Low Myoplasmic Mg^{2+} Potentiates Calcium Release during Depolarization of Frog Skeletal Muscle Fibers

VINCENT JACQUEMOND and MARTIN F. SCHNEIDER

From the Department of Biological Chemistry, University of Maryland, School of Medicine, Baltimore, Maryland 21201

ABSTRACT The role of intracellular free magnesium concentration ($[Mg^{2+}]$) in modulating calcium release from the sarcoplasmic reticulum (SR) was studied in voltage-clamped frog cut skeletal muscle fibers equilibrated with cut end solutions containing two calcium indicators, fura-2 and antipyrylazo III (AP III), and various concentrations of free Mg^{2+} (25 μM–1 mM) obtained by adding appropriate total amounts of ATP and magnesium to the solutions. Changes in AP III absorbance were used to monitor calcium transients, whereas fura-2 fluorescence was used to monitor resting calcium. The rate of release (R_{rel}) of calcium from the SR was calculated from the calcium transient and found to be increased in low internal $[Mg^{2+}]$. After correcting for effects of calcium depletion from the SR and normalization to SR content, the mean values of the inactivatable and noninactivatable components of R_{rel} were increased by 163 and 46%, respectively, in low Mg^{2+}. Independent of normalization to SR content, the ratio of inactivatable to noninactivatable components of R_{rel} was increased in low internal $[Mg^{2+}]$. Both observations suggest that internal $[Mg^{2+}]$ preferentially modulates the inactivatable component of R_{rel}, which is thought to be due to calcium-induced calcium release from the SR. This could also explain the observation that, in low internal $[Mg^{2+}]$, the time to the peak of the calcium transient for a 5-ms depolarizing pulse was not very different from the time to the peak of the $\Delta[Ca^{2+}]$ for a 10-ms pulse of the same amplitude. Finally, in low internal $[Mg^{2+}]$, the calcium transient elicited by a short depolarizing pulse was in some cases clearly followed by a very slow rise of calcium after the end of the pulse. The observed effects of reduced $[Mg^{2+}]$ on calcium release are consistent with a removal of the inhibition that the normal 1 mM myoplasmic $[Mg^{2+}]$ exerts on calcium release in skeletal muscle fibers.

INTRODUCTION

Activation of a skeletal muscle fiber is produced by electrical depolarization of the membranes of the transverse (T) tubular system. The change in T-tubular membrane potential causes the movement of charged voltage sensors (Schneider and Chandler, 1973) in the T-tubular membrane, which appears to activate calcium release (Melzer,

Schneider, Simon, and Szucs, 1986; Simon and Schneider, 1988; Simon and Hill, 1992) from the neighboring sarcoplasmic reticulum (SR), causing a rise in myoplasmic [Ca^{2+}] (Miledi, Parker, and Schalow, 1977; Blinks, Rudel, and Taylor, 1978). The T-tubular voltage sensors are now believed to be the dihydropyridine (DHP) receptors (Rios and Brum, 1987; Tanabe, Takeshima, Mikami, Flockerzi, Matsuo, Hirose, and Numa, 1987) located in the T-tubule membrane. Calcium release is believed to occur via channels in ryanodine receptors (Fleischer, Ogunbunmi, Dixon, and Fleer, 1985; Pessah, Francini, Scales, Waterhouse, and Casida, 1986; Imagawa, Smith, Coronado, and Campbell, 1987; Lai, Erickson, Rousseau, Liu, and Meissner, 1988) in the junctional SR membrane. Coupling between the DHP and ryanodine receptors may involve cytosolic domains in the DHP receptors (Tanabe, Beam, Adams, Niidome, and Numa, 1990). In addition to the T-tubular voltage sensor, a variety of myoplasmic constituents, including magnesium ions, may control or modulate the SR calcium release channels (cf. Fleischer and Inui, 1989).

Two main lines of results have suggested that intracellular free magnesium plays a significant role in the regulation of calcium release from the SR: (a) lowering the free Mg^{2+} elicits a spontaneous contraction of skinned muscle fibers (Stephenson, 1981; Herrmann-Frank, 1989; Lamb and Stephenson, 1991); and (b) the calcium release from heavy SR vesicles and the calcium release channel isolated from these vesicles or purified as the ryanodine receptor have all been shown to be inhibited by millimolar levels of Mg^{2+} (Meissner, 1984; Smith, Coronado, and Meissner, 1985, 1986; Meissner, Darling, and Eveleth, 1986; Lai et al., 1988; Moutin and Dupont, 1988).

To investigate the possible role of intracellular free Mg^{2+} in modulating the physiologically induced SR calcium release during depolarization of a skeletal muscle fiber, we carried out experiments on frog cut skeletal muscle fibers mounted in a double Vaseline-gap device using internal solutions containing various concentrations of free Mg^{2+}. Analysis of the changes in myoplasmic [Ca^{2+}] and [Mg^{2+}] elicited by membrane depolarization in the presence of low concentrations of internal Mg^{2+} suggests that in normal conditions, physiological levels of free internal Mg^{2+} tend to inhibit release of calcium from the SR during fiber depolarization.

METHODS

All methods of fiber preparation, solutions, electrical and optical recording, and calculation of resting [Ca^{2+}] and [Ca^{2+}] transients were as described in our preceding paper (Jacquemond and Schneider, 1992).

The rate of release (R_{rel}) of calcium from the SR was calculated from each Δ[Ca^{2+}] according to the general approach of Melzer, Rios, and Schneider (1984, 1987), using method 1 of Melzer et al. (1987). Details regarding most of the specific myoplasmic calcium binding sites, both rapidly and slowly equilibrating, used in the present model of calcium removal and the rationale for choosing their values appear in the preceding paper (Jacquemond and Schneider, 1992). Fits of the model to the decay of Δ[Ca^{2+}] after various pulses in the same fibers in control and low internal [Mg^{2+}] as used in the present release calculations were described in the preceding paper (Jacquemond and Schneider, 1992). In brief, in all of our calculations we assumed the calcium-specific binding sites on thin filament troponin C to be present at 250 μM (referred to myoplasmic water) and to have on and off rate constants of 1.3×10^8 M^{-1} s^{-1} and 10^3 s^{-1}. The calcium binding sites on the SR calcium pump were assumed to be present at 200

μM, to have a dissociation constant of 1 μM, and to be in instantaneous equilibrium with the myoplasmic $[Ca^{2+}]$. The rate of calcium transport by the SR calcium pump was assumed to be proportional to the degree of calcium occupancy of the pump sites. The value of the maximum transport rate of the SR calcium pump was adjusted to fit the decline of $\Delta[Ca^{2+}]$ after several pulses. Ca^{2+} and Mg^{2+} binding to myoplasmic parvalbumin (Parv) were assumed to occur with respective on rate constants of 1.6×10^8 and 4×10^4 M^{-1} s^{-1} and off rate constants of 1.5 and 8.0 s^{-1}. The concentration [Parv] of Parv binding sites was assumed to be 1 mM. In all cases the $[Mg^{2+}]$ in the fiber was assumed to be equal to the value calculated for the solution applied to the cut ends of the fiber and that value of $[Mg^{2+}]$ together with the measured resting $[Ca^{2+}]$ was used to calculate the calcium and magnesium occupancy of Parv in the resting fiber.

