9-1-1989

# The Voltage Sensor of Excitation-Contraction Coupling in Skeletal Muscle. Ion Dependence and Selectivity

Gonzalo Pizarro
*Rush University*

Robert H. Fitts
*Marquette University*, robert.fitts@marquette.edu

Ismael Uribe
*Rush University*

Eduardo Rios
*Rush University*

Published version. *Journal of General Physiology,* Vol. 94, No. 3 (September 1989): 405-428. DOI. 

Published September 1, 1989

# The Voltage Sensor of Excitation-Contraction Coupling in Skeletal Muscle

## *Ion Dependence and Selectivity*

GONZALO PIZARRO, ROBERT FITTS, ISMAEL URIBE, and EDUARDO RÍOS

From the Department of Physiology, Rush University School of Medicine, Chicago, Illinois 60612

ABSTRACT Manifestations of excitation-contraction (EC) coupling of skeletal muscle were studied in the presence of metal ions of the alkaline and alkaline-earth groups in the extracellular medium. Single cut fibers of frog skeletal muscle were voltage clamped in a double Vaseline gap apparatus, and intramembrane charge movement and myoplasmic $Ca^{2+}$ transients were simultaneously measured. In metal-free extracellular media both charge movement of the charge 1 type and Ca transients were suppressed. Under metal-free conditions the nonlinear charge distribution was the same in depolarized (holding potential of 0 mV) and normally polarized fibers (holding potentials between −80 and −90 mV). The manifestations of EC coupling recovered when ions of groups Ia and IIa of the periodic table were included in the extracellular solution; the extent of recovery depended on the ion species. These results are consistent with the idea that the voltage sensor of EC coupling has a binding site for metal cations—the "priming" site—that is essential for function. A state model of the voltage sensor in which metal ligands bind preferentially to the priming site when the sensor is in noninactivated states accounts for the results. This theory was used to derive the relative affinities of the various ions for the priming site from the magnitude of the EC coupling response. The selectivity sequence thus constructed is: Ca > Sr > Mg > Ba for group IIa cations and Li > Na > K > Rb > Cs for group Ia. $Ca^{2+}$, the most effective of all ions tested, was 1,500-fold more effective than $Na^+$. This selectivity sequence is qualitatively and quantitatively similar to that of the intrapore binding sites of the L-type cardiac Ca channel. This provides further evidence of molecular similarity between the voltage sensor and Ca channels.

Address reprint requests to Dr. Eduardo Rios, Department of Physiology Rush-Presbyterian-St. Luke's Medical Center, 1750 West Harrison Street, Chicago, IL 60612. Dr. Fitt's present address is Department of Biology, Marquette University, Milwaukee, WI 53233; and Dr. Uribe's present address is Department of Pharmacology, Centro de Investigación y Estudios Avanzados del Instituto Politécnico Nacional, Mexico, DF.

J. GEN. PHYSIOL. © The Rockefeller University Press · 0022-1295/89/09/0405/24 $2.00
Volume 94 September 1989 405–428

## INTRODUCTION

The effects of extracellular $Ca^{2+}$ on sekeletal muscle are multiple, and appear to be contradictory. Elevated extracellular $Ca^{2+}$ prolongs K contractures (Lüttgau, 1963; Caputo, 1972; Stéfani and Chiarandini, 1973), and $Ca^{2+}$-free conditions produce use-dependent paralysis (Graf and Schatzmann, 1984). This last phenomenon is similar to the D600-induced paralysis observed by Eisenberg et al. (1983), which involves suppression of charge movement positive to the resting potential (Hui et al., 1984) and increase in charge movement of the charge 2 type (Fill et al., 1988; Caputo and Bolaños, 1988). This dependence of EC coupling on extracellular calcium has been interpreted as evidence that $Ca^{2+}$ influx, either through the tubular Ca channel (Frank, 1980) or through a Na-Ca exchange reaction (Curtis, 1986), is the signal that triggers $Ca^{2+}$ release from the sarcoplasmic reticulum.

On the other hand, the ability of muscle fibers to twitch in $Ca^{2+}$-free Ringer (Armstrong et al., 1972) is contrary to a role of extracellular $Ca^{2+}$, as is the report of Miledi et al. (1984) that myoplasmic Ca transients elicited by voltage-clamp depolarization are unaffected by extracellular salines of different monovalent and divalent metal ionic composition.

This conflict was partly solved by the demonstration of involvement of $Ca^{2+}$ at the level of the voltage sensor of EC coupling. In two previous papers from this laboratory (Brum et al., 1988*a*, *b*) it was shown that low $[Ca^{2+}]_e$ suppressed $Ca^{2+}$ release from the sarcoplasmic reticulum, reduced charge movement of the charge 1 type, increased charge movement of the charge 2 type, and increased the rate at which the system entered the inactivated condition upon depolarization. This is evidence of a major role of $Ca^{2+}$ in the function of the voltage sensor. In the same studies, $Mg^{2+}$ was found to preserve the EC coupling functions when substituted for $Ca^{2+}$.

The experiments described in the present paper were designed to systematically test the ability of metallic ions other than $Ca^{2+}$ to sustain EC coupling. We found that ions of groups Ia and IIa of the periodic table were able to support EC coupling with a selectivity sequence strikingly similar to that of binding to the permeation sites of L-type Ca channels. When the cells were exposed to metal-free external solution, $Ca^{2+}$ release was abolished and intramembrane charge movement had the properties found in fibers inactivated by prolonged depolarization.

Some of the present results have been communicated in abstract form (Pizarro et al., 1987, 1988).

## METHODS

The experiments were performed in cut skeletal muscle fibers, singly dissected from the semitendinosus muscle of the frog *Rana pipiens*, voltage-clamped in a double Vaseline gap apparatus. The fiber was subjected to various patterns of pulse stimulation while two sets of measurements were carried out in parallel: (*a*) intramembrane charge movement was measured, in the presence of suitable impermeant ionic substituents and channel blockers, as the difference between membrane capacitive currents during test and control pulses (nonlinear capacitive current). The control pulses were applied to positive voltages, from a holding potential of 0 mV (Brum and Ríos, 1987). Total intramembrane charge moved was calculated by integration of the nonlinear capacitive current. Such integration can be carried out during the ON or the OFF transient (leading or trailing edge of the test pulse); following the rationale of

Brum et al. (1988*a*) the depolarizing edge of the applied pulses was always used for the integration, that is, ON areas for depolarizing, OFF areas for hyperpolarizing pulses. (*b*) $Ca^{2+}$ transients (time course of change in free myoplasmic calcium ion concentration) were derived from changes of optical absorption in the presence of the dye antipyrylazo III diffused intracellularly (methods described by Brum et al., 1988*b*). The flux of $Ca^{2+}$ release from the sarcoplasmic reticulum was derived from the $Ca^{2+}$ transients by a technique that involves an empirical characterization of the calcium removal process, described by Melzer et al. (1987) and Brum et al. (1988*b*). None of the interventions applied in the present work seemed to have a significant effect on the measured removal rates, beyond the well understood fact that the rates of removal depend on the amplitude and duration of previous Ca transients (Melzer et al., 1986).

### *Solutions*

Two sets of extracellular solutions were used (in addition to a "reference" solution, introduced by Kovacs et al., 1979). The first set included solutions devoid of metal cations; isotonic TEA $CH_3SO_3$ (referred to as "metal-free"), as well as a solution containing 2 mM of the divalent organic cation dimethonium as a dibromide (a kind gift of Stuart McLaughlin, State University of New York, Stony Brook), in addition to TEA $CH_3SO_3$. Solutions in the second set were designed to study the effect of a single metal ion on EC coupling. They contained either 100 mM of an ion of group Ia or 2 mM of an ion of group IIa as the sole metal. The osmolarity was set to 270 mosmol with TEA $CH_3SO_3$; the pH was adjusted to 7.0 with TEA hydroxide. The reference solution is the $Ca^{2+}$-containing member of this set. In all solutions but reference, EGTA was added in an amount that gave 0.1 mM of free acid in order to chelate contaminating $Ca^{2+}$ (estimated to be <20 μM). In some experiments, solutions containing 50 mM of ions of group Ia were used, in these experiments the effect of such solutions was compared with solutions containing 0.1 mM $Ca^{2+}$ as sole metal ion. The internal solution which diffused from the end pools into the cell via notches cut in the cell membrane, was that of Kovacs et al. (1979). The composition of all external solutions is given in Table I.

TABLE I

*Composition of Solutions*

| Name | $Ca_{Total}$ | $Ca^{2+}$ | $Me_{Total}$ | $Me_{free}$ | TEA | $CH_3SO_3$ | $EGTA_{free}$ |
|---|---|---|---|---|---|---|---|
| Reference | 2 | 2 | — | — | 128 | 132 | — |
| Metal-free | — | — | — | — | 130 | 130 | 0.1 |
| 160 $Na^{2+}$ | — | — | 160 | 160 | — | * | — |
| 2 $Me^{2+}$‡ | — | — | ¶ | 2 | 128 | 132 | 0.1 |
| 100 $Me^{+}$‖ | — | — | 100 | 100 | 30 | 130 | 0.1 |
| 0.1 $Ca^{2+}$ | 0.1 | 0.1 | — | — | 130 | 130.2 | — |
| 50 $Me^{+}$‖ | — | — | 50 | 50 | 80 | 130 | 0.1 |
| 2 dimethonium** | — | — | — | — | 128 | 128 | 0.1 |

All concentrations in millimolar. 10 μM TTX, 1 mM 2,4-diaminopyridine, and 5 mM TEA Tris-maleate added to all solutions. pH 7.0.

*80 mM $SO_4^{2-}$ as anion, no $CH_3SO_3$.

‡Solutions with Sr, Mg, or Ba.

¶Total Me and EGTA calculated to have 2 mM [$Me^{2+}$] and 0.1 mM free EGTA; metals added as hydroxides, titrated with $CH_3SO_3$.

‖Solutions with Li, Na, K, Rb, or Cs.

**2 mM dimethonium, added as bromide.

The temperature did not vary more than 1°C during an experiment and was between 8 and 13°C.

