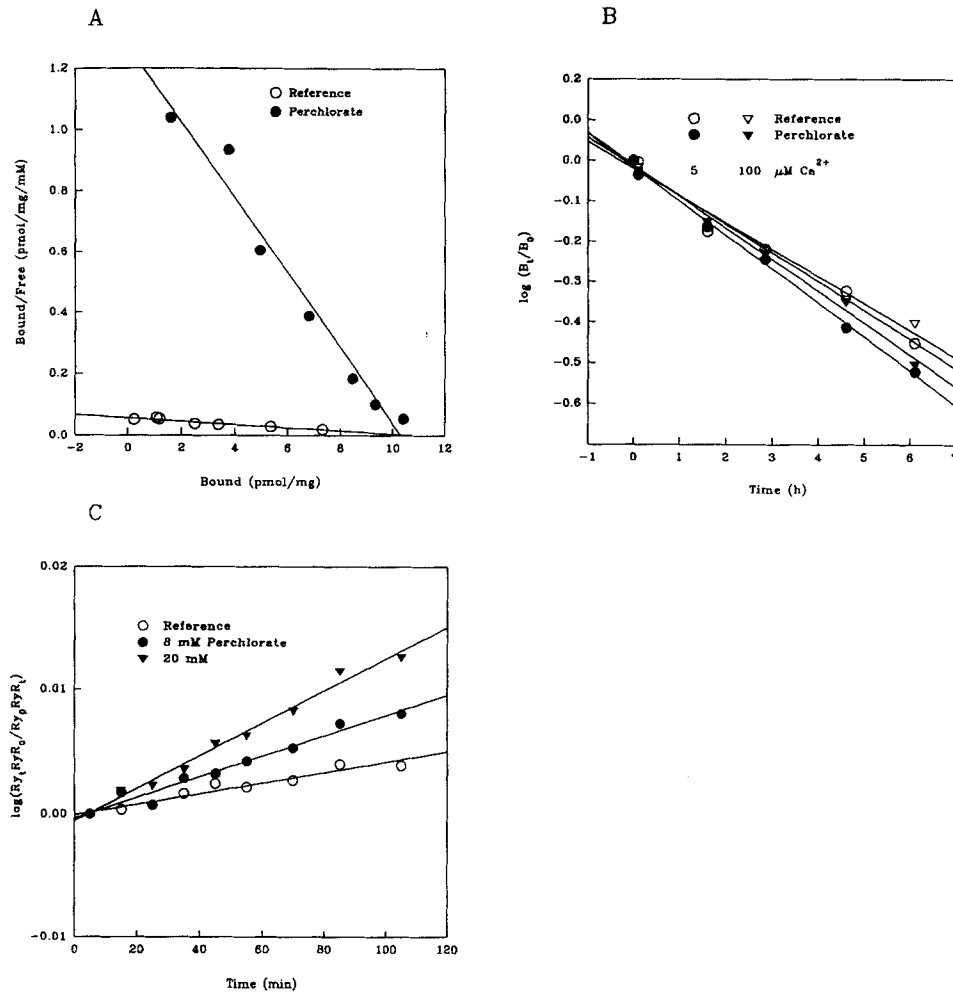


FIGURE 11. ClO_4^- increases ryanodine binding affinity to the triad-enriched fraction by increasing the association rate constant. (A) Equilibrium binding constants were determined by Scatchard analysis in media containing 10 mM NaPIPES (pH 7.2), 5 mM AMP, 5 mM NTA, 175 μM CaCl_2 (5 μM $[\text{Ca}^{2+}]$), and either 100 mM NaCl (reference, open circles) or 100 mM ClO_4^- at 12°C. Shown is one representative experiment. In reference $K_D = 178$ nM and $B_{\text{max}} = 10.7$ pmol/mg, and in 100 mM ClO_4^- $K_D = 8$ nM and $B_{\text{max}} = 10.3$ pmol/mg. (B) Time course of dissociation, measured at 25°C in the binding medium described in A, containing either 5 mM NTA and 175 μM CaCl_2 ($[\text{Ca}^{2+}] = 5$ μM) or 2.2 mM CaCl_2 ($[\text{Ca}^{2+}] = 100$ μM). The data are plotted to rectify the time dependence of an irreversible first-order dissociation $B_t = B_0 e^{-k^- t}$, where B_t is [ryanodine] bound at time t , B_0 is [ryanodine] bound at time 0, and k^- is the dissociation rate constant. The values of k^- are derived from the regression lines shown, of equation $\log B_t/B_0 = -(k^-/2.3) t$, and given in units of 10^{-3} min^{-1} . In 5 μM $[\text{Ca}^{2+}]$ (circles) k^- was 2.88 in reference (open symbols) and 3.13 in 100 mM ClO_4^- (filled symbols). In 100 μM $[\text{Ca}^{2+}]$ (triangles) k^- was 2.91 in reference and 2.43 in 100 mM ClO_4^- . (C) Time course of [^3H]ryanodine binding, measured at 25°C in the binding medium described in A, in the absence and presence of ClO_4^- . The data are plotted to rectify the time dependency of an irreversible

