

Surface Density of Calcium Ions and Calcium Spikes in the Barnacle Muscle Fiber Membrane

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ABSTRACT The effects of various divalent cations in the external solution upon the Ca spike of the barnacle muscle fiber membrane were studied using intracellular recording and polarizing techniques. Analysis of the maximum rate of rise of the spike potential indicates that different species of divalent cations bind the same membrane sites competitively with different dissociation constants. The overshoot of the spike potential is determined by the density of Ca (Sr) ions in the membrane sites while the threshold membrane potential for spike initiation depends on the total density of divalent cations. The order of binding among different divalent and trivalent cations is the following: La^{+++} , UO_2^{++} > Zn^{++} , Co^{++} , Fe^{++} > Mn^{++} > Ni^{++} > Ca^{++} > Mg^{++} , Sr^{++}

As described in previous papers (Hagiwara, Naka, and Chichibu, 1964; Hagiwara and Naka, 1964; Hagiwara and Nakajima, 1966 *a*) all-or-none spike potentials are found in the barnacle muscle fiber when the internal Ca^{++} concentration is reduced below a certain level. The experimental results show that these spikes are produced by the increase in permeability of the membrane to Ca ions, hence, they can be called "Ca spikes" in contrast to Na spikes, in which the permeability to Na ion plays the major role. The behavior of the overshoot of the Ca spike during the alteration of the external Ca^{++} concentration agrees with what is expected of a Ca electrode in the range of low external Ca^{++} concentrations; i.e., the change of overshoot with a tenfold change in the Ca^{++} concentration is about 29 mv. However, as the Ca^{++} concentration increases the rate of change decreases and the overshoot tends to saturate. In other words, the behavior of the overshoot shows a significant deviation from what is found in a Ca electrode at high Ca concentrations.

When the external Ca^{++} concentration is increased the various parametric membrane potentials in the current-voltage relation of excitable membranes, such as the threshold membrane potential for the spike, shift towards a more

positive membrane potential level. This effect of Ca^{++} is often called the stabilizing action (Frankenhaeuser and Hodgkin, 1957). The stabilizing action of Ca is common to both Ca and Na spikes.

Frankenhaeuser and Hodgkin (1957) have suggested that the stabilizing action of Ca ions can be explained by their adsorption at the outer edge of the fiber membrane. When Ca ions are adsorbed, their density at the membrane surface may not be proportional to the Ca ion concentration of the external solution. If the Ca ions at the membrane are important for spike initiation this may explain the discrepancy in the overshoot–Ca concentration relation from that expected in a Ca electrode.

The purpose of the present work is to analyze the overshoot of the spike, the maximum rate of rise of the spike, and the threshold membrane potential for spike initiation under various conditions. A basic assumption implicit in the analyses is that divalent cations are adsorbed on the sites at the outer edge of the membrane.

MATERIALS AND METHODS

Large specimens of *Balanus nubilus* obtained from the Pacific coast of California were used.

Preparation of the single muscle fiber and general experimental procedure were as described previously (Hagiwara and Naka, 1964). The “internal” solution used for the intracellular injection had the following composition: EGTA (ethylene glycol bis (*b*-aminoethylether)-*N,N'*-tetraacetic acid) 100 mM; methanesulfonic acid, 180 mM; KOH, 400 mM; Tris-maleate, 20 mM, and sucrose, 349 mM, and the pH was adjusted to 6.9 by an additional small amount of methanesulfonic acid. The composition of the normal barnacle saline was NaCl, 461.5 mM; KCl, 8 mM; CaCl_2 , 20 mM; MgCl_2 , 12 mM; Tris-maleate–NaOH, 10 mM, and the solution had a pH of 7.7. The composition of Ca, Sr, or Mg-saline was CaCl_2 , SrCl_2 , or MgCl_2 , 340 mM; KCl, 8 mM; Tris-maleate–NaOH, 10 mM, and the pH was also 7.7. In order to obtain solutions of desired Ca, Sr, and Mg concentration these solutions were mixed with the Ca–Mg–free solution (NaCl, 509.5 mM; KCl, 8 mM; Tris-maleate–NaOH, 10 mM) in an appropriate proportion. To obtain solutions containing CoCl_2 , MnCl_2 , NiCl_2 , LaCl_3 , FeCl_2 , ZnCl_2 , and $\text{UO}_2(\text{NO}_3)_2$ a varying amount of these salts was added to the solution. This resulted in the solution becoming slightly hypertonic but the concentration of the salt was usually small so that the change in tonicity can be neglected.