### *Liquid Junction Potentials*

Given the variety of external solutions used, liquid junction potential changes were a concern. These changes were measured by shorting the voltage-measuring compartment to the middle compartment and measuring the voltage difference between pellets connected to these via KCl pools and agar bridges. The junction potentials changed by <5 mV in all cases as different solutions were passed through the middle compartment; correction for these changes in the actual experiments was considered unnecessary.

### *Homogeneity of Voltage Control*

Some of the extracellular solutions used contained elevated concentrations of ions that permeate the membrane; inhomogeneities in tubular polarization, induced by the higher conductance, could explain some of the inhibitory effects of these solutions. To evaluate the magnitude of this problem we estimated the cable parameter $r_m$ (membrane resistance per unit length of fiber) in the different solutions. This was carried out by using the methods described by Brum and Ríos (1987), which are minor modifications of general methods and theory introduced by Irving et al. (1987). The results obtained in two fibers were later confirmed using a recently introduced four Vaseline gap voltage clamp (Ríos et al., 1989), which permits a more direct determination of cable parameters; a full description of it is currently in preparation.

In two especially complete sets of measurements, the membrane resistance of two fiber segments (of 800 μm in length and 80 and 76 μm in diameter) was measured in most solutions of interest. In the following we list the results in the segment that gave the lowest values of resistance; the resistance measured with a negative going pulse (20 mV) is given first, and is followed by the value obtained in the same solution with a positive-going pulse of the same voltage, which was always greater. The membrane resistance was 1.01 (1.11) MΩ.cm in reference. In 100 mM $Na^+$ it decreased to 0.481 (0.673 MΩ.cm; in 100 mM $Li^+$ the resistance was similar, 0.441 (0.557) MΩ.cm. In 100 mM $K^+$ it underwent the largest decrease, to 0.233 (0.284) MΩ.cm. Using this value an estimation can be made of the T-tubular space constant. Since this value of $r_m$ is substantially greater than that of a similar fiber in Ringer's (Adrian et al., 1979) we expect a maximum deviation from spatial homogeneity of less than the value found in that paper, 4%. Following the distributed model of the T system introduced there, the space constant of the T system can be calculated as:

$$\lambda_T = \sqrt{(\bar{G}_L/\bar{G}_W)} \quad (1)$$

where $\bar{G}_L$ is effective radial conductivity of the lumen of the T system (S $cm^{-1}$) and $\bar{G}_W$ is conductance of the T membranes per unit volume of fiber. $\bar{G}_L$ is calculated by Adrian et al. (1969) as $G_L\rho\sigma$, where $G_L$ is the conductivity of the luminal solution, $\rho$ is the fractional volume of the T system and $\sigma$ a network factor, inversely related to tortuosity. $G_L$ is assumed to be equal to that of the extracellular solution. An approximate value at 20°C was calculated in the present case by adding terms corresponding to the equivalent conductivities at infinite dilution of the main ionic components ($1.48 \times 10^{-2}$ S/cm, from tabulated values in Weast, 1985) and correcting for finite dilution with the exact factor required for such correction in the case of pure KCl (1/1.35). The final estimate is $1.09 \times 10^{-2}$ S/cm. It should be slightly less at the temperature of the experiments and is therefore very close to $10^{-2}$ S/cm, the value used by Adrian et al. (1969). Since the geometry of the fiber is the same, and the value of $G_L$ is similar, $\bar{G}_L$ will be approximately the same as in the paper of reference, $1.5 \times 10^{-5}$ S/cm.

Published September 1, 1989



$\bar{G}_w$ is related simply to $r_m$ provided that all membranes are homogeneously polarized, an assumption known to hold approximately. Assuming that the membrane conductance is distributed between surface and T membranes proportionally to area, $\bar{G}_w$ is calculated as the ratio of measured fiber conductance ($3.74 \times 10^{-7}$ S) and volume of fiber under clamp ($4 \times 10^{-6}$ cm$^3$), multiplied by the fractional area of the T system (6/7). The value of $\lambda_T$ thus calculated is 137 $\mu$m, and the maximum deviation expected in the value of voltage during steady depolarization is <2.2% (Eq. 10 of Adrian et al., 1969).

The calculations of Adrian et al. (1969) followed here may give an underestimate of cable decay along the T system. Schneider (1970) found that his measurements of input impedance could be reconciled with a similar model if the resistivity of the T lumen was two or three times greater than that of the extracellular solution, and this was interpreted later as greater tortuosity in the network (Mathias et al., 1977); using a twofold greater tortuosity in the calculation of the space constant for radial decay of voltage, the maximum voltage inhomogeneity is close to 4%. In summary, there is spatial inhomogeneity in polarization of the T membranes, but it is probably small even in the high $K^+$ solution.

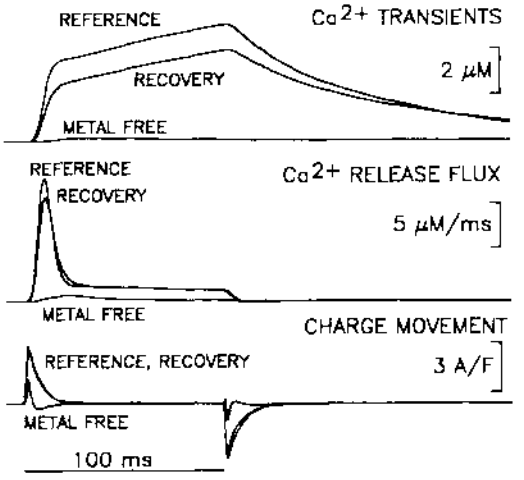


FIGURE 1. Effect of metal-free solution on $Ca^{2+}$ transients and charge movement. Myoplasmic $Ca^{2+}$ transients (*A*), $Ca^{2+}$ release flux (*B*), and charge movement current (*C*) elicited by a 100-ms pulse to 0 mV recorded in steady state in the presence of reference extracellular solution, before exposure to metal-free (traces labeled "reference"), in metal-free solution (composition given in Table I traces labeled "metal-free") and after readmission of reference solution ("recovery"). Fiber 318; diameter, 80 $\mu$m; linear capacitance, 7.18 nF; dye concentration between 482 $\mu$M and 671; temperature, 10°C.

## RESULTS

### *Fibers Become Refractory in a Metal-free External Solution*

Figs. 1 and 2 show details of the effect, first reported by Brum et al. (1988*a*) of a metal ion–free solution. Fig. 1 shows records of Ca transients, $Ca^{2+}$ release flux, and intramembrane charge movement, obtained under stationary conditions at different stages of the experiment. Fig. 2 plots peak $Ca^{2+}$ release flux and total charge moved during individual pulses in the course of the experiment. The experiment started in reference solution (2 mM $Ca^{2+}$ as sole metal ion). Upon changing to metal-free both charge movement and $Ca^{2+}$ release were readily suppressed. The effect was reversed by returning to the initial 2 mM $Ca^{2+}$ conditions. (An incomplete recovery was not uncommon; in the experiment shown the charge movement recovered completely

but Ca release flux apparently did not. This may result from changes in dye concentration, imperfectly corrected in the computation of $[Ca^{2+}]_i$; it might also reveal a slow decaying trend during the experiment). In the lower panel of Fig. 2 the values of holding current are also shown, to prove fiber integrity during and after the metal-free treatment. Similar results were obtained in three other fibers. These results show that a metal-free solution abolishes EC coupling without fiber injury, suggesting that external metal ions are involved at some stage of the EC coupling process.

To discard a surface charge screening effect as the cause of the suppression of EC coupling when $Ca^{2+}$ was withdrawn, the organic divalent cation dimethonium was substituted for $Ca^{2+}$. This ion is able to screen negative fixed charges on the surface of lipid bilayers without significantly binding to them (McLaughlin et al., 1983). The result of such an experiment is shown in Fig. 3; in the dimethonium-containing,

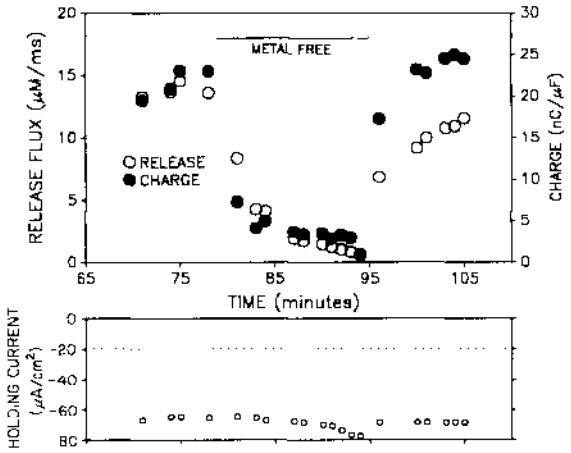


FIGURE 2. Time course of the effect of a metal-free solution. (*Top*) Intramembrane charge movement (*filled circles*) and peak $Ca^{2+}$ release flux (*open circles*) in response to 100-ms voltage-clamp pulses to 0 mV, vs. time during the experiment. The holding potential was −90 mV; the pulses were preceded by a 100-ms prepulse to −70 mV. The period of perfusion with metal-free solution is indicated by a horizontal bar. (*Bottom*) Holding current plotted on the same time scale. Same fiber and set of measurements as in Fig. 1.

metal-free solution charge 1 was suppressed, and the $Ca^{2+}$ transient was abolished, very much as observed with the isotonic TEA $CH_3SO_3$ extracellular solution (Figs. 1 and 2). Additionally, the experiment explored the behavior of charge 2 in the dimethonium-containing solution; as shown in Fig. 3 *B*, charge 2, defined as the charge moved by a pulse to a voltage more negative than −90 mV, increased as compared with the reference situation; a similar phenomenon has been observed by Brum et al. (1988*a*) in metal-free solution.