t tests. The Ca^{2+} permeability enhanced by ClO_4^- is inhibited by ruthenium red, as shown by $^{45}\text{Ca}^{2+}$ efflux in inhibitory media (circles) with or without ClO_4^- .

Effects of Perchlorate on [^3H]Ryanodine Binding

High affinity [^3H]ryanodine binding has been shown to monitor protein conformational changes associated with channel opening (Fleischer, Ogunbunmi, Dixon, and Fleer, 1985; Pessah, Stambuk, and Casida, 1987; Chu, Díaz-Muñoz, Hawkes, Brush and Hamilton, 1990; Meissner and El-Hashem, 1992; see also review by Lai and Meissner, 1989). [^3H]Ryanodine binding was used to analyze in more detail the effects of ClO_4^- on the RyR. Fig. 10 shows that ClO_4^- increased [^3H]ryanodine binding in a Ca^{2+} -dependent manner, with the most dramatic increases occurring in the 1–5 μM [Ca^{2+}] range. In this experiment, a less than saturating concentration of [^3H]ryanodine (5 nM) was used so that detection of differences in ryanodine binding affinity and/or number of binding sites was improved.

Since the ClO_4^- -induced increase in binding might have been due to a change in affinity and/or number of high affinity sites, we determined K_D and maximum [^3H]ryanodine binding capacity (B_{max}) under equilibrium binding conditions in media containing 5 μM [Ca^{2+}]. Scatchard plots (Fig. 11 *A*) indicate that K_D decreased from 166 (32) nM in reference to 6.3 (2.4) nM in 100 mM ClO_4^- , while B_{max} remained essentially the same at 7.9 (0.7) pmol/mg in reference and 10.3 (1.0) pmol/mg in 100 mM ClO_4^- ($n = 3$), indicating that the ClO_4^- -induced ryanodine binding increase (demonstrated in Fig. 10) was due to an increase in affinity.

The effects of ClO_4^- on the RyR were also analyzed by determining the association (k^+) and dissociation (k^-) rate constants of high affinity ryanodine binding in the presence of varying [ClO_4^-] and [Ca^{2+}]. Fig. 11, *B* and *C*, shows that binding data are well fitted assuming simple second-order association and first-order dissociation kinetics. At 5 and 100 μM Ca^{2+} , the dissociation rate constant was not changed from a reference value of $2.8 \times 10^{-3} \text{ min}^{-1}$ by 100 mM ClO_4^- , while the association rate constant increased 2-fold in 8 mM, 3-fold in 20 mM, and 22-fold in 100 mM ClO_4^- . These rate constants are summarized in Table III, along with others determined at various [Ca^{2+}]. At a concentration of 20 mM, ClO_4^- significantly increased the association rate constant at 5 μM [Ca^{2+}] ($P < 0.02$), whereas the increases at 50, 100, and 250 μM [Ca^{2+}] had $P > 0.05$. [Ca^{2+}] below 5 μM , at which the effects on ryanodine binding appeared greatest (as indicated in Fig. 10), resulted in ON rates in the reference condition that were too slow to be measured accurately. The same happened at [Ca^{2+}] in the millimolar range.