An additional calcium binding site (X), with a dissociation constant of 1 μM, was also included in all removal and release calculations (Jacquemond and Schneider, 1992). In reduced internal $[Mg^{2+}]$ the off rate constant (0.5–5.0 s^{-1}) and concentration (50–200 μM) for the extra site were varied within the indicated ranges to obtain a best fit to the decay of $\Delta[Ca^{2+}]$ after several pulses (Jacquemond and Schneider, 1992). In the control fibers the fit was extremely good even without using the extra site, so the values of the parameters of the site could not be determined in control (Jacquemond and Schneider, 1992). The values used for the off rate constant (2.84 s^{-1}) and site concentration (158 μM) for each control fiber were set equal to the mean values determined in the fibers in low internal $[Mg^{2+}]$ (Jacquemond and Schneider, 1992).

In calculating the rate of calcium release (R_{rel}) from the SR, the removal model parameters determined from the decline of $[Ca^{2+}]$ after various pulses (above) were assumed to apply during the pulse. R_{rel} was calculated as the rate of change of free $[Ca^{2+}]$ plus the calculated rate of change of calcium bound to all sites in the removal model plus the calculated rate of transport of calcium into the SR via the SR calcium pump.

RESULTS

Rate of Calcium Release from the SR in the Presence of Low Mg^{2+}

Fig. 1 presents the rate of calcium release (R_{rel}) from the SR in response to a 120-ms depolarizing pulse to -20 mV (bottom) in a control fiber (1 mM internal $[Mg^{2+}]$, left column) and in a fiber equilibrated with a low $[Mg^{2+}]$ internal solution (25 μM, right column). The calcium transients and the removal model fits to the decay of $\Delta[Ca^{2+}]$ used to calculate these release records were presented in Fig. 6, E and F, of the preceding paper (Jacquemond and Schneider, 1992). The rate of release of calcium from the SR was calculated from the free calcium transient (see Methods) using the removal model including the extra site X both in control and in low internal $[Mg^{2+}]$. The R_{rel} records in both control and low internal $[Mg^{2+}]$ (Fig. 1, top row) were similar in exhibiting an early peak followed by a rapid decline and then a slower phase of decline. Previous studies under the control conditions indicated that the rapid decline in release after the peak is probably due to inactivation of calcium release, whereas the slower phase of decline of release is probably due to depletion of calcium from the SR (Schneider, Simon, and Szucs, 1987; Schneider and Simon, 1988). Since the fast and slow phases of decline of R_{rel} were also observed in low internal $[Mg^{2+}]$ (Fig. 1, top row, right), they may be assumed to arise from inactivation and depletion, respectively, in low internal $[Mg^{2+}]$ as in control. Two differences between R_{rel} in control and in low internal $[Mg^{2+}]$ that were characteristic of most control and low Mg^{2+} fibers were that the relative rate of the slow phase of decline of R_{rel} was faster in

low internal [Mg^{2+}] than in control, and the final level of R_{rel} at the end of the pulse was lower in low internal [Mg^{2+}].

To quantitate the slow decline of R_{rel}, a single exponential was fit to the slow phase of decay of R_{rel} during the latter part of each 120-ms pulse to -20 mV (constant set equal to 0 for fit; fits not shown). The resulting mean ± SEM values for the rate
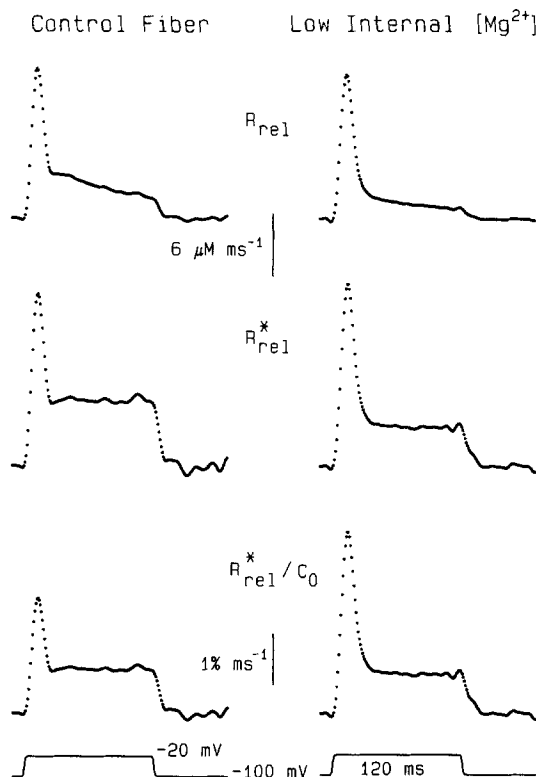

FIGURE 1. Rate of release of calcium from the SR in a control fiber (1 mM internal [Mg^{2+}], *left panel*) and in a fiber with low internal [Mg^{2+}] (25 μM, *right panel*). First row, rate of release of calcium from the SR (R_{rel}) calculated from the Δ[Ca^{2+}] records in Figs. 4 and 6 of Jacquemond and Schneider, 1992. *Second row*, rate of calcium release (R^*_{rel}) after correcting for the effects of depletion of calcium from the SR, assuming the SR calcium content before the pulse to be (if dissolved in the myoplasmic water) 1,200 μM for the control and 800 μM for the low [Mg^{2+}] fiber. *Third row*, rate of calcium release (R^*_{rel}/C_0) after correction for calcium depletion and normalization to the SR content C_0 determined from the correction for depletion. *Fourth row*, membrane potential. The rate of release was calculated as described in the text and in Methods. For the control fiber the value of the SR pump V_{max} selected in the least-squares fit to the decay of the calcium transients was equal to 3,293 μM s^{-1}. An extra calcium binding site X was included at a concentration of 158 μM with an on rate constant of 2.8×10^6 M^{-1} s^{-1} and an off rate constant of 2.8 s^{-1}. These values for the extra site X are the means of the values determined for X in low internal [Mg^{2+}] since the properties of X could not be determined in control conditions (Jacquemond and Schneider, 1992). For the fiber in low internal [Mg^{2+}] the fitted pump rate was 889 μM s^{-1} and 100 μM of the extra calcium binding site was added with $k_{on} = 1 \times 10^6$ M^{-1} s^{-1} and $k_{off} = 1$ s^{-1}. Same fibers and conditions as in Figs. 4 and 6 of Jacquemond and Schneider (1992).