### *The Metal-free Effect Is Similar to Inactivation*

In a fiber inactivated by maintained depolarization to 0 mV the distribution of mobile charge is shifted to negative potentials with respect to the distribution in normally polarized fibers (Brum and Ríos, 1987; Caputo and Bolaños, 1988). Upon repolarization the fibers "reprime"; the ability to release $Ca^{2+}$ and to contract

Published September 1, 1989



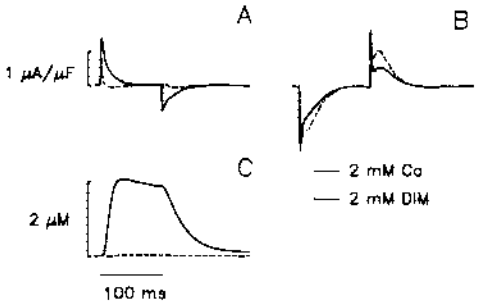


FIGURE 3. Dimethonium failed to support EC coupling. (*A*) Charge movement current in response to a 100-ms pulse to 0 mV from a prepulse level of −70 mV. (*C*) The corresponding myoplasmic $Ca^{2+}$ transients. (*B*) Charge movement elicited by a negative-going pulse to −160 mV from the same prepulse level; *solid line*, records in reference solution; *dashed line*, recorded in an extracellular medium with 2 mM dimethonium substituted for $Ca^{2+}$. Fiber 334; diameter, 95 μm; linear capacitance, 15.5 nF; dye concentration between 535 and 569 μM; temperature, 12°C.

returns, and the charge distribution undergoes a shift to more positive voltages. As the changes in the distribution are similar under prolonged depolarization and in metal-free conditions we studied the combined effects of both interventions. Fig. 4 presents $Q$ vs. $V$ curves in metal-free at holding potentials 0 and −80 mV. The charge distribution in the depolarized fiber was fit with a Boltzmann function with parameters $Q_{max}$ = 30.24 nC/μF, $\bar{V}$ = −113.5 mV, and $k$ = 16.9 mV. Thus, it had properties similar to those described by Brum and Ríos (1987) for depolarized fibers in physiological extracellular $[Ca^{2+}]$. When the holding potential was changed to −80 mV the charge distribution remained unaltered (Boltzmann parameters: $Q_{max}$ = 34.5 nC/μF, $\bar{V}$ = −119.2 mV, $k$ = 17.3 mV). By comparison, in reference solution, parameters for polarized fibers were $Q_{max}$ = 38.3 nC/μF, $\bar{V}$ = −36.1 mV, and $k$ = 18.6 mV (Caputo and Bolaños, 1989).

Thus, in a metal-free medium, fibers were unable to reprime and had the same charge distribution as fibers in normal $Ca^{2+}$ inactivated by depolarization. These

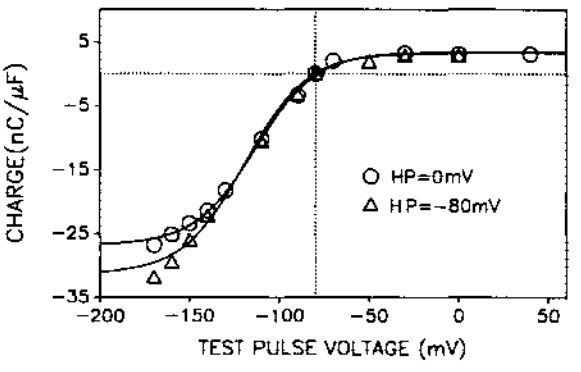


FIGURE 4. Voltage dependence of charge movement under metal-free conditions. Intramembrane charge moved by 100-ms pulses to test potentials in the abscissa, measured at holding potentials of 0 mV (*circles*) and −80 mV (*triangles.*). The curves represent best fit two-state canonical ("Boltzmann") distribution functions with parameters given in the text. Fiber 279; diameter, 70 μm; capacitance, 8.7 nF; temperature, 13°C.

results suggest that the primary effect of the metal-free solution is a modification of the voltage sensor, which becomes inactivated, that is, undergoes changes usually associated with prolonged depolarization.

*EC Coupling Persists If Enough Metal Cations Are Present*

In general, low $Ca^{2+}$ experiments in the literature have been done replacing $Ca^{2+}$ with some other divalent cation (usually $Mg^{2+}$) in order to maintain the solution's ability to screen "fixed charges" (Lüttgau and Spiecker, 1979). Armstrong et al. (1972) demonstrated conservation of twitches in a $Na^+$-containing $Ca^{2+}$-free saline. That the absence of $Ca^{2+}$ did not abolish EC coupling in those experiments suggests that the other cations permitted EC coupling to some degree. Specifically, the result of Armstrong et al. (1972) could be explained if extracellular $Na^+$ played a role similar to $Ca^{2+}$ at the voltage sensor. An experimental test of this hypothesis is shown in Fig. 5. Charge movement and $Ca^{2+}$ release were not abolished by changing

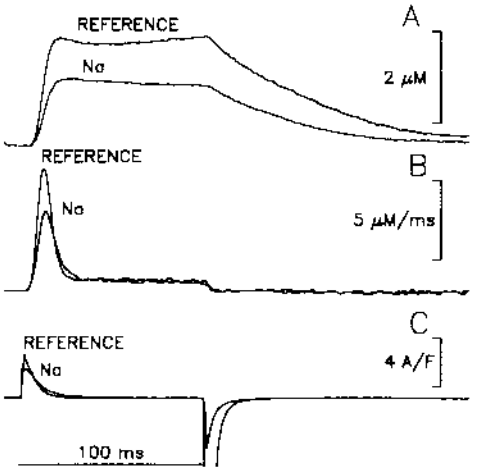


FIGURE 5. $Na^+$ supports EC coupling. Myoplasmic $Ca^{2+}$ transients (*A*), $Ca^{2+}$ release flux (*B*), and intramembrane charge movement currents (*C*) elicited by a 100-ms pulse to 0 mV, in reference solution and in a $Ca^{2+}$-free extracellular saline containing 160 mM $Na^+$ (traces labeled "Na"). Fiber 238; diameter, 60 μm; capacitance, 5.3 nF; dye concentration, 209 and 312 μM; temperature, 12°C.

the external solution from reference (records labeled *Ca*) to a solution with 160 mM $Na^+$, although the kinetics of charge movement were altered and $Ca^{2+}$ release was reduced. The large inward current at the end of the pulse in 160 mM $Na^+$ is probably carried by $Na^+$ flowing through the Ca channel rendered nonspecific by the absence of $Ca^{2+}$ (Almers et al., 1984). Similar results were observed in two other fibers. These results opened the possibility that many other ions, in addition to $Ca^{2+}$ and $Na^+$, would support EC coupling. In particular, the other elements in groups Ia and IIa of the periodic table were likely candidates.

$Ca^{2+}$ transients in response to a fixed voltage clamp depolarization (to 0 mV, 100 ms in duration) were measured in different solutions containing only one metal ionic species of group IIa. Experiments began in reference (2 mM $Ca^{2+}$), then the external solution was changed successively to the other test solutions containing equal concentrations of free divalent cations. Bracketing runs in reference were recorded, and used to offset fiber rundown. A total of six fibers were used; in all but

Published September 1, 1989



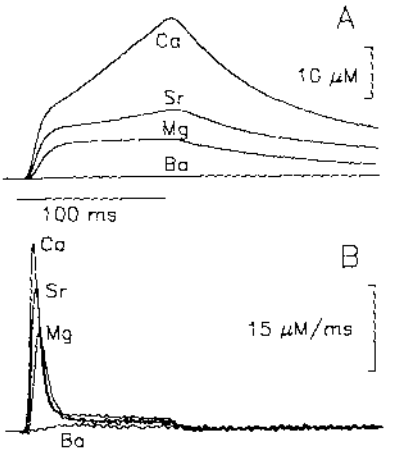


FIGURE 6. All ions of group IIa support EC coupling. Myoplasmic $Ca^{2+}$ transients (*A*) and $Ca^{2+}$ release flux (*B*) in response to 100-ms pulses to 0 mV in solutions containing, as sole metal cation, 2 mM of the ion listed next to each record. Fiber 301; diameter, 76 μm; linear capacitance, 7.8 nF; dye concentration between 306 and 365 μM; temperature, 9°C.

one all four solutions were tested in the same fiber. Records from a typical experiment are shown in Fig. 6; the upper panel represents Ca transients in the presence of the metal cation indicated; Ca transients were elicited in all four solutions tested, but they were of different magnitude. The lower panel represents corresponding $Ca^{2+}$ release fluxes. The peak values of $Ca^{2+}$ release flux were in the sequence Ca > Sr > Mg > Ba.

Fig. 8 *A*, representing a segment of the same experiment, plots stationary values of peak release flux in each solution against time during the experiment. Values of total holding membrane current, plotted in the lower graph, document that the solution changes did not affect resting membrane conductance.

A similar protocol was used to study the effect of group Ia ions, in this case solu-

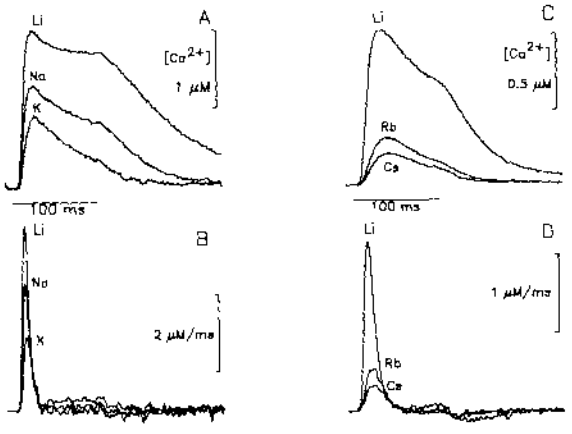


FIGURE 7. All ions of group Ia support EC coupling. Myoplasmic $Ca^{2+}$ transients (*A* and *C*) and $Ca^{2+}$ release flux (*B* and *D*) in response to 100-ms pulses to 0 mV in solutions containing, as sole metal cation, 100 mM of the ion listed next to each record. Records in 100 mM $Li^+$, that serve as basis for normalization of these results in Fig. 9, were obtained repeatedly, bracketing measurements in other solutions. Fiber 296; diameter, 81 μm; capacitance, 10.2 nF; dye concentration between 209 and 539 μM; temperature, 10°C.

tions contained 100 mM of the test ion as the sole metal, and slightly more negative holding potentials were used (−90 to −100 mV, instead of −75 to −90 mV, used in the previous series, cf. Discussion). All ions were tested in every one of four fibers. Typical $Ca^{2+}$ transients and corresponding $Ca^{2+}$ release flux records are shown in Fig. 7, and the time course of the same experiment is shown in Fig. 8 *B*. The sequence Li > Na > K > Rb > Cs was always observed.