The isolated skeletal muscle Ca release channel is partially activated by either Ca^{2+} or adenine nucleotides (Lai and Meissner, 1989). The effect of ClO_4^- on [^3H]ryanodine binding to the Ca^{2+} -free and nucleotide-activated channel was determined in the presence of 5 mM EGTA, high levels of triad vesicles, and [^3H]ryanodine, with or

second-order association. The values of the association rate constant k^+ were derived from the regression lines shown, of equation $\log [(Ry_t \times RyR_0)/(Ry_0 \times RyR_t)] = k^+ [\log e / (Ry_0 - RyR_0)] t$, where Ry_0 and RyR_0 are [ryanodine] and [ryanodine receptor] at time zero, and Ry_t and RyR_t are the concentrations at time *t*. The k^+ values (in units of $10^{-6} \text{ min}^{-1} \text{ nM}^{-1}$) were 5.2 in reference (*open circles*), 9.7 in 8 mM ClO_4^- (*filled circles*), and 16 in 20 mM ClO_4^- (*filled triangles*).

without the ATP analogue AMP-PCP (5 mM) in the binding medium. After allowing the reaction to approach equilibrium in the presence of AMP-PCP, the reference ryanodine binding level of 0.04 pmol/mg protein (SEM = 0.02, $n = 4$) was found to increase to 0.07 (0.01) pmol/mg in 20 mM ClO_4^- . This was significant ($P < 0.05$). In 100 mM ClO_4^- , ryanodine binding increased to 0.20 (0.04), a highly significant change ($P < 0.001$). These results suggest that Ca^{2+} is not required for activation of the channel. ClO_4^- (100 mM) also appeared to increase [^3H]ryanodine binding several-fold in the absence of AMP-PCP, but the binding levels were too close to background to be measured accurately.

In summary, we conclude from these observations that ClO_4^- primarily affects ryanodine binding to the RyR by increasing the association rate constant and that

TABLE III
Effects of Perchlorate on Ryanodine Binding to Triad-enriched Fractions and Purified RyR Protein

Preparation	$[\text{ClO}_4^-]$	$[\text{Ca}^{2+}]$	k^-	k^+	K_D
	mM	μM	10^{-3} min^{-1}	$10^{-6} \text{ nM}^{-1} \text{ min}^{-1}$	nM
TR*	—	5	2.6 ± 0.2	5.7 ± 1.6	456
TR*	8	5	2.4 ± 0.6	$9.7 \pm 2.0^{\ddagger}$	247
TR*	20	5	2.5 ± 0.3	$17 \pm 4.0^{\ddagger}$	147
TR*	100	5	2.8 ± 0.3	130 ± 30	22
TR*	—	50	2.7 ± 0.1	90 ± 20	30
TR*	20	50	2.9 ± 0.1	140 ± 10	21
TR*	—	100	2.9 ± 0.3	74 ± 5.0	39
TR*	20	100	2.5 ± 0.4	87 ± 7.0	28
TR*	—	250	2.4 ± 0.1	38 ± 2.0	63
TR*	20	250	2.8 ± 0.3	52 ± 1.0	54
RyR §	—	5	3.6 ± 0.2	110 ± 50	33
RyR §	20	5	3.0 ± 0.5	$230 \pm 80^{\ddagger}$	13

All values are averages of three experiments.

*Triad-enriched vesicular fraction.

‡ Significantly different from reference value placed above ($P < 0.02$ by paired Student's t test; in all other cases $P > 0.05$).

§ Purified ryanodine receptor protein reconstituted into liposomes.

Perchlorate (Na salt) was added to media in substitution for NaCl.

Ca^{2+} is not required for activation by ClO_4^- , even though the largest effects are seen in 1–5 μM $[\text{Ca}^{2+}]$.

The effects of 20 mM ClO_4^- on the [^3H]ryanodine association rate constant were also determined using purified RyR protein reconstituted into liposomes. The results are summarized in the bottom entries of Table III. As was the case with triad-enriched vesicles, 20 mM ClO_4^- caused a significant increase of the rate constant in 5 μM $[\text{Ca}^{2+}]$. One additional interesting result of this experiment is that the reference value of the association rate constant was much greater in the purified reconstituted than in the native preparation, while the dissociation rate constant remained the same. This suggests that the purified RyR channel has a substantially greater intrinsic tendency to open than the native channel. It also helps understand the need to go to

very low $[Ca^{2+}]$ in order to lower P_o sufficiently in the previously described set of experiments with purified channels reconstituted in planar bilayers.