constant for the slow phase of decline of R_{rel} were 7.6 ± 1.0 s^{-1} in control ($n = 23$) and 10.8 ± 1.3 s^{-1} in low internal [Mg^{2+}] (25, 58, and 134 μM; $n = 19$), indicating a 42% increase in the rate constant in low Mg^{2+}. If the slow decline of R_{rel} were due only to depletion of calcium from the SR (Schneider et al., 1987) at constant SR release activation, the mean rate constants for the slow decline should be propor-

tional to the mean SR calcium permeability in each condition. In this case the mean SR permeability during the slow decline of R_{rel} was 42% larger in low Mg^{2+} than in control. Even though the mean value of the steady SR permeability during the pulses was 42% larger in low internal [Mg^{2+}] than in control, the mean value of R_{rel} at the end of the same pulses was less than half as large in low internal [Mg^{2+}] (1.52 ± 0.16 μM ms^{-1}) than in control (3.26 ± 0.15 μM ms^{-1}). This indicates that at the end of the pulses the SR content must have been considerably smaller in low internal [Mg^{2+}] than in control. Assuming R_{rel} to be proportional to both SR permeability and SR content, if the mean permeability were 1.42 times higher in low Mg^{2+} than control (above), the mean SR content at the end of the pulses in low Mg^{2+} must have been only ~0.33 (=1.52/[3.26 × 1.42]) times the SR content at the end of the pulses in control. To relate this value to the relative SR contents in the resting fibers at the start of the pulses it is necessary to consider the extent of calcium depletion from the SR during the pulses.

The R_{rel} records were corrected for calcium depletion from the SR as previously described (Schneider et al., 1987; Schneider, Simon, and Klein, 1989) using the equation

$$R^*_{rel} = R_{rel}C_0/(C_0 - \int R_{rel}\, dt) \tag{1}$$

where R^*_{rel} denotes a release record corrected for depletion, R_{rel} and R^*_{rel} are both functions of time, and C_0 is the SR calcium content at the start of the pulse in units of the equivalent myoplasmic calcium concentration that would be produced if the entire SR calcium content were present as free calcium in the myoplasmic water. The value for C_0 in each fiber was selected so as to produce a constant level of R^*_{rel} during the latter part of each pulse (Schneider et al., 1987, 1989). The R^*_{rel} records for the fibers in Fig. 1 (second row) show that the depletion correction eliminated the slow phase of decline of R_{rel} (Fig. 1, top row) in both the control and the low internal [Mg^{2+}] fibers. In general, the correction for depletion was relatively larger at the end of the pulse in low internal [Mg^{2+}] than in control, indicating a greater relative degree of depletion of calcium from the SR during the pulses in low internal [Mg^{2+}] than in control. The mean values of the percent of the initial SR calcium content present at the start of the pulse that still remained at the end of a 120-ms pulse to −20 mV obtained from the relative size of the depletion correction at the end of the pulses were 37 ± 3% ($n = 23$) in control and 25 ± 3% ($n = 19$) in low internal [Mg^{2+}] (same fibers as above). These values have two implications. First, since a larger fraction of the initial SR content was released in low [Mg^{2+}] than in control, the average SR permeability during the pulses must have been greater in low internal [Mg^{2+}] than in control. Second, if the SR calcium content at the start of the pulses had been the same in control and in low internal [Mg^{2+}], the SR content at the end of the pulses in low internal [Mg^{2+}] would have been 0.68 (=25/37) times the value in control. Since the ratio of contents at the end of the pulses was actually found to be 0.33 (above), the SR calcium content in the resting fibers at the start of the pulses in low internal [Mg^{2+}] must have been only ~49% (=0.33/0.68) of the resting SR calcium content at the start of the pulses in control conditions.

The values of C_0 obtained from the depletion correction indicate that the SR calcium content before the pulses was lower in fibers exposed to low internal [Mg^{2+}]

solution than in the control fibers. In Fig. 1, C_0 was 1,200 μM in the control fiber and 800 μM in the fiber in 25 μM internal [Mg^{2+}]. The mean values of C_0 at the start of each pulse obtained from the depletion correction in each fiber are shown in Fig. 2 as a function of [Mg^{2+}] in the internal solution. These results confirm that the mean SR content at the start of the pulses was decreased in low internal [Mg^{2+}] compared with control.

The rate of release of calcium from the SR should be proportional to both the SR calcium permeability and the driving force for calcium efflux. Assuming the driving force to be proportional to the SR calcium content, the effects of differences in driving force on the measured rate of release would be removed by expressing release relative to SR content. The resulting R^*_{rel}/C_0 records would give the time course of SR calcium release permeability independent of SR content and corrected for calcium depletion during the pulse. The third row of Fig. 1 presents the R^*_{rel}/C_0
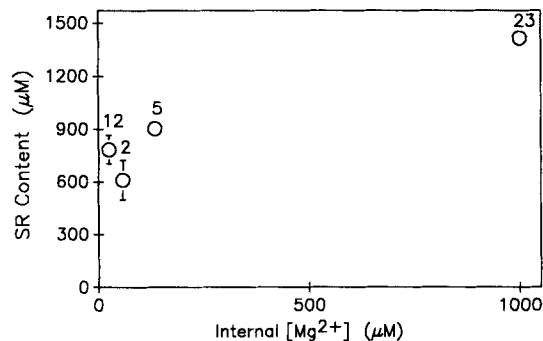

FIGURE 2. Mean value (± SEM) of the SR calcium content as a function of internal [Mg^{2+}], assuming [Mg^{2+}] in the central portion of the fiber to be the same as in the end pools. The SR content C_0 in each fiber was determined by assuming various values for C_0 and selecting that value that produced the most steady final level of R_{rel} after correction for depletion of calcium from the SR during the pulse. The number of fibers used in each set of conditions is indicated above each corresponding data point here and in all other figures presenting mean values. All records were obtained from calcium transients elicited by a 120-ms pulse to −20 mV from a holding potential of −100 mV. At 134 μM and 1 mM [Mg^{2+}] the SEM was less than or equal to the size of the point. Same fibers and pulses as in Figs. 2 and 3 of Jacquemond and Schneider (1992).

records calculated from the R^*_{rel} records in the second row. Since C_0 was larger in control than in low internal [Mg^{2+}], the control R^*_{rel}/C_0 record was relatively smaller compared with the R^*_{rel}/C_0 in low internal [Mg^{2+}] (Fig. 1, row 3) than was the case for the R^*_{rel} records not normalized to C_0 (Fig. 1, row 2).