As shown in the lower plot of Fig. 8 *B*, substantial increments in holding current were observed with $K^+$, $Rb^+$, and $Cs^+$; a direct measurement of $r_m$ described in Methods showed that $r_m$ decreased fourfold in the worst case (100 mM $K^+$). With this figure the spatial inhomogeneity of the steady membrane polarization was estimated as 4% or less. The other monovalent ions caused less inhomogeneity, and the solutions with divalent ions did not cause any measurable change in cable parameters.

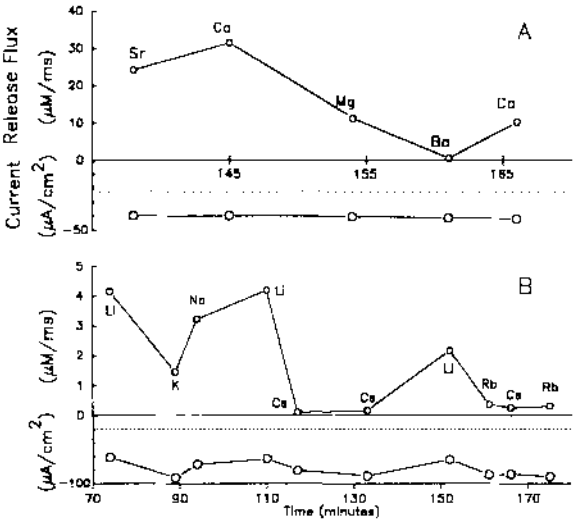


FIGURE 8. A comparison of the effect of the different test solutions on $Ca^{2+}$ release flux and holding currents. Steady-state values of peak $Ca^{2+}$ release flux and holding current in each test solution. (*A*) Group IIa ions; same fiber and records as in Fig. 6. (*B*) group Ia ions; same fiber and records as in Fig. 7.

Fig. 9 summarizes the experiments described above: peak $Ca^{2+}$ release flux is plotted against radius of the divalent (left) or monovalent ions. The values of release flux were normalized to the values obtained in the same fiber with the most effective ion ($Ca^{2+}$ among the divalents; $Li^+$ for the monovalents). The observed sequence for group Ia ions corresponds to Eisenman's high field strength series (sequence XI, Eisenman, 1962; Eisenman and Horn, 1983). The sequence for group IIa differs from the high field strength sequence VI of Sherry (cited by Diamond and Wright, 1969) in the inversion of the order of $Sr^{2+}$ and $Mg^{2+}$; the difference observed between $Sr^{2+}$ and $Mg^{2+}$ in the present experiments was, however, not significant.

### *Ion-binding Affinities of the Priming Site*

We have previously proposed a state model of the voltage sensor of EC coupling, the molecule that underlies charge movement, in which extracellular $Ca^{2+}$ binds to the sensor in a state-dependent manner. The essential aspect of this model is that

Published September 1, 1989



noninactivated states of the voltage sensor are assumed to bind $Ca^{2+}$ with high affinity, whereas the inactivated states bind negligibly. As a result, the noninactivated states are effectively stabilized by $Ca^{2+}$; conversely, their probability becomes negligible in the absence of extracellular $Ca^{2+}$ and the sensors inactivate.

The model accounts for the present results provided that other ions in addition to $Ca^{2+}$ are allowed to bind to the priming site, in the same state-sensitive manner. In the framework of this model, the observation of Ca transients—and $Ca^{2+}$ release—of different size, in the presence of different ions at a fixed holding potential and concentration, can be explained as a consequence of different affinities for the priming site: ions that bind with higher affinity occupy and stabilize in noninactivated states a greater fraction of sensors. This extension of the model is elaborated in the Discussion, where a general relationship between inactivation, holding potential, and metal cation concentration is formulated quantitatively. To justify the design of the next set of experiments, we will use from there the result, Eq. 5, that the amplitude of the response of the voltage sensor to a given depolarization (charge movement) is linearly dependent on the fraction of sensors bound to the

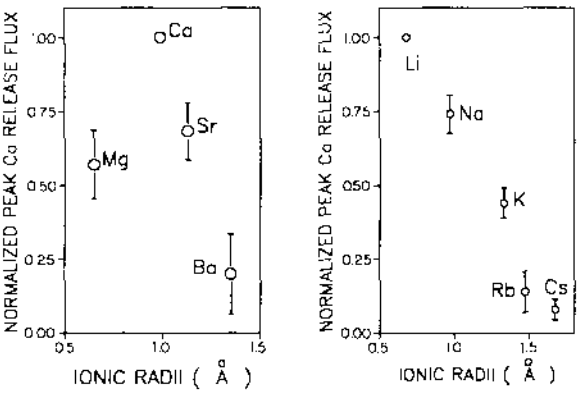


FIGURE 9. Ability of ions of group Ia and IIa to support EC coupling. Peak $Ca^{2+}$ release flux normalized to that observed with the most effective ion in each fiber, plotted against ionic radius. (*Left*) Collected results for alkaline-earth cations, mean ± SEM ($n = 6$). (*Right*) Alkaline ions ($n = 4$).

metal ion present, and that this fraction is proportional to the affinity of the binding sites for the ion *provided that the sites are far from saturated.* In turn, the response of the voltage sensor to a pulse will translate to $Ca^{2+}$ release flux approximately linearly, as discussed by Melzer et al., 1986, and Brum et al., 1988*a*.

It is therefore essential for having a linear relationship between $Ca^{2+}$ release and affinity for the ion to put the system in a low-saturation situation. This was not the case in the experiments that generated Fig. 9, which therefore does not give a quantitative measure of relative affinities. The linear regime can be achieved either by reducing the concentration of the metal ligand, or by inducing partial inactivation through relatively depolarized holding potentials.

Figs. 10 and 11 document experiments to evaluate relative affinities of the monovalent ions in respect to $Ca^{2+}$. The experiments were performed at lower concentrations of $Ca^{2+}$ (0.1 mM) and the test monovalent (50 mM); a higher concentration of dye was used to improve detection of the small signals. The linearity of the system was tested by the additivity of the effects when both $Ca^{2+}$ and the test ion were present at the same time in the external solution. This is shown for the case of $Na^{+}$

Published September 1, 1989



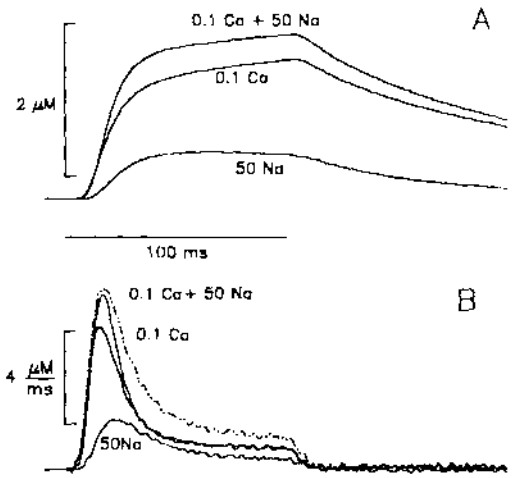


FIGURE 10. A comparison of the ability of $Ca^{2+}$ and $Na^+$ to support EC coupling in conditions of low saturation. Myoplasmic $Ca^{2+}$ transients (*A*) and $Ca^{2+}$ release flux (*B*) in response to a 100-ms pulse to 0 mV, recorded in solutions containing as sole metal ions 0.1 mM $Ca^{2+}$, 50 mM $Na^+$, or 0.1 mM $Ca^{2+}$ plus 50 mM $Na^+$, as indicated by the labels. In *B*, the sum of the records obtained in solutions containing a single species of test ion is also shown as a dashed trace. Fiber 395; diameter, 67 μm; capacitance, 6.5 nF; dye concentration between 777 and 907 μM; temperature, 10°C.

vs. $Ca^{2+}$ in Fig. 10. The solid trace labeled "0.1 Ca + 50 Na" is the experimental record, the dashed trace is the sum of the records obtained in the solutions containing a single ligand species. Using this approach, the relative affinities were obtained as the ratio between peak release flux in $Ca^{2+}$ and peak release flux in test monovalent (3.0 in Fig. 10), divided by the ratio of the concentrations (0.1 mM/50 mM = 0.002). Analogous experiments were carried out to evaluate relative affinities of the alkaline-earth cations with respect to $Ca^{2+}$. When a small release flux persisted even in metal-free (Fig. 11) this value was subtracted before computing ratios.

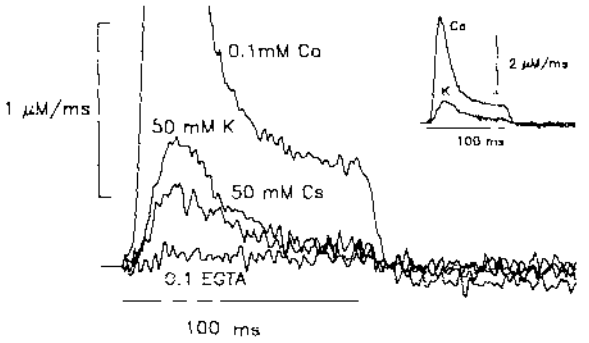


FIGURE 11. Relative ability of $K^+$ and $Cs^+$ to support EC coupling. $Ca^{2+}$ release flux elicited by a 100-ms pulse to 0 mV, recorded in $Ca^{2+}$-free solutions containing 50 mM $K^+$ or 50 mM $Cs^+$, as indicated by the labels. The release flux obtained in the presence of 0.1 mM $Ca^{2+}$ is much greater and out of scale. The response obtained in metal-free is also shown (record labeled 0.1 EGTA). The same records of release flux in 0.1 mM $Ca^{2+}$ and 50 mM $K^+$ are compared on a different scale in the inset. Fiber 392; diameter, 80 μm; capacitance, 8.2 nF; dye concentration between 440 and 720 μM; temperature, 12°C.