Intracellular stimuli were applied through the injection pipette while the membrane potential was recorded with a 3 M KCl-filled glass micropipette. When the potential change was recorded in the fiber which had not been treated with EGTA, a double wire electrode was introduced longitudinally through the fiber after the withdrawal of the injection pipette. Changes in the membrane potential were recorded through one wire while the stimulus was applied through the other. This was to avoid possible mechanical artefact due to contraction. The rate of rise of the

spike potential was obtained by differentiating the potential change with a circuit having a time constant of 100 μ sec.

All experiments were performed at room temperature, 22–25°C.

RESULTS

1. *Surface Ca Density and Spike Potential*

Fig. 1 shows spike potentials obtained with an EGTA-treated muscle fiber during increase of Ca concentration in the external Mg-free solution. The overshoot potentials of these spikes are plotted against the logarithm of the

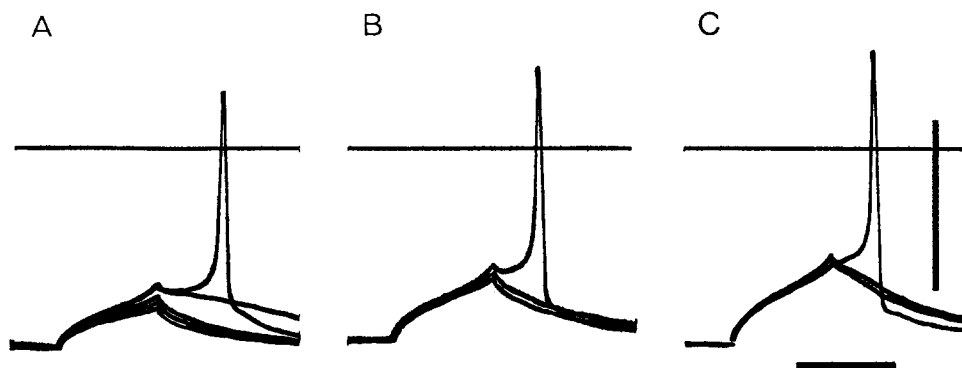


FIGURE 1. Spike potential of barnacle muscle fiber injected with EGTA solution. Ca concentration was 20, 40, and 80 mM in A, B, and C, respectively, and Mg concentration, 0. The upper trace in each record shows the reference potential level. Outward current pulses of an intensity just below or above the threshold were applied and potential changes for these pulses were superposed in each record. Voltage calibration, 50 mv, and time calibration, 50 msec.

external Ca concentration in Fig. 2. The overshoot increases with a slope of approximately 29 mv for a tenfold increase in concentration in the range of low Ca concentrations. However, the rate decreases as the concentration increases and the overshoot tends to approach a saturation level at high concentrations unlike the behavior predicted for a Ca electrode. In this experiment the CaCl_2 concentration was increased by replacing the NaCl in the solution with osmotically equivalent amounts of CaCl_2 . The increase in Ca concentration was associated with an increase in the ionic strength of the solution, which may have resulted in a change in the activity coefficient of Ca ions. Activity coefficients estimated from ionic strength are 0.207, 0.219, and 0.223 for Ca concentrations of 200, 40, and 10 mM respectively. This indicates that the linearity is not significantly improved even when the activity coefficient is taken into consideration.