To obtain an indication of effects of ClO_4^- on the DHPRs present in triad-enriched membranes, the binding of $[^3H]PN200-110$ to this fraction was measured. When equilibrium binding and association rates were quantified (in media described in Materials and Methods containing up to 100 mM ClO_4^- , no effects of ClO_4^- were found.

We attempted to obtain additional information regarding the mechanism of activation of the RyR by ClO_4^- . One possibility considered was that the interior of the large RyR complex was less accessible to ClO_4^- than Cl^- (or other ions in the physiological situation), thereby causing dehydration of the receptor protein. This possibility was tested by determining the $[^3H]$ ryanodine association rate constant k^+ in the presence of 5% polyethylene glycol (PEG), which has an osmolarity equivalent to 20 mM $NaClO_4$ and should cause at least as much dehydration as ClO_4^- . The result is included in Table IV. Dehydration by PEG did not increase k^+ . A small but

TABLE IV
Effect of Chaotropes on Ryanodine Binding Kinetics

Chaotrope	(mM)	Other agent	(mM)	Association constant (k^*) ($10^{-6} \text{ nM}^{-1} \text{ min}^{-1}$)
—	—	—	—	5.4 ± 1.6
ClO_4^-	20	—	—	$17 \pm 4.0^*$
—	—	PEG	5%	4.0 ± 1.1
ClO_4^-	100	—	—	130 ± 30
SCN^-	100	—	—	250 ± 150
I^-	100	—	—	210 ± 30
—	—	DTT	5	2.9 ± 0.6
ClO_4^-	100	DTT	5	$51 \pm 5.0^*$

In all cases the chaotropic anions were added as sodium salts. Then NaCl was added for a total $[Na]$ of 100 mM. Media also contained 0.6 mg protein/ml, 25 nM $[^3H]$ ryanodine, 5 μM Ca^{2+} , and 20 mM NaPIPES, pH 7.2

*Significantly different from reference value placed above.

significant decrease was observed instead, which may be due to a decrease in NaCl concentration (from 100 to 80 mM) in these experiments.

Another mechanism considered was oxidation of the RyR by ClO_4^- or by a contaminant present in the ClO_4^- solution. Association rate constants were therefore also measured in the presence of a strongly reducing agent (5 mM dithiothreitol, DTT). The result is shown in Table IV. In the presence of DTT, 100 mM ClO_4^- increased k^+ to an extent (~ 20 -fold) similar to that seen in the absence of DTT. It therefore seems unlikely that ClO_4^- exerts its activating effects by oxidizing the channel.

A major question posed by the effects of ClO_4^- is whether they are associated with the chaotropic properties of the anion. As discussed by González and Ríos (1993), ClO_4^- occupies an extreme position in the lyotropic series, where anions are ranked according to their protein solubilization and precipitation properties (Hofmeister, 1888; Collins and Washabaugh, 1985). Although the mechanism of the protein

solubilization effect is still a matter of speculation (González and Ríos, 1993), it is of great interest to establish whether the EC coupling effects are common to other anions that are close to ClO_4^- in the Hofmeister series. Therefore, we tested the effects of SCN^- and I^- , which have comparable chaotropic strength. The values of the association rate constant of [^3H]ryanodine binding in the presence of these ions at 100 mM, averaged in three experiments, are listed in Table IV. The differences with the value measured in reference condition are highly significant and are very similar to that in the same concentration of ClO_4^- .