Low Internal [Mg^{2+}] Preferentially Potentiates the Inactivatable Component of SR Calcium Release Permeability during Depolarization

The dependence of the mean values of the peak and the steady level of R^*_{rel} on the [Mg^{2+}] in the internal solution are presented in the upper plot of Fig. 3. Both values were obtained from release records corrected for depletion of calcium from the SR during the pulse. The mean peak value of R^*_{rel} was slightly larger in low internal [Mg^{2+}] than in control, but the mean value of the steady level was about the same at each internal [Mg^{2+}]. However, the SR calcium content was decreased in low internal

$[Mg^{2+}]$ compared with control, which would tend to decrease both R_{rel} and R^*_{rel} in low Mg^{2+}. To correct for differences in SR content, the rate of calcium release was systematically normalized to the initial SR calcium content determined for each fiber, giving R^*_{rel}/C_0 records that are proportional to the SR calcium permeability. The mean values of the peak and the steady level of the rate of release corrected for depletion and expressed now as R^*_{rel}/C_0 (i.e., in percent of the SR calcium content) are plotted in the lower panel of Fig. 3, again as a function of the internal $[Mg^{2+}]$. When normalized to SR content to correct for differences in driving force, both the peak and the steady level of R^*_{rel}/C_0 in low internal $[Mg^{2+}]$ were significantly larger than in 1 mM internal $[Mg^{2+}]$. However, the relative increase was considerably greater for the peak than for the steady level. For all fibers in low internal $[Mg^{2+}]$ (25, 58, and 134 μM; $n = 19$) the mean peak R^*_{rel}/C_0 was 115% larger than in control
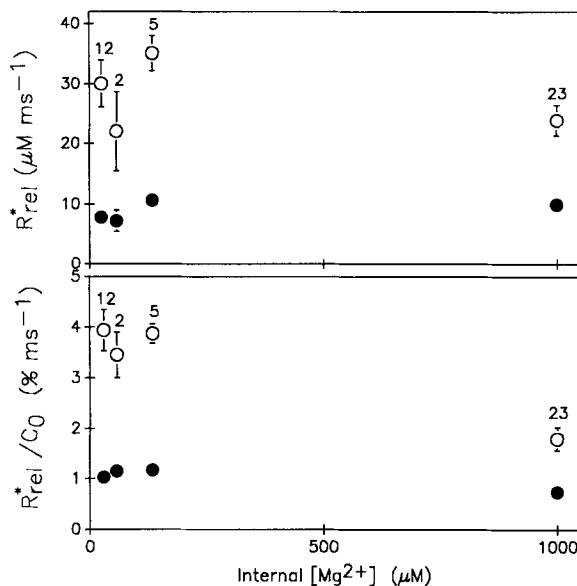

FIGURE 3. Rate of calcium release from the SR for a 120-ms pulse to -20 mV (HP $= -100$ mV) as a function of internal $[Mg^{2+}]$. *Upper plot,* mean value ($\pm$ SEM) of the peak (O) and steady level ($\bullet$) of the depletion-corrected rate of release (R^*_{rel}) versus internal $[Mg^{2+}]$. *Lower plot,* mean value ($\pm$ SEM) of the peak (O) and steady level ($\bullet$) of the depletion-corrected release expressed in percent of the SR calcium content (R^*_{rel}/C_0) versus internal $[Mg^{2+}]$. Same fibers and pulses as in Fig. 2.

($n = 23$), whereas the mean steady level was only 46% larger (all values corrected for calcium depletion and expressed relative to SR calcium content).

The early peak and rapid decline of R^*_{rel} are due to inactivation of the "inactivatable" component of release, whereas the steady level of R^*_{rel} is due to the noninactivatable component. Using the difference $P - S$ between the peak (P) and the steady level (S) of the rate of release as a measure of the inactivatable component of release, and using values of P and S from R^*_{rel}/C_0 (i.e., corrected for depletion and normalized to SR content [Fig. 3, bottom]), the inactivatable component of release was increased by 163% in low internal $[Mg^{2+}]$. This percent increase is 3.5 times larger than the 46% increase in the noninactivatable component of release determined from the increase in steady level. Since these values were obtained from R^*_{rel} normalized to SR content (Fig. 3, bottom), they represent the relative increase in

permeability of the inactivatable $(P - S)$ and noninactivatable (S) components of release in low $[Mg^{2+}]$ compared with control.

A good approximation of the ratio of the inactivatable to the noninactivatable components of R^*_{rel} is given by the expression $P/S - 1$, where P and S are both determined after correction for calcium depletion from the SR (Simon, Klein, and Schneider, 1991). Fig. 4 presents the mean ± SEM values of $P/S - 1$ for the fibers in Figs. 2 and 3. The value of $P/S - 1$ was increased by almost a factor of 3 in 25 μM internal $[Mg^{2+}]$ compared with control. Thus, lowering internal $[Mg^{2+}]$ strongly potentiated the inactivatable component of R^*_{rel} compared with the noninactivatable component. It should be noted that the value of $P/S - 1$ for each fiber is independent of any scaling of P and S to the value of the SR content since P and S would both be scaled by the same factor, which would cancel in taking the ratio P/S. The conclusion that the inactivatable component is relatively potentiated in low internal $[Mg^{2+}]$ (Fig. 4) is therefore completely independent of the normalization of P and S to SR content used in relation to the lower panel of Fig. 3.
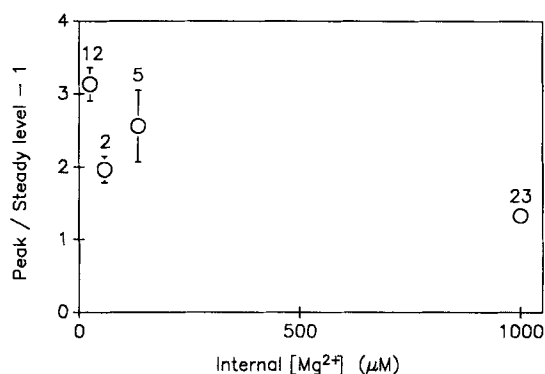

FIGURE 4. Ratio of the inactivatable to the noninactivatable components of release (= peak/steady level − 1) versus the internal $[Mg^{2+}]$ (mean values ± SEM; n indicated above each corresponding data point). All release records were corrected for depletion of calcium from the SR before determining peak/steady level. Same fibers and pulses as in Figs. 2 and 3.