Because of the high ionic strength of the solution, the activity coefficient cannot be calculated from the conventional equation derived from Debye-Hückel theory. The ionic strength was calculated for each solution and then the mean activity coefficient f_i for the CaCl_2 in the solution was found in the table (Robinson and Stokes, 1959) for the same ionic strength. The activity coefficient of Ca^{++} in the corresponding solution was taken as f_i^2 .

If it is assumed that Ca ions are adsorbed at sites at the outer edge of the fiber membrane and that the density of the sites is finite, the density of Ca^{++}

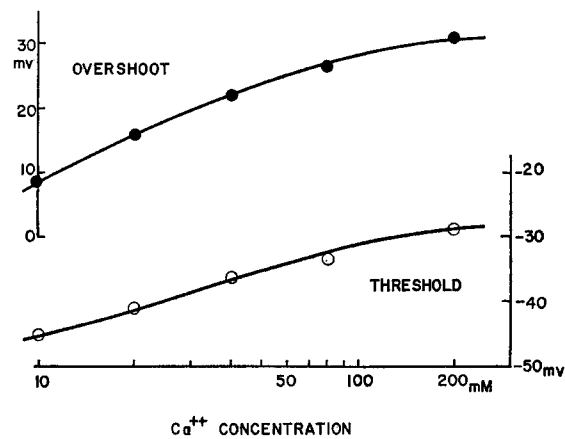


FIGURE 2. Relationships between the overshoot (filled circles) and the threshold membrane potential (open circles) of spike potential, and the external Ca concentration. The external solution contained no magnesium.

at the membrane surface in moles cm^{-2} , ρ_{Ca} , will be given by the Langmuir equation, i.e.

$$\rho_{\text{Ca}}/\rho_{\text{Ca max}} = [\text{Ca}^{++}]_{\text{out}}/([\text{Ca}^{++}]_{\text{out}} + k_{\text{Ca}}) \quad (1)$$

Here $\rho_{\text{Ca max}}$ represents the maximum surface density of Ca^{++} which can be achieved when $[\text{Ca}^{++}]_{\text{out}}$ approaches infinity and k_{Ca} is the dissociation constant of Ca ions at the site. When this membrane undergoes an excitation the rate of Ca^{++} entry through the membrane should be proportional to ρ_{Ca} if other conditions, especially the membrane potential, are constant. In the present experiment the maximum rate of rise of the spike potential was taken as a measure of the rate of Ca^{++} entry since it should be proportional to the inward membrane current at the time when the rate becomes maximal. The membrane potential at the maximum rate of rise was shifted in the positive direction with increasing $[\text{Ca}^{++}]_{\text{out}}$ and the shift paralleled the threshold membrane potential for spike initiation. The relation between the threshold

membrane potential and the external Ca concentration obtained in Mg-free media is shown in the lower part of Fig. 2. The result indicates that the membrane potential at the maximum rate of rise of spike potential significantly changes with external Ca concentration. Therefore the maximum rate of rise should not be proportional to ρ_{Ca} under this condition. In order to find a condition in which the membrane potential at the maximum rate of rise is independent of $[Ca^{++}]_{out}$ the effect of magnesium upon the threshold was studied. When $MgCl_2$ was added to the external solution by replacing an osmotically equivalent amount of $NaCl$ in the solution the threshold membrane potential as well as the membrane potential at the maximum rate of rise shifted in the positive direction even when the Ca^{++} concentration was unaltered. The change of the threshold membrane potential by Mg ions increased with increasing Mg concentration at a given Ca concentration and

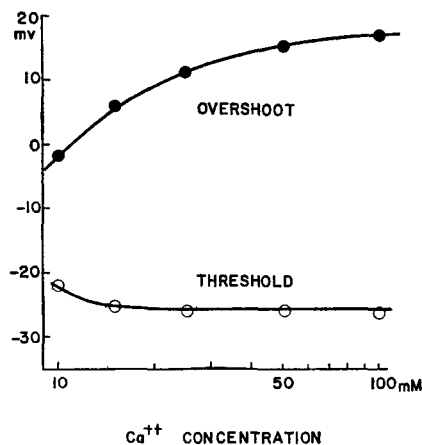


FIGURE 3. Relationships between the overshoot (filled circles) and the threshold membrane potential (open circles) of spike potential, and the external Ca concentration. The external solution contained 100 mM Mg throughout.