DISCUSSION

The main results of these experiments are two. The first, a negative result, is that the anion ClO_4^- applied to DHPRs of skeletal muscle in artificial bilayers and to cardiac Ca channels in situ had none of the large effects that it had on the charge movements of the skeletal muscle in situ. The effects on muscle, described in detail in the previous article, include a leftward shift of voltage dependence (by some 15 mV), a substantial increase in the steepness of the Q vs. V distribution, and a substantial slowing of kinetics, particularly at low voltages. ClO_4^- had none of those effects on the Ca and gating currents studied in this article. It only caused a minor leftward shift of $Q(V)$ in cardiac muscle and an increase in the activity of the skeletal muscle Ca channel, but only at high voltages, at which there is no major effect of ClO_4^- in situ. The central voltage of the \bar{P}_o vs. V dependence was actually shifted in the positive direction. These modest effects are generally consistent with, and confirmatory of, the previously reported lack of effects of ClO_4^- on I_{Ca} measured in situ (Feldmeyer and Lüttgau, 1988; Gyorke and Palade, 1992; but see Gallant and Lentz, 1992, reporting a -10 -mV shift of I_{Ca} activation in mouse muscle).

Confirming these negative results, measurements of [^3H]PN200-110 binding to the DHPR sites abundant in the triad-enriched membrane fraction failed to demonstrate any effect of ClO_4^- . Had ClO_4^- altered the fraction of DHPRs in the inactivated states, an increase in either affinity (Bean, 1984) or number (Schwartz, McCleskey, and Almers, 1985) of the binding sites would have been expected. The negative result, however, is of limited significance as the transmembrane potential and electrical integrity of the T membrane moieties in the vesicular preparation are unknown. ClO_4^- may have failed to have an effect because it does not affect primarily the DHPRs. Alternatively, the membrane potential may be collapsed in this preparation, causing complete inactivation of the voltage sensors (and maximal binding of dihydropyridines) in the reference solution.

The second main result of this work, a positive one, is that ClO_4^- increased the open probability of SR release channels. This result was obtained with three different preparations and techniques: as an increase in activity of single channels in planar bilayers, as an increase in efflux of radioactive Ca^{2+} from triad-enriched vesicular fractions, and as an increase in the association rate constant of high affinity [^3H]ryanodine binding (in both vesicular fractions and purified protein reconstituted into liposomes). The observations are all consistent with a direct stimulation of channel activity by ClO_4^- .

One interesting feature of the agonistic effect of ClO_4^- is that it was strongly affected by the level of free Ca^{2+} at the myoplasmic side of the channel. The effect on

the P_o of channels in bilayers could only be demonstrated if the $[Ca^{2+}]$ was reduced to tens of nanomolar, but this may have been due to the reduced variance in current achieved by inhibiting channel activity. It becomes very clear in the ryanodine binding experiments, however, that Ca^{2+} reduces the effect of ClO_4^- when it is present at high concentrations. The greatest relative increase in tendency to opening caused by ClO_4^- (50-fold) was found at a $[Ca^{2+}]$ of 1 μM . However, a significant increase in ryanodine binding affinity of the receptor was also observed at $[Ca^{2+}] < 10^{-8} M$, when nucleotides were present. It is therefore likely that ClO_4^- may affect the release channel at the $[Ca^{2+}]$ prevailing in the myoplasm at rest. This is consistent with the tentative conclusions of González and Ríos (1993), based on their estimates of evolution of SR calcium content in cut fibers exposed to ClO_4^- .

Considering the existing literature and ideas about the ClO_4^- effect, these results are surprising. From the early work of Lüttgau, Gottschalk, Kovacs, and Fuxreiter (1983), later confirmed by Huang (1986), Csernoch, Kovacs, and Szücs (1987), Dulhunty, Zhu, Patterson, and Ahern (1992), and Gyorke and Palade (1992), it is known that ClO_4^- has profound effects on the voltage sensor. These, plus other studies discussed later concentrating more on the Ca^{2+} release function (Stephenson, 1989; Fill and Best, 1990), point to the early steps of voltage sensing, rather than the release channel, as targets of ClO_4^- .

The general failure of ClO_4^- to affect the various forms of DHPRs studied here is amenable to two different interpretations. The trivial one is that ClO_4^- does not affect the cardiac DHPR because of structural differences with the skeletal DHPR, and that it does not affect the Ca current in bilayers because this function of the skeletal DHPR may not reflect effects at the level of charge movement that may occur in the isolated DHPR.¹ Thus, we may have failed to find effects on isolated systems because we were studying the wrong molecule (the cardiac DHPR) or the wrong function (I_{Ca} in the skeletal DHPR).