Even though the inactivatable component $(P - S)$ of R^*_{rel} was relatively potentiated compared with the noninactivatable component (S) in low internal $[Mg^{2+}]$, its time course was not appreciably altered. The mean time to peak R^*_{rel} was 15.2 ± 0.6 ms for the control fibers and 15.7 ± 0.4 for all 19 fibers in low internal $[Mg^{2+}]$. From a single exponential plus constant fit to the declining phase of the depletion-corrected release (R^*_{rel}) records starting 5 ms after the time of peak R^*_{rel}, the mean rate constant for inactivation of release was found to be 159 ± 36 s^{-1} in control and 158 ± 16 s^{-1} in the 19 fibers with low internal $[Mg^{2+}]$.

Slowed Turn-off of the Calcium Release Induced by Short Depolarizing Pulses in the Presence of Low Internal Free Mg^{2+}

The preceding results indicate that the inactivatable component of R^*_{rel} was potentiated considerably more than the noninactivatable component in low internal $[Mg^{2+}]$. Recent experiments involving injection of calcium buffers into skeletal fibers have indicated that the inactivatable component of release may correspond to a calcium-induced component of calcium release (Jacquemond, Csernoch, Klein, and Schneider, 1991). In other studies using caffeine to potentiate calcium-induced calcium

release, the effects of caffeine were most apparent for short depolarizing pulses (Simon, Klein, and Schneider, 1989; Klein, Simon, and Schneider, 1990). We therefore investigated the effects of low internal $[Mg^{2+}]$ using short depolarizing pulses.

Fig. 5 shows calcium transients elicited by pulses of 5, 10, and 30 ms duration to 0 mV in a control fiber (1 mM internal free Mg^{2+}, left panel) and in a fiber equilibrated with an internal solution containing 25 μM free Mg^{2+} (right panel). The maximal amplitude of the calcium transients was about four times larger for the fiber in low internal $[Mg^{2+}]$ than for the control fiber, but this was usually not the case (Fig. 2 of
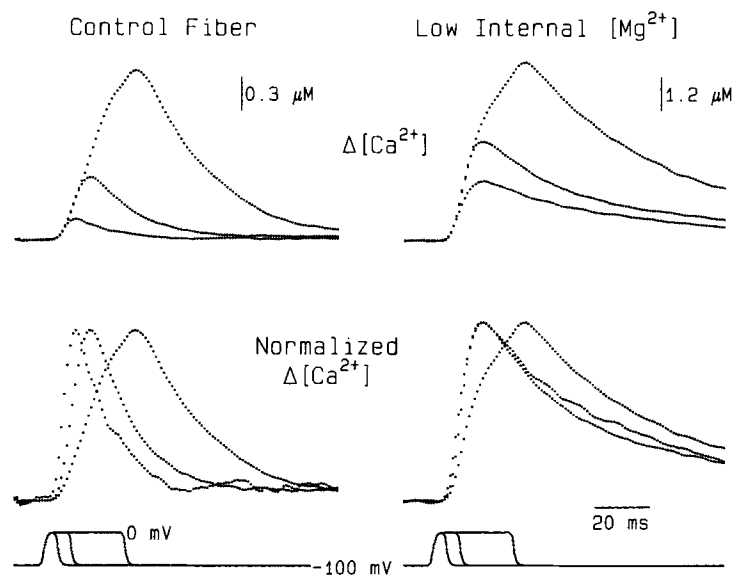

FIGURE 5.　Effect of low internal $[Mg^{2+}]$ on the time course of the calcium transients for pulses of short duration. *Upper row*, $\Delta[Ca^{2+}]$ elicited by a 5-, 10-, and 30-ms pulse to 0 mV (HP = −100 mV) in a control fiber (1 mM internal $[Mg^{2+}]$, *left records*) and in a fiber with low internal $[Mg^{2+}]$ (25 μM, *right records*). *Middle row*, same records as above normalized to the same maximum amplitude. *Third row*, membrane potential. Control: fiber 767, AP III concentration 509–518 μM, resting $[Ca^{2+}]$ 54–59 nM, fura-2 K_D 102 nM, sarcomere length 4.6 μm, 9°C. Low $[Mg^{2+}]$: fiber 759, AP III concentration 365–379 μM, resting $[Ca^{2+}]$ 50–53 nM, fura-2 K_D 47 nM, sarcomere length 4.1 μm, 9°C.

Jacquemond and Schneider, 1992). The second row of Fig. 5 shows the calcium transients normalized to the same maximal amplitude. In control (second row, left), the time to the peak of the calcium transient elicited by the 5-ms pulse was definitely shorter than in the case of the 10-ms pulse. In contrast, in low internal $[Mg^{2+}]$ (second row, right) there was no significant difference between the time to peak $\Delta[Ca^{2+}]$ for the 5- and 10-ms pulses.

Fig. 6 presents values for the time to peak $\Delta[Ca^{2+}]$ as a function of pulse duration for all sequences in each fiber in which pulses of 5, 10, and 30 ms were applied to 0 mV in control (left; 6 sequences from 3 fibers) or in low internal $[Mg^{2+}]$ (right; 11

sequences from 6 fibers). In each panel values from a single sequence in a given fiber are connected by straight lines. The diamonds represent results from different fibers in which only a single sequence was monitored in each fiber, whereas a different symbol (other than the diamond) is used to represent results from each fiber having more than one sequence. In almost all control fibers there was a definite increase in time to peak $\Delta[Ca^{2+}]$ when the pulse duration was increased from 5 to 10 ms (Fig. 6, left). In contrast, in most of the fibers in 25 μM internal $[Mg^{2+}]$ there was little or no difference in the time to peak $\Delta[Ca^{2+}]$ for the 5- and 10-ms pulses (Fig. 6, right). The mean value ($\pm$ SEM) of the difference between the time to the peak $\Delta[Ca^{2+}]$ elicited by a 10- and a 5-ms pulse ($t_{10} - t_5$) to 0 mV was 0.55 $\pm$ 0.8 ms in the presence of 25 μM internal $[Mg^{2+}]$ ($n = 11$, 6 fibers) and 4.2 $\pm$ 0.4 ms in the presence of 1 mM internal $[Mg^{2+}]$ ($n = 6$, 3 fibers). No such effect of low internal $[Mg^{2+}]$ could be detected for pulses of longer duration. For instance, for the same fibers, the mean difference between the time to peak $\Delta[Ca^{2+}]$ for a 30- and a 10-ms pulse to 0 mV