Direct, pairwise comparisons were performed in this way between $Ca^{2+}$ and the ions $Sr^{2+}$, $Na^+$, $K^+$, and $Cs^+$. In all cases the affinity ratios were evaluated from ratios of peak release in the presence of the given ion, and averaged values of bracketing peak releases in $Ca^{2+}$. Values for the other ions were derived from comparisons with $Sr^{2+}$ (for the divalents) or $Na^+$. The relative affinities evaluated in this way are collected in Table II. The values were all normalized to the ion of lowest affinity, $Cs^+$. The values are plotted vs. ionic radius in Fig. 12; in the Table and the figure the relative affinities are compared with (and are very similar to) permeability ratios for the L-type cardiac Ca channel, derived from reversal potentials by Tsien et al. (1987).

### *The Contribution of Na at Physiological Concentrations*

Since both $Ca^{2+}$ and $Na^+$ are fundamental components of physiological extracellular solutions, we asked to what extent is EC coupling sustained by extracellular $Na^+$

TABLE II

*Relative Affinities of the Priming Site*

| X | Priming Site Affinity (X) / Affinity (Cs) | Cardiac L Channel Permeability (X) / Permeability (Cs) |
|---|---|---|
| Ca | 6,800 | 4,200 |
| Sr | 4,050 | 2,800 |
| Mg | 3,300 | 0 |
| Ba | 1,200 | 1,700 |
| Li | 6.8 | 9.9 |
| Na | 4.8 | 3.6 |
| K | 2.5 | 1.4 |
| Rb | 1.4 | — |
| Cs | 1 | 1 |

Affinities of the skeletal muscle priming site, compared with permeability ratios of the cardiac L-type Ca channel as calculated by Tsien et al. (1987) from reversal potentials in biionic conditions. The values of relative affinity for Sr, Na, K, and Cs were obtained from direct comparison with Ca in conditions of low saturation (Figs. 10 and 11). The values for Mg and Ba were obtained from comparison with Sr; affinity of Li and Rb from comparisons with Na. All values are plotted in Fig. 12.

in physiological conditions. The effect of adding 130 mM $Na^+$ to an extracellular medium containing 2 mM $Ca^{2+}$ is shown in Fig. 13: the peak $Ca^{2+}$ release flux increased by approximately 6%, suggesting that $Na^+$ plays a minor role, as expected given the differences in affinity and the degree of saturation achieved with 2 mM $Ca^{2+}$.

## DISCUSSION

The experiments described support the hypothesis of Brum et al. (1988*a*) that the voltage sensor of EC coupling has an essential $Ca^{2+}$ binding site; they show that other ions can bind to this site and permit quantitation of relative ion-site affinities.

Published September 1, 1989



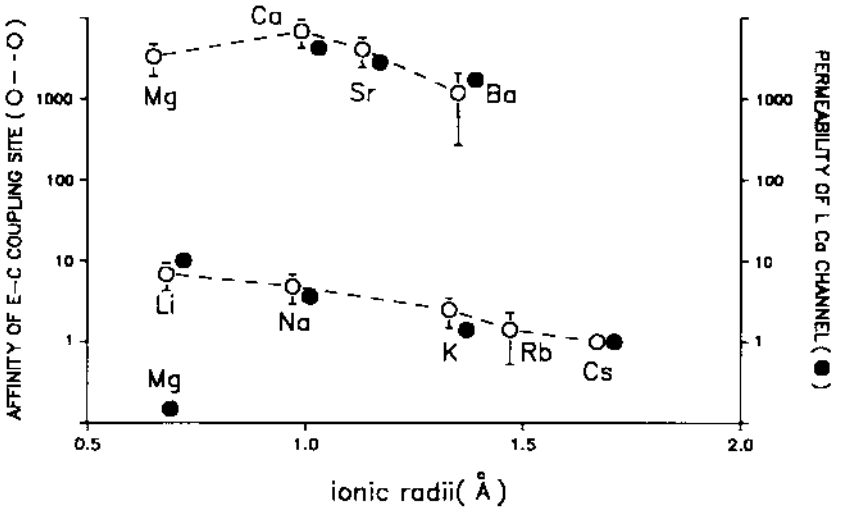


FIGURE 12. Relative binding affinities of the priming site. (*Open symbols*) Affinities of the priming site for the ion indicated, normalized to that of $Cs^+$; the values are as in Table II, obtained as described in the text and the table legend. (*Filled symbols*) Relative permeabilities of the cardiac L-type Ca channel, as reported by Tsien et al. (1987).

### *The Voltage Sensor Requires Metal Ions*

Metal-free conditions suppressed $Ca^{2+}$ transients and charge 1. In contrast, charge 2, the charge that moves at large negative potentials, was increased in the absence of metals. 90% of the effect (Fig. 2) occurred within 6 min of the solution change (both onset and recovery), and charge movement and $Ca^{2+}$ release were affected in parallel. This observation of fast, parallel reductions in charge movement and $Ca^{2+}$

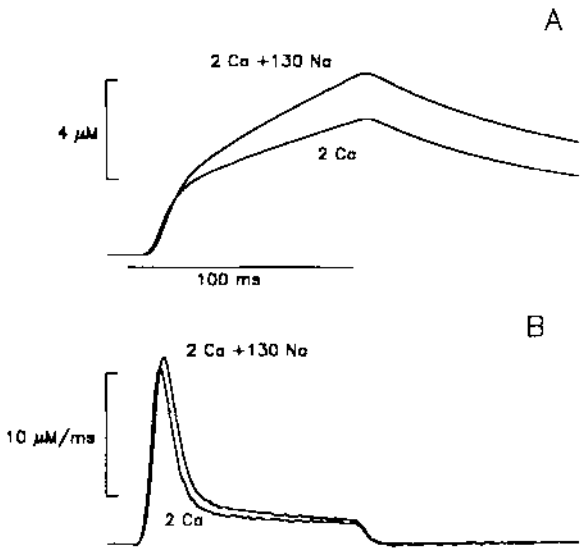


FIGURE 13. EC coupling in physiological concentrations of $Ca^{2+}$ and $Na^+$. $Ca^{2+}$ transients (*A*) and $Ca^{2+}$ release flux (*B*) elicited by 100-ms pulses to 0 mV, recorded in reference solution (traces labeled "2 Ca") and in a solution containing 2 mM $Ca^{2+}$ and 130 mM $Na^+$ (traces labeled "2 Ca + 130 Na"). Fiber 395; diameter, 67 μm; capacitance, 6.5 nF; dye concentration 974 to 1,030 μM; temperature, 10°C.

Published September 1, 1989



release flux indicates that the effect of the metal-free solution is exerted primarily at the voltage sensor of EC coupling; it also rules out a major role of $Ca^{2-}$ depletion.

*Ions Do Not Operate through Electrostatic Screening*

Metal-free solutions containing the organic divalent cation dimethonium, at the same concentration of divalent cations as reference, did not support EC coupling. This result rules out another trivial explanation of the effect of metal-free, a reduction of the transmembrane microscopic potential, due to reduced screening of negative surface charges by an extracellular solution with no divalent cations. The dimethonium experiments do not rule out an electrostatic effect due to specific binding of those ions that support EC coupling to negative fixed charges localized very close to the voltage sensor. This last "fixed charge" model is still insufficient, as it does not explain the fact that $Ca^{2+}$ shifts the voltage dependence of inactivation of EC coupling, but not its activation (Lüttgau and Spiecker, 1979; Brum et al., 1988*a*, *b*). This feature of the effect can be accounted for by a model in which $Ca^{2+}$ and other ligands bind preferentially to noninactivated states (Brum et al., 1988*a*).

*The Lack of Metal Ions Favors Inactivated States*

The voltage distribution of intramembrane mobile charge remains unchanged after polarization in metal-free solutions; this is consistent with the idea that metal ions act at the voltage sensor. Additionally, this is the main indication that the metal-free treatment induces disabled states analogous to the inactivated states usually reached through prolonged depolarization. Another indication is that 70% of the recovery of charge movement after reexposure to $Ca^{2+}$ in the external medium occurs in the first 2 min (Fig. 2). This is similar to the time constant of repriming of charge movement after repolarization to a negative holding potential (47 s at 12°C, Pizarro et al., 1987).

*Alkaline and Alkaline-Earth Ions Support EC Coupling*

The centerpiece of this study is a comparison of EC coupling functions in the presence of different alkaline and alkaline-earth ions. $Ca^{2+}$ transients could be recorded in all metal ions tested, although the size of the transients varied by orders of magnitude.

Here we faced an important experimental problem: it was impossible to record charge movement quantitatively in most of the different test solutions, because of the existence of superimposed ionic currents through the Ca channel in $Ca^{2+}$-free conditions. This problem cannot be circumvented by adding Ca channel blockers to the $Ca^{2+}$-free solutions because all Ca antagonists tested also affect the voltage sensor (Ríos and Brum, 1987; Fill et al., 1988; Pizarro et al., 1988). Despite this limitation, we found at least three examples of parallel changes of $Ca^{2+}$ release and charge movement caused by changes in the extracellular metal ions: one is the reduction in charge 1 and $Ca^{2+}$ release in metal-free solution; another is the observation of slightly slowed ON charge movement in the presence of $Na^{+}$ as sole metal (Fig. 5), underlying a smaller release flux; a third is the good correlation reported by Brum et al. (1988*a*, *b*) between alterations of charge movement and $Ca^{2+}$ release flux when the extracellular $Ca^{2+}$ is replaced by $Mg^{2+}$. In view of these facts, plus the

rapid onset and reversion of the effects of all ionic substitutions, it seems justified to interpret the changes in EC coupling observed as a consequence of changes in the voltage sensors.