Still, for reasons that follow we think the negative results surprising and significant. First, the failure of ClO_4^- to affect cardiac charge movement cannot be readily assigned to structural differences between the molecules. All pharmacological agents studied so far, including five different types of Ca channel antagonists (Pizarro, Brum, Fill, Fitts, Rodríguez, Uribe, and Ríos, 1988; Romey, García, Rieger, and Lazdunski, 1988; reviewed by Ríos and Pizarro, 1991) are active on both cardiac and skeletal DHPRs. Moreover, conversion of charge 1 to charge 2 upon voltage-dependent inactivation occurs in both (Shirokov et al., 1992), and metal cations, which affect the skeletal voltage sensor from the outside (Pizarro, Fitts, Uribe, and Ríos, 1989), antagonizing voltage-dependent inactivation and charge 1 to charge 2 conversion, have a similar effect on L-type channel charge movement in heart cells (Shirokov, Levis, Shirokova, and Ríos, 1993). ClO_4^- is, in fact, the first agent that acts on skeletal but not cardiac muscle charge movement. This selectivity is made more

¹ A trivial variant of this possibility is that the lack of effects of ClO_4^- is due to structural differences among different animal species, since the effects on charge movement and Ca release were studied on amphibian muscle, and the studies on isolated DHPRs were performed on mammalian preparations. However, kinetic effects of ClO_4^- on charge movement, similar to those described in the first article of this series, have been found on mouse myotubes as well (Adams, B., personal communication).

surprising by the conclusion below, namely, that the effect of ClO_4^- is relatively nonspecific, shared with multiple inorganic anions of simple structure, and requiring high concentrations (millimolar).

The failure of ClO_4^- to affect voltage dependence of Ca channels in bilayers may also be trivial. The link between intramembrane charge movement and T tubular Ca current (I_{Ca}) is well established in skeletal muscle; the pharmacology is very similar (Pizarro et al., 1988) and they reappear in parallel with expression of DHPRs in dysgenic mice (Adams, Tanabe, Mikami, Numa, and Beam, 1990; Beam, Adams, Niidome, Numa, and Tanabe, 1992). Most probably activation of I_{Ca} requires charge movement. However, I_{Ca} activates at higher voltages and more slowly. Therefore, ClO_4^- may fail to shift the voltage dependence of I_{Ca} simply because it does not affect slower steps involved in its activation.

On the other hand, the differences in kinetic effects are surprising: 20 mM ClO_4^- failed to make channel closing slower in the bilayer experiments, while at 8 mM it increased markedly the time constant of OFF charge movement in cut fibers (González and Ríos, 1993) and at 40 mM it prolonged the closing of the release channels to ~ 50 s (Ríos, Karhanek, Ma, and González, 1993). The trivial explanation of this difference would be the existence of a ClO_4^- -insensitive, minor component of charge movement that moves rapidly to close the channel at the OFF.

A Hypothesis

The trivial explanations cannot be ruled out. The most intriguing possibility, however, is that ClO_4^- sensitivity in the voltage sensors requires the physiological interaction of EC coupling, either because the anion binds to a site on the T tubule sensor that is modified by the interaction or because it acts on the release channel, affecting the sensor only secondarily. This explanation is consistent with the positive results, showing effects on SR release channel activity, $^{45}\text{Ca}^{2+}$ efflux, and ryanodine binding. It is consistent with the observation in the previous article that the anion does not slow the kinetics of charge 2, as charge 2 is believed to correspond to the voltage sensors inactivated by depolarization and is thus devoid of the functional interaction. It is also consistent with the main observation of the previous article, that ClO_4^- effects are not limited to a shift in the properties of the voltage sensor but include an alteration of the transfer function, which is to be expected if ClO_4^- alters the release channel. In terms of the discussion in the previous article, these results are consistent with target sites of type III (on the release channel).