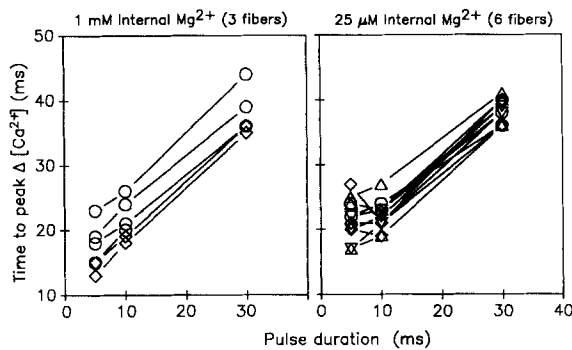FIGURE 6. Dependence of the time to peak of $\Delta[Ca^{2+}]$ on the duration of a depolarizing pulse to 0 mV in control (*left panel*, fibers with 1 mM internal $[Mg^{2+}]$) and in low internal $[Mg^{2+}]$ (*right panel*, fibers equilibrated with 25 μM Mg^{2+}). A different symbol was used for measurements done on each fiber except for the diamonds, which correspond to different fibers in which only a single sequence of short pulses was used. One determination at each pulse duration was made for each fiber represented by the diamonds. The time to peak $\Delta[Ca^{2+}]$ was measured from the start of each depolarizing pulse.

($t_{30} - t_{10}$) was 16.0 $\pm$ 0.6 ms in 25 μM internal $[Mg^{2+}]$ ($n = 11$) and 16.4 $\pm$ 0.5 in 1 mM internal $[Mg^{2+}]$ ($n = 5$). A continued rise in free calcium indicates that release must be exceeding removal. Thus, these results suggest that the release of calcium continued for a longer time after the end of a 5-ms pulse in 25 μM internal $[Mg^{2+}]$ than in the 1 mM internal $[Mg^{2+}]$.

Low Internal $[Mg^{2+}]$ Can Induce a Long-lasting Ultra-slow Transient Change in Free Calcium in Response to a Short Depolarization

Fig. 7 shows the changes in free calcium and in fura-2 percent saturation elicited by a 10-ms pulse to +20 mV in a control fiber (left panel) and by pulses of 3, 5, and 10 ms of the same amplitude in a fiber internally equilibrated with a 25 μM free Mg^{2+} solution (right panel). It must be noticed that the recordings were continued over a prolonged period of time of $\sim$14 s after the end of the pulses. The control records (left) show that, although the resting calcium was elevated (indicated by a high resting level of fura-2 saturation), the transient rise in free Ca^{2+} returned quite rapidly to its

resting level. In 25 μM internal [Mg²⁺] (right), the rise in free Ca²⁺ in response to a depolarization was clearly followed by an ultra-slow transient increase in free Ca²⁺ lasting several seconds after the end of the pulse. Surprisingly, the shortest pulse (3 ms) elicited the slowest and most prolonged slow change in free Ca²⁺. For unknown reasons these slow calcium changes were hardly reproducible from fiber to fiber. They were unquestionably observed in three fibers where they could definitely not be confused with a slow movement artifact on two grounds: (*a*) the intrinsic signal
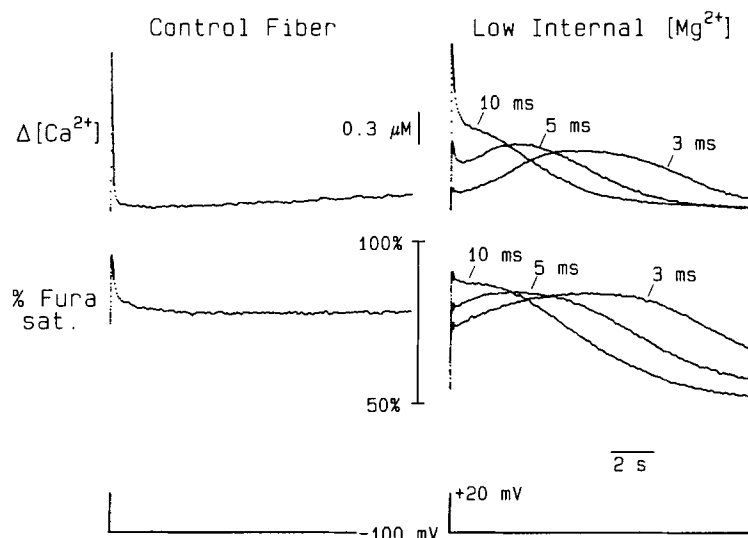

FIGURE 7. The amazing ultra-slow bumps following the calcium transient in the presence of low internal [Mg²⁺] (*right panel;* control fiber without bumps on the left). *Upper row,* Δ[Ca²⁺] from AP III; *middle row,* percent fura-2 saturation; *lower row,* membrane potential. The control fiber was stimulated with a 10-ms pulse to +20 mV (HP = −100 mV). The fiber internally equilibrated with 25 μM [Mg²⁺] was stimulated with pulses of 3, 5, and 10 ms (respectively indicated at the corresponding Δ[Ca²⁺] and fura-2 records) to +20 mV (HP = −100 mV). Records were taken 80 min after applying the internal solution at the ends in control and 84 min after applying internal solution in low [Mg²⁺]. Control: fiber 764, AP III concentration 555 μM, resting [Ca²⁺] 373 nM, fura-2 K_D 128 nM, sarcomere length 4.2 μm, 12°C. Low internal [Mg²⁺]: fiber 718, AP III concentration 480–501 μM, resting [Ca²⁺] 235–333 nM, fura-2 K_D 187 nM, sarcomere length 4.1 μm, 10°C (same fiber as low internal [Mg²⁺] fiber in Fig. 1 of Jacquemond and Schneider [1992] but records were taken later in the experiment).

recorded at 850 nm, which is usually very sensitive to the movements of the fiber (Klein, Simon, Szucs, and Schneider, 1988), did not show any sign of such slow changes (not shown); and (*b*) similar slow changes were simultaneously recorded from the fura-2 fluorescence as shown in Fig. 7, whereas the fluorescence signal is usually not distorted by the movement of the fiber (Klein et al., 1988). Such slow rises in [Ca²⁺] indicate that the SR must have continued to release calcium long after the end of these short pulses.