*State-dependent Binding to the Priming Site*

Brum et al. (1988*a*, *b*) explained the effects of $Ca^{2+}$ as the result of binding to a priming site. In this theoretical framework, the present results are evidence that all the metal ions tested bind to the priming site. In contrast, the fact that the organic ions dimethonium and TEA do not support EC coupling, means that they cannot access the site or have negligible affinity for it.

The values of $Ca^{2+}$ release flux in the presence of different ions may be used to evaluate relative affinities of the priming site for the various ions. For this purpose the model of Brum et al. (1988*a*) is reformulated below, allowing binding of a metal ion Me to the voltage sensor.

$$\begin{array}{ccc} \text{Inactivated}^* & \xleftarrow{K_2} & \text{Inactivated} \\ \downarrow K_1 & & K_3 \uparrow \\ \text{Resting} + \text{Me} & \xrightarrow[K_5]{} & \text{Active} + \text{Me} \\ \downarrow K_4 & & K_6 \uparrow \\ \text{Resting:Me} & \xrightarrow[K_7]{} & \text{Active:Me} \end{array}$$

(Scheme 1)

Aside from slight differences in nomenclature, this model is identical to that of Brum et al. (1988*a*); the equilibrium constants are defined in the direction indicated by the arrows. The metals are assumed to have equal affinity for both "ground" states (Resting and Active); this aspect of the model was proposed by Brum et al. (1988*a*) because substitution of Mg for Ca does not change the voltage dependence of activation, even though that of inactivation is shifted substantially (Lüttgau and Spiecker, 1979).

At a normally polarized holding potential and in a physiological concentration of $Ca^{2+}$, most of the sensors are in the $Ca^{2+}$-bound resting state (Resting:Me); a large depolarizing pulse will drive most of them to Active:Me and cause a near maximal charge movement and $Ca^{2+}$ release. In the normal resting situation, addition of more ions (for instance greater $[Ca^{2+}]_e$) will cause little change in the fraction of metal-bound sensors, and therefore little change in peak $Ca^{2+}$ release (but major changes in voltage dependence of inactivation, as shown by Brum et al., 1988*a*, *b*). Addition of a large quantity of $Na^+$ to the physiological 2 mM $Ca^{2+}$ will likewise increase only slightly the fraction of sensors in the resting states and the response to a large pulse, as confirmed in the experiment of Fig. 13.

To use the amplitude of the EC coupling response as a measure of affinity, a low-saturation situation is necessary. This is formally defined below.

In a normally polarized fiber in the presence of low concentrations of ionic ligand the sensors will be distributed between the inactivated states and the resting states;

Published September 1, 1989



the state diagram simplifies to:

$$\begin{array}{l} \text{Inactivated*} \xleftarrow{K_2} \text{Inactivated} \\ \quad\downarrow K_r \\ \text{Resting} + \text{Me} \\ \quad\downarrow K_a \\ \text{Resting:Me} \end{array}$$

(Scheme 2)

Resting and Resting:Me are the states available to undergo the activating transition, generating the movement of charge 1 and the release response. Their fractional occupancies are calculated using the equilibrium and conservation equations of the model:

$$K_2 \equiv \frac{[\text{Inactivated*}]}{[\text{Inactivated}]} = \exp\left[-(V - V_2)z_2\beta\right] = \exp(-\varphi) \tag{1}$$

$$K_r \equiv \frac{[\text{Resting}]}{[\text{Inactivated*}]} \tag{2}$$

$$K_a \equiv \frac{[\text{Resting:Me}]}{[\text{Resting}]\cdot[\text{Me}]} \tag{3}$$

$$[\text{Inactivated}] + [\text{Inactivated*}] + [\text{Resting}] + [\text{Resting:Me}] = 1 \tag{4}$$

In these equations $V$ is membrane holding potential, $V_2$ is the central voltage of the distribution of inactivated states (and is also the central voltage of charge 2, −113.5 mV in the measurements of Fig. 3), $z_2$ is the apparent valence of the inactivated states, $\beta \equiv e/kT \simeq {}^1/_{24}$ mV, $e$ is elementary charge, and $k$ is the Boltzmann constant. $\varphi$ is the normalized voltage $(V - V_2)z_2\beta$. Again, from the measurements of charge 2, $z_2\beta \simeq 17.5$ mV.

By sequential substitutions in Eq. 4 the desired expression is obtained:

$$[\text{Resting:Me}] + [\text{Resting}] = \frac{1 + [\text{Me}]K_a}{1 + [\text{Me}]K_a + [1 + \exp(\varphi)]/K_r} \tag{5}$$

The available charge 1, proportional to [Resting:Me] + [Resting], will depend linearly on the affinity constant $K_a$ provided that $[\text{Me}]K_a \ll [1 + \exp(\varphi)]/K_r$. $K_r$ can be evaluated from the limiting case [Me] = 0; this is the metal-free condition, exemplified by the record of $Ca^{2+}$ release labeled "0.1 EGTA" in Fig. 11. The peak value of $Ca^{2+}$ release is barely above noise level, not greater than 0.03 of the value in 0.1 mM $Ca^{2+}$. From records in 2 mM $Ca^{2+}$ obtained earlier, the value in 0.1 EGTA was estimated to be <0.005 of the maximum. Therefore the amplitude of the response (relative to the maximum response), Eq. 5, becomes:

$$K_r/\{K_r + 1 + \exp[(-100 + 113.5)/17.5]\} < 0.005, \tag{5a}$$

giving an upper bound of 0.02 for the repriming constant $K_r$. Inserting a value of $K_r$

of at most 0.02 in Eq. 5 the condition of linearity may be restated as:

$$[\mathrm{Me}]K_a \ll 50\{1 + \exp[(V - 113.5\ \mathrm{mV})/17.5\ \mathrm{mV}]\}. \quad (6)$$

In this case the following approximation holds for the magnitude of the response:

$$[\mathrm{Resting{:}Me}] + [\mathrm{Resting}] \simeq \frac{1 + [\mathrm{Me}]K_a}{50\{1 + \exp[(V + 113.5\ \mathrm{mV})/17.5\ \mathrm{mV}]\}}, \quad (7)$$

which is linear in both [Me] and $K_a$.

The upper bound of 0.02 for the repriming constant $K_r$, means that voltage sensors have an extremely low intrinsic tendency toward the resting state, and that only the presence of ionic ligands makes the repriming process advance measurably. In that sense, binding to the ion causes, or is necessary for repriming (the induced-fit phenomenon, as described by Colquhoun, 1973).

The condition of linearity is violated at high [Me]. The system can always be forced to satisfy the condition by making the holding potential ($V$ in Eq. 5) more positive. This procedure was used for instance in the experiments with 2 mM of alkaline-earths, to offset their much greater affinity.

### *Relative and Absolute Affinities*

The view taken here, that the magnitude of the EC coupling manifestations is a measure of occupancy of the priming site by ions is, admittedly, an unproven interpretation. A conclusive proof could be the generation of a number of curves of $Ca^{2+}$ release or charge movement vs. concentration, differing in dissociation constants but not in saturation value for the different metal ions. Fig. 5 shows that the EC coupling manifestations are far from their maxima even at the highest $[Na^+]$ tested (160 mM); therefore, a complete release vs. concentration curve was not feasible for group Ia ions.

A study of concentration dependence with $Ca^{2+}$ and other divalent cations is currently in progress (abstract by Ríos et al., 1988). A rough estimate of affinity constant can be obtained from the experiments in conditions of low saturation. [Resting:Me] evaluated from the magnitude of Ca release flux in the experiment of Fig. 10 is ≃0.2 in 0.1 mM $[Ca^{2+}]_e$ (that is, the peak of $Ca^{2+}$ release flux is ≃20% of the maximum in high $[Ca^{2+}]_e$, not shown). From the approximate Eq. 7, introducing the value −100 mV for the holding potential $V$, $[\mathrm{Me}]K_a \sim 32$, or $K_a \simeq 3 \times 10^5\ \mathrm{M}^{-1}$.

### *A Comparison with $Ca^{2+}$ Channels*

A remarkable finding of this study is the similarity between the selectivity sequence of the voltage sensor and that of permeability of the L-type Ca channel. The observed sequence for ions of group Ia, Li > Na > K > Rb > Cs, is the same reported by Hess et al. (1986) for the cardiac Ca channel and by Coronado and Smith (1987) for Ca channels reconstituted from T tubules of rat skeletal muscle, based on reversal potential measurements. For group IIa cations, there are reversal potential measurements that give permeabilities in the sequence Ca > Sr > Ba ≫ Mg (Hess et al., 1986) and this agrees with the present results, with the notable exception of Mg.

Published September 1, 1989



Our quantitative estimates of relative affinities are given in Table II and Fig. 12. together with relative permeabilities of the cardiac L channel, estimated by Tsien et al. (1987) from reversal potential measurements using constant field theory. The values of relative affinity have large probable errors due to fiber to fiber variability. Additionally, the monovalent ions $Rb^+$, $Cs^+$, and especially $K^+$, cause spatial inhomogeneities in voltage, as discussed in Methods (the upper bound of 4% estimated for the spatial inhomogeneity seems small, however, a 4-mV change in the holding potential could have a nonnegligible inactivating effect in some situations; no attempt has been made to estimate errors in the affinity due to this effect). In spite of these problems, the agreement between affinities of the priming site and channel permeabilities is striking.

This similarity is consistent with a number of other evidences that the voltage sensor of EC coupling and the L-type Ca channel are structurally related, namely, that they have in common a dihydropyridine binding site (Lamb, 1986; Rakowsky et al., 1987; Ríos and Brum, 1987; Dulhunty and Gage, 1988; Gamboa-Aldeco et al., 1988; Fill and Best, 1989), a D-600 binding site (Eisenberg et al., 1983; Hui et al., 1984; Berwe et al., 1987; Melzer and Pohl, 1987; Caputo and Bolaños, 1989; Pizarro et al., 1988*a*), and a benzothiazepine binding site (Walsh et al., 1987; Gamboa-Aldeco et al., 1988); plus other common aspects discussed by Ríos and Brum (1987) and Pizarro et al. (1988*a*). More recently Tanabe et al. (1988) reported that injection of a plasmid carrying the cDNA of the rabbit skeletal muscle dihydropyridine receptor protein restored contractility, together with slow membrane $Ca^{2+}$ currents, to muscle cells from mice with muscular dysgenesis; this result is evidence that the encoded protein is both an essential component of EC coupling and a $Ca^{2+}$ channel.