These results help decide whether these target sites are on the release channel proper or on sites in between sensor and channels, that is, sites that are not present or are altered when the voltage sensor is not present. Comparison of the ryanodine binding properties of the triad-enriched and the purified RyR preparations (Table III) demonstrates that ryanodine binding affinity is much greater in the purified preparation. The relative effect of 20 mM ClO_4^- , however, is similar in both (a two- to threefold increase in affinity), suggesting that the binding of ClO_4^- to the RyR neither requires nor is strongly altered by the interaction between voltage sensor and release channel. In disagreement with this suggestion, however, Gyorke and Palade (1992) failed to detect an effect of ClO_4^- on the activation of release in crayfish muscle. This muscle is considered analogous to cardiac muscle, a paradigm of Ca^{2+} -mediated EC

coupling, and the lack of effect of ClO_4^- suggests that the anion does not require the specific interaction of EC coupling in vertebrates to have its effect. The issue of the requirements for ClO_4^- effects thus remains unsettled.

The Effects of Perchlorate Are “Chaotropic”

The present results shed some light on the mechanism of the ClO_4^- effect. An important issue is whether the effect is related to the protein solubilization properties of the anion, to its ability to dissociate protein complexes, and to other properties related to its position in the Hofmeister series. For expediency we will keep the term chaotropic to refer to mechanisms common to Hofmeister's chaotropes, even though, as discussed in the previous article (González and Ríos, 1993), there is no agreement on what these mechanisms are.

To establish whether the EC coupling effects of ClO_4^- are chaotropic, we compared the effects with those of other ions, especially SCN^- and I^- , which are close to ClO_4^- in the Hofmeister series and in other chaotropic rankings (Collins and Washabaugh, 1985). SCN^- and I^- are known to potentiate EC coupling, acting equally well when applied from inside the cells, and slowing OFF charge movements (Delay, García, and Sánchez, 1990). In this sense, the effect of ClO_4^- can be termed chaotropic. The effectiveness of SCN^- and I^- to increase ryanodine binding, demonstrated here, is additional evidence that these ions share with ClO_4^- the same potentiating mechanism (ClO_4^- , however, may have additional effects not observed with the other chaotropes [Huang, 1986]). Recently, a modulation of Ca release channel activity has also been reported for gluconate and NO_3^- (Hasselbach and Migala, 1992). NO_3^- is a chaotrope (Collins and Washabaugh, 1985) and has potentiating effects on EC coupling (Delay et al., 1990). Replacement of Cl^- by gluconate decreased Ca^{2+} release from and [^3H]ryanodine binding to heavy SR vesicles, whereas NO_3^- was stimulatory. In summary, since the effects of ClO_4^- on EC coupling and on the release channel are common to all chaotropes studied, they must be related to the defining properties of these ions, including their ability to cause dissociation of protein complexes into subunits. This suggests that EC coupling depends critically on interactions within protein complexes.

The observation of ClO_4^- effects on the release channel appears to contradict prior works on two accounts. First, Stephenson (1989) observed that the anion does not alter unstimulated ^{45}Ca efflux, interpreting this observation as evidence against a primary effect on the release pathway. However, it is possible that an increase in resting ^{45}Ca went unnoticed, as Fill and Best (1990) found that ClO_4^- elicits contractures of skinned fibers without stimulation (see also results in high [ClO_4^-] in the following article of this series). Also in apparent contradiction to these findings, Fill and Best (1990) found no effect of ClO_4^- on the caffeine sensitivity of skinned fibers. This was interpreted as an indication that the anion does not primarily affect the release channel. A different interpretation is possible: perhaps the effect of ClO_4^- on the release channel does not interfere with the mechanism of activation by caffeine, in the same way as the activation of the release channel by AMP-PCP does not radically change the concentration dependence of its activation by Ca^{2+} (inferred from ryanodine binding) in recent measurements of Zimanyi and Pessah (1991) on skeletal and cardiac mammalian SR vesicles.

In summary, both the essential absence of effects of ClO_4^- on DHPRs and their activating effect on Ca release channels, demonstrated in this article, are against widely held ideas but do not contradict previous results. In these observations we see evidence, albeit indirect, of an allosteric interaction between voltage sensors and release channels of EC coupling in skeletal muscle. A plausible model for such an interaction is developed in the next article.

This work was supported by grants from the National Institutes of Health to M.M. Hosey (HL-23306), E. Ríos (AR-32808), and G. Meissner (AR-18687). J. Ma was the recipient of a Muscular Dystrophy Association postdoctoral fellowship.

Original version received 29 July 1992 and accepted version received 20 May 1993.

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