DISCUSSION

Previous studies using the "physiological" concentration of 1 mM internal [Mg^{2+}] have indicated that there are two components of calcium release from the SR during voltage-clamp depolarization of frog cut skeletal fibers. The R_{rel} wave form exhibits an early peak followed by a rapid decline and then a much slower phase of decline. The slow phase of decline of R_{rel} appears not to be due to any change in SR calcium channel activity but rather to depletion of calcium from the SR (Schneider et al., 1987, 1989), whereas the rapid decline is believed to be due to inactivation of the SR calcium release system (Baylor, Chandler, and Marshall, 1983; Schneider and Simon, 1988; Schneider et al., 1989). After correction for calcium depletion from the SR, the release (R^{*}_{rel}) waveform declines rapidly from its early peak to a steady level that is maintained during a 100- to 200-ms pulse. The inactivatable component (=peak − steady level) of R^{*}_{rel} is selectively abolished both by calcium buffers that eliminate the [Ca^{2+}] transient (Jacquemond et al., 1991) and by prepulses that elevate [Ca^{2+}] (Schneider et al., 1987; Schneider and Simon, 1988), leading to the conclusion that the inactivatable component may be both activated (Jacquemond et al., 1991) and inactivated (Baylor et al., 1983; Baylor and Hollingworth, 1988; Schneider and Simon, 1988; Simon et al., 1991) by Ca^{2+}. The steady (noninactivatable) component of R^{*}_{rel} during depolarization is independent of myoplasmic calcium buffering (Jacquemond et al., 1991) and remains after inactivation is complete (Schneider and Simon, 1988; Simon et al., 1991). Both the inactivatable and noninactivatable components of R^{*}_{rel} appear to activate with the same time course (Simon and Schneider, 1988). These observations have led to the hypothesis that the T-tubule voltage sensor directly controls the activation of the noninactivatable component of release and that the resulting local elevation of [Ca^{2+}] causes the activation of the inactivatable component via calcium-induced calcium release (Rios and Pizarro, 1988; Jacquemond et al., 1991).

Increased Release Activation in Low Internal [Mg^{2+}]

In the present experiments we found that in low internal [Mg^{2+}] the R_{rel} wave form exhibits the same characteristics of an early peak followed by a rapid and then a slower phase of decline as observed in control. However, after correcting for calcium depletion from the SR and normalizing to SR content the mean value of the inactivatable component (=peak − steady level) of the resulting R^{*}_{rel}/C_0 records for a 120-ms pulse to −20 mV was 163% larger in low internal [Mg^{2+}] than in control. This represents an increase of 163% in the SR permeability underlying the inactivatable component of release. In contrast, the noninactivatable (steady) component of R^{*}_{rel}/C_0 for the same pulses was only increased by 46% in low internal [Mg^{2+}] compared with control.

An increase in SR calcium release permeability as observed here in low internal [Mg^{2+}] could in principle be due to changes in either the T-tubule voltage sensor or the SR calcium release channel. Since Mg^{2+} is known to act directly at the SR calcium channel to inhibit channel opening and calcium release (Smith, Coronado, and Meissner, 1985; Meissner et al., 1986), the simplest interpretation of the present results is that lowering internal [Mg^{2+}] increased release by decreasing an inhibitory

effect of Mg^{2+} on the SR release channel. However, since we did not monitor intramembrane charge movements routinely in these experiments we do not know whether the voltage sensor was completely unaffected by changes in internal $[Mg^{2+}]$. One point that does seem clear is that a simple shift in the voltage dependence of charge movement in low internal $[Mg^{2+}]$ would not be sufficient to account for the observed increase in R_{rel}^*/C_0 in low internal $[Mg^{2+}]$. In control conditions (1 mM $[Mg^{2+}]$) the ratio of peak to steady level of R_{rel}^* is relatively independent of membrane potential for intermediate and large depolarizations (Melzer et al., 1984). In the present experiments both the peak and the steady level of R_{rel}^*/C_0 were increased in low internal $[Mg^{2+}]$, but the relative potentiation of the peak was considerably greater than that of the steady level. This is not consistent with a simple shift in voltage dependence of charge movement. Finally, since R_{rel}^* during depolarization can be potentiated to a similar extent by low concentrations of caffeine without alteration of charge movement (Klein et al., 1990), it is not unreasonable to suppose that the present results were due to effects of Mg^{2+} on the SR release channel rather than on the T-tubule voltage sensor.

The 46% larger steady release permeability in low internal $[Mg^{2+}]$ compared with control obtained from the R_{rel}^*/C_0 records (i.e., after correction for depletion and normalization to SR content) is in excellent agreement with the 42% larger steady SR permeability in low internal $[Mg^{2+}]$ compared with control obtained from the mean rate constant of the slow phase of decline of the R_{rel} records from the same fibers before correction for calcium depletion. The close agreement of these two measures of the relative increase in steady SR permeability, one based on the depletion correction of the entire R_{rel} record including both the early peak and the slow final phase of R_{rel} and the other made without the depletion correction and considering only the final phase of R_{rel}, provides further support for the interpretation that the slow phase of decline in the uncorrected R_{rel} records was indeed due to calcium depletion from the SR (Schneider et al., 1987). The agreement also tends to support the validity of our procedure of correcting R_{rel} for calcium depletion using a constant level of R_{rel}^* during the latter part of each corrected release record as the criterion for correct selection of the initial SR calcium content (Schneider et al., 1987, 1989).

Mg^{2+} Inhibition of Release Activation

The increase in release activation observed here in low internal $[Mg^{2+}]$ is probably due to removal of the inhibition that the 1 mM $[Mg^{2+}]$ in the control solution normally exerts on SR release activation under control conditions. Assuming the inhibition by Mg^{2+} to have been totally removed in the low internal $[Mg^{2+}]$ solutions used here, the relatively large (163%) increase in the mean value of the inactivatable component ($=P - S$) of R_{rel}^*/C_0 in low internal $[Mg^{2+}]$ would indicate that the inhibition due to the 1 mM internal $[Mg^{2+}]$ in the control solution decreased the inactivatable component of release permeability to 38% ($= 100\% \times 1/2.63$) of its noninhibited value. Expressed in terms of inhibition, 1 mM $[Mg^{2+}]$ produced a 62% inhibition of the inactivatable component of release permeability. If the Mg^{2+} inhibition were actually not totally removed in the low internal $[Mg^{2+}]$ solutions used here, the degree of inhibition calculated for 1 mM $[Mg^{2+}]$ would be even greater. Thus 62% should be a lower bound on the percent inhibition of the inactivatable

component of release due to 1 mM [Mg^{2+}]. This degree of inhibition would be consistent with appreciable Mg^{2+} binding at an inhibitory site for the inactivatable component in 1 mM [Mg^{2+}]. The fractional occupancy would be 62% if Mg^{2+} binding to a single site inactivated each channel, and would be even greater if binding to more than one site (Meissner et al., 1986; Klein et al., 1991) were involved in producing inhibition.