### *The Nature of the Priming Site*

The permeability sequence of the L channel has been interpreted as determined by binding of the permeant ion to an intrapore site; ions that bind well have a high permeability, and a low conductance (reviewed in Tsien et al., 1987 for cardiac muscle and Almers et al., 1986 for skeletal muscle). The simplest interpretation of the similarity with the binding selectivity of the priming site is that the priming site corresponds to the intrapore binding site of the Ca channel.

This interpretation is in conflict with the observation that $Mg^{2+}$ can support EC coupling but does not permeate the cardiac channel. The discrepancy may be due to tissue differences, as $Mg^{2+}$ carries measurable currents in skeletal muscle $Ca^{2+}$ channels (Almers et al., 1984). Additionally, the low permeability of $Mg^{2+}$ may be a consequence of high rejection, rather than poor binding (discussed by Lansman et al., 1986), as revealed by the fact that $Mg^{2+}$ is a substantial blocker of $Ca^{2+}$ channel currents in skeletal (Almers et al., 1984), cardiac (Lansman et al., 1986; Matsuda, 1986), and mouse neoplastic B lymphocytes, in which its affinity for a blocking site was estimated at $^1/_{15}$ that of $Ca^{2+}$ (Fukushima and Hagiwara, 1985).

### *Comparison with Previous Work*

The present results and model agree with previous observations and help clarify points that remained obscure in the literature. All the effects of low extracellular

$Ca^{2+}$ on K contractures, including the 0 $Ca^{2+}$ "paralysis" of Graf and Schatzmann (1984), are explained by an increase in the equilibrium probability of the inactivated states or in the rate of the transition to inactivated states (discussed in detail by Brum et al., 1988*a*). The conservation of twitches in the absence of extracellular $Ca^{2+}$ observed by Armstrong et al. (1972) is a consequence of the ability of $Na^+$ to bind to the site. In apparent conflict with our findings, Miledi et al. (1984) reported complete independence of EC coupling on the extracellular ionic composition. They performed two kinds of interventions, $Na^+$ replacement (by $Li^+$, $K^+$, and various organic cations) and modification of divalent cation composition ($Ca^{2+}$ withdrawal, high $Ca^{2+}$, and high $Mg^{2+}$). All $Na^+$ substitution experiments were carried out in the presence of 2 mM $[Ca^{2+}]_e$; in the $Ca^{2+}$ withdrawal experiments, 2 mM $Ca^{2+}$ were replaced by 5 mM $Mg^+$ in the presence of 120 mM $Na^+$; the high $Ca^{2+}$ experiments were done in the presence of 120 mM $Na^+$ and the high $Mg^{2+}$ was applied in the presence of 120 mM $Na^+$ and 2 mM $Ca^{2+}$. All interventions reducing the concentration of ions were thus performed in the presence of other metal ions that support EC coupling. Furthermore, Miledi et al. (1984) used short voltage clamp pulses (≤30 ms) that do not produce significant voltage-dependent inactivation (Brum et al., 1988*a*), therefore they missed the differences in kinetics of inactivation induced by the ionic changes. None of the interventions thus performed are expected to have dramatic effects on EC coupling, their results are readily explained by our interpretation.

The present results demonstrate antagonism between $Ca^{2+}$ and voltage-dependent inactivation. Taken together with the mechanism of Ca antagonists as drugs that bind to and stabilize inactivated states, they imply that $Ca^{2+}$ and other cations should antagonize binding and pharmacological actions of these drugs. Pizarro et al. (1988*a*) have reported direct antagonism between $Ca^{2+}$ and nifedipine effects. A number of reports document the inhibition by $Ca^{2+}$ of binding of tritiated benzothiazepines and phenylalkylamines to dihydropyridine receptors from mammalian muscle (Galizzi et al., 1984, 1985); the $K_{0.5}$ for this effect is 5 $\mu$M, the affinity decreases for the other alkaline-earths in the sequence Ca > Sr > Ba > Mg. This is essentially as predicted by our model and the rough estimate of $K_a$ given above. The situation is more complicated regarding the dihydropyridines, as their binding requires the presence of extremely low concentrations of $Ca^{2+}$ (reviewed by Glossmann and Striessnig, 1988) and there is evidence of inhibition of nitrendipine binding at very high $[Ca^{2+}]$ (Fosset et al., Ervasti et al., 1989).

### *Consequences for Channel Gating*

This paper and others from our laboratory provide evidence of the existence of a metal-binding site on the voltage sensor of EC coupling that is essential for its function. When the site is not occupied by a metal ligand the sensor is inactivated and therefore unable to signal the sarcoplasmic reticulum to release $Ca^{2+}$. In view of the chemical nature of the voltage sensor these interactions between ion binding and voltage-dependent gating should apply to $Ca^{2+}$ channels as well; that is, $Ca^{2+}$ and other ions should antagonize voltage-dependent inactivation. It has long been known that divalent cations oppose inactivation in $Na^+$ channels of the squid axon (Shoukimas, 1978) and a similar effect has been demonstrated in L-type cardiac

$Ca^{2+}$ channels (Kass and Krafte, 1987). This effect appears as a shift to higher voltages of the inactivation curve at higher $[Ca^{2+}]_e$; it has been explained as screening or binding and neutralization of negative charges on the external surface of the membrane. Based on the similarity between voltage sensors and $Ca^{2+}$ channels it is also possible that ions bound within the permeation path oppose voltage-dependent inactivation of the channels in a more specific manner; this would happen for instance if the permeant ions had to be dislodged from the pore to permit the conformational change of inactivation.

Metal binding has been recognized for decades as a promoter of conformational changes in proteins. Recently, it has been shown to regulate voltage-driven transitions in channel proteins (Armstrong and Matteson, 1986; Matteson and Swenson, 1986; Nelson et al., 1984). The present work suggests an analogous role of metal binding in a channel-like protein.

We are grateful to Dr. Mónica Rodríguez for help at various stages of the experiments, to Dr. Stuart McLaughlin for a generous gift of dimethonium dibromide, and to Dr. Fredric Cohen for thoughtful reading and suggestions.

This work was supported by National Institutes of Health grant Ar-32808 to E. Rios, National Research Service Award AR-07575 to R. Fitts, and a Muscular Dystrophy Association basic research grant to E. Rios.

*Original version received 16 November 1988 and accepted version received 10 April 1989.*

## REFERENCES

Adrian, R. H., W. K. Chandler, and A. L. Hodgkin. 1969. The kinetics of mechanical activation in frog muscle. *Journal of Physiology.* 204:207–230.

Almers, W., E. W. McCleskey, and P. T. Palade. 1984. A non-selective cation conductance in frog muscle membrane blocked by micromolar external calcium ions. *Journal of Physiology.* 353:565–583.

Almers, W., E. W. McCleskey, and P. T. Palade. 1986. The mechanism of ion selectivity in calcium channels of skeletal muscle membrane. *In* Membrane Control (Fortschritte der Zoologie, Band 33) H. Luttgau, editor. Gustav Fischer Verlag, Stuttgart, New York. 61–73.

Armstrong, C., F. Bezanilla, and P. Horowicz. 1972. Twitches in the presence of ethyleneglycol bis(β-aminoethyl ether)-*N,N'*-tetracetic acid. *Biochimica et Biophysica Acta.* 267:605–608.

Armstrong, C. M., and D. R. Matteson. 1986. The role of calcium ions in the closing of K channels. *Journal of General Physiology.* 87:817–832.

Berwe, D., G. Gottschalk, and H. Ch. Luttgau. 1987. The effects of the Ca-antagonist gallopamil (D600) upon excitation-contraction coupling in toe muscles of the frog. *Journal of Physiology.* 385:693–708.

Brum, G., R, Fitts, G. Pizarro, and E. Ríos. 1988*a*. Voltage sensors of the frog skeletal muscle membrane require calcium to function in excitation-contraction coupling. *Journal of Physiology.* 398:475–505.

Brum, G., E. Ríos, and E. Stéfani. 1988*b*. Effects of extracellular calcium on calcium movements of excitation-contraction coupling in frog skeletal muscle fibres. *Journal of Physiology.* 398:441–473.

Brum, G., and E. Ríos. 1987. Intramembrane charge movement in skeletal muscle fibres. Properties of charge 2. *Journal of Physiology.* 389:489–517.

Caputo, C. 1972. The time course of potassium contractures of single muscle fibres. *Journal of Physiology.* 223:483–505.

Caputo, C., and P. Bolaños. 1988. Effect of D600 and $La^{+++}$ on charge movement in depolarized muscle fibers. *Biophysical Journal.* 53:604*a*. (Abstr.)

Caputo, C., and P. Bolaños. 1989. Effects of D-600 on intramembrane charge movement of polarized and depolarized frog muscle fibers. *Journal of General Physiology.* 94:43–64.

Colquhoun, D. 1973. The relation between classical and cooperative models for drug action. *In* Drug Receptors. A. P. Rand, editor. University Park Press, London. 149–182.

Coronado, R., and J. S. Smith. 1987. Monovalent ion current through single calcium channels of skeletal muscle transverse tubules. *Biophysical Journal.* 51:497–502.

Curtis, B. A. 1986. Is Ca/H exchange involved in t-sr coupling? *Biophysical Journal.* 49:189*a*. (Abstr.)

Diamond, J. M., and E. M. Wright. 1969. Biological membranes: the physical basis of ion and nonelectrolyte selectivity. *Annual Review of Physiology.* 31:581–646.

Dulhunty, A. F., and P. W. Gage. 1988. Effects of extracellular calcium concentration and dihydropyridines on contraction in mammalian skeletal muscle. *Journal of Physiology.* 399:63–80.