The noninactivatable component of release permeability was either 46% larger (from steady levels of depletion-corrected R^{*}_{rel}/C_0, i.e., after depletion correction and normalization to SR content) or 42% larger (from rate constants for the slow phase of decay of R_{rel} before depletion correction) in low internal [Mg^{2+}] than in control. These values indicate that 1 mM [Mg^{2+}] caused inhibition of the noninactivatable component of R^{*}_{rel}/C_0 to ≤68% (= 100% × 1/1.46) or to ≤70% (= 100% × 1/1.42) of its uninhibited value. Thus the noninactivatable component of release was inhibited by at least 32 or 30% in 1 mM [Mg^{2+}]. Similar effects of Mg^{2+} have been observed using SR vesicles fused into lipid bilayers. In bilayers containing multiple SR calcium release channels, 1 mM Mg^{2+} decreased the macroscopic Ba^{2+} current activated by 2 μM Ca^{2+} and 3.6 mM nonhydrolyzable ATP analogue to 60% of the conductance in the absence of Mg^{2+} (Smith et al., 1986), which is similar to the effects of 1 mM Mg^{2+} determined here on both the inactivatable and noninactivatable components of release.

Inhibition of the noninactivatable component by Mg^{2+} might seem to be at odds with the fact that the noninactivatable component was not inactivated by calcium. However, a minor change in the above model for control of release might account for the apparent discrepancy. The noninactivatable component of release might in fact be susceptible to inactivation by divalent cation binding to an inactivation site, but that site might have a much lower affinity for both Ca^{2+} and Mg^{2+} than the site controlling the inactivatable component of R_{rel}. In that case low internal [Mg^{2+}] might remove a small inhibition of steady release due to removal of a small amount of Mg^{2+} binding to the inactivation site for the steady component. No inactivation of the steady release would occur during the pulse if the Ca^{2+} affinity of the site were sufficiently low that significant Ca^{2+} occupancy of the site did not occur even during the [Ca^{2+}] transient. Alternatively, Mg^{2+} might inhibit release by two different mechanisms, a rapid direct channel block which reduces the apparent single channel conductance (Smith et al., 1986) and which could modulate both the noninactivatable and the inactivatable components of release, and an inhibition due to binding to a modulatory site which affects only the inactivatable component. In this case the fractional suppression of the inactivatable component of release could correspond to the product of the fractional suppression due to each mechanism of inhibition. Either of the above interpretations would explain why elevation of myoplasmic [Mg^{2+}] to 10 mM can completely abolish release during T-tubule depolarization (Lamb and Stephenson, 1991).

The present studies on voltage-clamped cut fibers complement and extend the recent studies of Lamb and Stephenson (1991) on the effects of cytosolic [Mg^{2+}] on contraction of skinned fibers with sealed T-tubules which could be depolarized by changes in the solution ionic composition. In those studies, reducing [Mg^{2+}] to 50 μM caused a contracture similar to that due to T-tubule depolarization and resulted

in complete depletion of calcium from the SR, thus eliminating the possibility of testing the effect of 50 μM [Mg^{2+}] on calcium release during depolarization (Lamb and Stephenson, 1991). In contrast, in the present studies on voltage-clamped fibers in which the T-system was not disrupted, the SR was only about half depleted of calcium in low internal [Mg^{2+}], thus permitting the detection of potentiation of release during T-tubule depolarization in low [Mg^{2+}]. In the skinned fibers contraction could be elicited simply by decreasing [Mg^{2+}] in the bathing solution (Lamb and Stephenson, 1991). Using this approach it was possible to demonstrate calcium release due to reduced [Mg^{2+}] in skinned fibers with depolarized T-tubules in which the T-tubule voltage sensor should be inactivated (Chandler, Rakowski, and Schneider, 1976), thus establishing that the Mg^{2+} effect was occurring beyond the level of the T-tubule voltage sensor (Lamb and Stephenson, 1991).

Lamb and Stephenson (1991) proposed a model for control of calcium release that could account for their observations. In their model, Mg^{2+} binding to a regulatory site would inhibit opening of the SR release channel, whereas Ca^{2+} binding to the same site would promote opening. This competition for the same site would account for the inhibitory effects of Mg^{2+} on calcium-induced calcium release. Activation by fiber depolarization would be produced by the activated T-tubule voltage sensor interacting with the SR release channel to decrease the Mg^{2+} affinity of the regulatory site. Although this model could account for the observations on calcium release in skinned fibers, which were made on a time scale of seconds or tens of seconds (Lamb and Stephenson, 1991), it remains to be established whether the model is sufficient to account for the effects on the inactivatable and noninactivatable components of release monitored in the present and other voltage-clamp studies in which release was followed during depolarization on a time scale 2–3 orders of magnitude faster than in the skinned fiber experiments.

In the context of the Lamb and Stephenson (1991) model the noninactivating component of release, which appears to be activated during depolarization by a mechanism that is independent of Ca^{2+} (Jacquemond et al., 1991), would correspond to the decreased inhibition of release due to Mg^{2+} dissociation from the regulatory site during depolarization. Mg^{2+} dissociation would result directly from the decreased Mg^{2+} affinity of the regulatory site produced by activation of the voltage sensor during depolarization. The inactivatable component of release, which requires Ca^{2+} for activation (Jacquemond et al., 1991), would correspond to increased activation of release due to Ca^{2+} binding to the regulatory site after Mg^{2+} had dissociated.

According to this formulation, the inactivatable and noninactivatable components of R_{rel} would maintain a constant ratio to each other if a constant fraction of the sites that lost Mg^{2+} during depolarization acquired Ca^{2+}. This expectation is fulfilled for intermediate and large amplitude depolarizing pulses, where activation is considerably faster than inactivation. For such pulses, the relative sizes of the inactivatable $(P - S)$ and noninactivatable (S) components are roughly constant, even though the absolute size of R_{rel} could vary several-fold for pulses of various amplitudes (Melzer et al., 1984). However, in the present experiments, the relative size of the inactivatable component compared with the noninactivatable component was clearly larger in low internal [Mg^{2+}] than in control for pulses to −20 mV. Whether this relative

potentiation of the inactivatable component in low [Mg^{2+}] can be incorporated into the Lamb and Stephenson (1991) model will require specification of Mg^{2+} and Ca^{2+} dissociation constants and quantitative testing of the inactivatable and noninactivatable components predicted by the model for several conditions of [Mg^{2+}] and various amplitude depolarizations. Alternatively, a more complicated model along the lines of that presented above may be required to account for the potentiating effect of low internal [Mg^{2+}] on both the inactivatable and the noninactivatable components of release.

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