Eisenberg, R. S., R. T. McCarthy, and R. L. Milton. 1983. Paralysis of frog skeletal muscle fibres by the calcium antagonist D600. *Journal of Physiology.* 341:495–505.

Eisenman, G. 1962. Cation selective glass electrodes and their mode of operation. *Biophysical Journal.* 2(Suppl. 2):259–323.

Eisenman, G., and R. Horn. 1983. Ionic selectivity revisited: the role of kinetic and equilibrium processes in ion permeation through channels. *Journal of Membrane Biology.* 76:197–225.

Ervasti, J., M. Mickelson, and C. Louis. 1989. Altered transverse tubule dihydropyridine receptor binding in malignant hyperthermia. *Journal of Biological Chemistry.* in review.

Fill, M. D., and P. M. Best. 1989. Block of contracture in skinned frog skeletal muscle fibers by calcium antagonists. *Journal of General Physiology.* 93:429–449.

Fill, M., R. Fitts, G. Pizarro, M. Rodríguez, and E. Ríos. 1988. Effects of Ca agonists and antagonists on E-C coupling in skeletal muscle fibers. *Biophysical Journal.* 53:603*a*. (Abstr.)

Fosset, M., E. Jaimovich, E. Delpont, and M. Lazdunski. 1983. [$^3$H]Nitrendipine receptors in skeletal muscle. Properties and preferential localization in transverse tubules. *Journal of Biological Chemistry.* 258:6086–6092.

Frank, G. B. 1980. The current view of the source of trigger calcium in excitation-contraction coupling in vertebrate skeletal muscle. *Journal of Biochemistry and Pharmacology.* 29:2399–2406.

Fukushima, T., and S. Hagiwara. 1985. Currents carried by monovalent cations through calcium channels in mouse neoplastic $\beta$ lymphocytes. *Journal of Physiology.* 358:255–284.

Galizzi, J.-P., M. Fosset, and M. Lazdunski. 1984. Properties of receptors for $Ca^{2+}$-channel blocker verapamil in transverse-tubule membranes of skeletal muscle. *European Journal of Biochemistry.* 144:211–215.

Galizzi, J.-P., M. Fosset, and M. Lazdunski. 1985. Characterization of the $Ca^{2+}$ coordination site regulating binding of $Ca^{2+}$ channel inhibitors d-*cis*-diltiazem, (±)bepridil and (−)desmethoxyverapamil to their receptor site in skeletal muscle transverse tubule membranes. *Biochemical and Biophysical Research Communications.* 132:49–55.

Gamboa-Aldeco, R., M. Huerta, and E. Stéfani. 1988. Effect of Ca channel blockers on K contractures in twitch fibres of the frog (*Rana pipiens*). *Journal of Physiology.* 397:389–399.

Glossmann, H., and J. Striessnig. 1988. Calcium channels. *Vitamins and Hormones.* 44:155–328.

Graf, F., and H. J. Schatzmann. 1984. Some effects of removal of external calcium on pig striated muscle. *Journal of Physiology.* 349:1–13.

Hess, P., J. B. Lansman, and R. W. Tsien. 1986. Calcium channels selectivity for divalent and

monovalent cations. Voltage and concentration dependence of single channel current in ventricular heart cells. *Journal of General Physiology.* 88:293–320.

Hui, C. S., R. L. Milton, and R. S. Eisenberg. 1984. Charge movement in skeletal muscle fibers paralyzed by the calcium-entry blockers D-600. *Proceedings of the National Academy of Sciences.* 81:2582–2585.

Irving, M., J. Maylie, N. L. Sizto, and W. K. Chandler. 1987. Intrinsic optical and passive electrical properties of cut frog twitch fibers. *Journal of General Physiology.* 89:1–41.

Kass, R. S., and D. Krafte. 1987. Negative surface charge density near heart Ca channels. Relevance to block by dihyropyridines. *Journal of General Physiology.* 89:629–644.

Kovács, L., E. Ríos, and M. F. Schneider. 1979. Calcium transients and intramembrane charge movement in skeletal muscle fibres. *Nature.* 279:391–396.

Lamb, G. 1986. Components of charge movement in rabbit skeletal muscle. The effects of tetracaine and nifedipine. *Journal of Physiology.* 376:85–100.

Lansman, J. B., P. Hess, and R. W. Tsien. 1986. Blockade of current through single calcium channels by $Cd^{2+}$, $Mg^{2+}$, and $Ca^{2+}$. Voltage and concentration dependence of calcium entry into the pore. *Journal of General Physiology.* 88:321–348.

Lüttgau, H. Ch. 1963. The action of calcium ions on potassium contractures of single muscle fibres. *Journal of Physiology.* 168:679–697.

Lüttgau, H. Ch., and W. Spiecker. 1979. The effects of calcium deprivation upon mechanical and electrophysiological parameters in skeletal muscle fibres of the frog. *Journal of Physiology.* 296:411–429.

Mathias, R. T., R. S. Eisenberg, and P. Valdiosera. 1977. Electrical properties of frog skeletal muscle fibers interpreted with a mesh model of the tubular system. *Biophysical Journal.* 17:57–93.

Matsuda, H. 1986. Sodium conductance in calcium channels of guinea-pig ventricular cells induced by removal of external calcium ions. *Pflügers Archiv.* 407:465–475.

Matteson, D. R., and R. P. Swenson, Jr. 1986. External monovalent cations that impede the closing of K channels. *Journal of General Physiology.* 87:795–816.

McLaughlin, A., W. K. Eng, G. Vaio, T. Wilson, and S. McLaughlin. 1983. Dimethonium, a divalent cation that exerts only a screening effect on the electrostatic potential adjacent to negatively charged phospholipid bilayer membranes. *Journal of Membrane Biology.* 76:183–193.

Melzer, W., and B. Pohl. 1987. Effects of D600 on the voltage sensor for Ca release in skeletal muscle fibres of the frog. *Journal of Physiology.* 390:151P.

Melzer, W., E. Ríos, and M. F. Schneider. 1986. The removal of myoplasmic free calcium following calcium release in frog skeletal muscle. *Journal of Physiology.* 372:261–292.

Melzer, W., E. Ríos, and M. F. Schneider. 1987. A general procedure for determining calcium release in skeletal muscle fibers. *Biophysical Journal.* 51:849–864.

Melzer, W., M. F. Schneider, B. Simon, and G. Szucs. 1986. Intramembrane charge movement and Ca release in frog skeletal muscle. *Journal of Physiology.* 373:481–511.

Miledi, R., I. Parker, and P. H. Zhu. 1984. Extracellular and excitation-contraction coupling in frog twitch muscle fibres. *Journal of Physiology.* 351:687–710.

Nelson, M. T., R. J. French, and B. K. Krueger. 1984. Voltage-dependent calcium channels from brain incorporated into planar lipid bilayers. *Nature.* 308:77–80.

Pizarro, G., G. Brum, M. Fill, R. Fitts, M. Rodríguez, I. Uribe, and E. Ríos. 1988*a*. The voltage sensor of skeletal muscle excitation-contraction coupling: a comparison with $Ca^{2+}$ channels. *In* The Calcium Channel, Structure, Function and Implications. M. Morad, W. Nayler, S. Kazda, and M. Schramm, editors. Springer-Verlag, Berlin, Heidelberg. 138–156.

Pizarro, G., R. Fitts, and E. Ríos. 1988*b*. Selectivity of a cation binding membrane site essential for EC coupling in skeletal muscle. *Biophysical Journal.* 53:645*a*. (Abstr.)

Pizarro, G., R. Fitts, G. Brum, M. Rodríguez, and E. Ríos. 1987. Simultaneous recovery of charge movement and Ca release in skeletal muscle. *Biophysical Journal.* 51:101*a* (Abstr.)

Rakowski, R. F., E. Olszewska, and C. Paxson. 1987. High affinity effect of nifedipine on K-contracture in skeletal muscle suggest a role for calcium channels in skeletal muscle. *Biophysical Journal.* 51:550.

Ríos, E., and G. Brum. 1987. Involvement of dihydropyridine receptors in excitation-contraction coupling in skeletal muscle. *Nature.* 325:717–720.

Ríos, E., G. Brum, and F. Stéfani. 1986. EC coupling effects of interventions that reduce slow Ca current suggest a role of T-tubule Ca channels in skeletal muscle function. *Biophysical Journal.* 49:13*a* (Abstr.)

Ríos, E., G. Pizarro, and G. Brum. 1989. A four gap voltage clamp improves measurements of EC coupling events in frog skeletal muscle. *Biophysical Journal.* 55: 237*a*. (Abstr.)

Ríos E., M. Rodríguez, G. Pizarro, R. Fitts, and G. Brum. 1988. Complete separation of charges 1 and 2 in frog skeletal muscle fibers. *Biophysical Journal.* 53:645*a*. (Abstr.)

Schneider, M. F. 1970. Linear electrical properties of the transverse tubules and surface membrane of skeletal muscle fibers. *Journal of General Physiology.* 56:640–671.

Shoukimas, J. J. 1978. Effect of calcium upon sodium inactivation in the giant axon of *Loligo pealei*. *Journal of Membrane Biology.* 38:271–289.

Stéfani, E., and D. Chiarandini. 1973. Skeletal muscle: dependence of potassium contractures on extracellular calcium. *Pflügers Archiv.* 343:143–150.

Tanabe, T., K. G. Beam, J. A. Powell, and S. Numa. 1988. Restoration of excitation-contraction coupling and slow calcium current in dysgenic muscle by dihydropyridine receptor cDNA. *Nature.* 336:134–139.

Tsien, R. W., P. Hess, E. W. McCleskey, and R. L. Rosenberg. 1987. Calcium channels: mechanisms of selectivity, permeation and block. *Annual Review of Biophysics and Biophysical Chemistry.* 16:265–290.

Walsh, K. B., S. H. Bryant, and A. Schwartz. 1987. Suppression of charge movement by calcium antagonists is not related to calcium channel block. *Pflügers Archiv.* 409:217–219.

Weast, R. C. 1985. Handbook of Chemistry and Physics. 66th Ed. CRC Press, Boca Raton, FL. D167–D169.