at a given Mg concentration it increased with decreasing Ca concentration. The threshold membrane potential was, therefore, less dependent on $[Ca^{++}]_{out}$ when the latter was altered in Mg media. The relation between the Ca concentration and the threshold membrane potential in a media containing 100 mM $MgCl_2$ is shown in Fig. 3. Fig. 3 also shows the relation between the overshoot and the Ca concentration. In the presence of this Mg^{++} concentration the threshold membrane potential is fairly constant in the range of 10–100 mM Ca concentration. This, therefore, constitutes a favorable experimental condition in which the maximum rate of rise should be proportional to ρ_{Ca} .

For the adsorption of ions the dissociation constant k in equation (1) becomes

$$k = k' \exp\left(\frac{zF\psi}{RT} - \frac{w}{RT}\right) \quad (\text{Stern, 1924; Grahame, 1947})$$

Here R , T , and F have the usual meaning, z is the valency of ion, ψ the potential of the membrane surface, taking the reference in the interior of the external bathing solution, and w , the chemical work necessary to remove 1 mole of adsorbed ions from the surface to the interior of the solution. Although there is no way at present to measure ψ directly it seems reasonable to assume that the change of ψ is parallel to the change of threshold membrane potential (see Discussion). Since under the present experimental condition the threshold membrane potential was kept constant regardless of the concentration of Ca ions, ψ is likely to be independent of the external Ca^{++} concentration. In other words k can be considered as constant regardless of the Ca^{++} concentration.

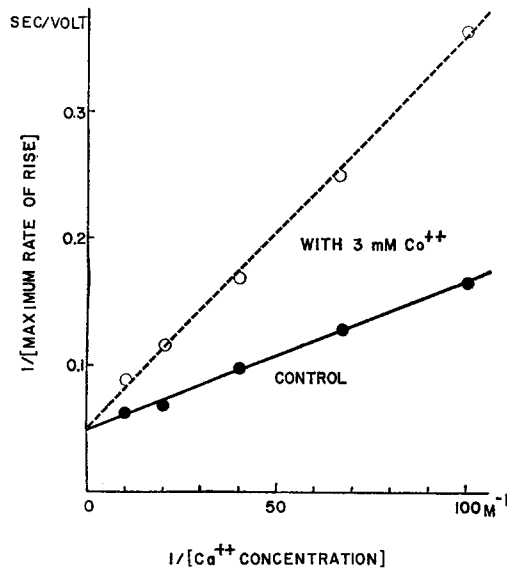


FIGURE 4. Demonstration of competitive type of inhibition of Co ions upon the Ca spike. Two sets of plots were obtained from the same fiber with (open circles) and without (filled circles) 3 mM CoCl_2 .

If it is assumed that the maximum rate of rise, T , is proportional to ρ_{Ca} equation (1) becomes

$$T/T_{\text{max}} = [\text{Ca}^{++}]_{\text{out}} / ([\text{Ca}^{++}]_{\text{out}} + k_{\text{Ca}}^*) \quad (2)$$

k_{Ca}^* and T_{max} being the dissociation constant of Ca^{++} to the membrane site in the presence of 100 mM MgCl_2 and T for $\rho_{\text{Ca max}}$ respectively. Theoretically in the equations the activity of Ca ion should be taken instead of concentration. Since each solution contains 100 mM MgCl_2 , the change in Ca concentration does not cause a great change in the ionic strength. A change in the Ca concentration from 10 to 100 mM is associated with a change in the ionic strength from 0.69 to 0.83 M. The activity coefficient of Ca ion is relatively insensitive to a change in ionic strength in this range. Calculations indicate that the change is less than 4%. For this reason the concentration of Ca ions was used instead of the activity in the present analysis. Equation (2) can also be written as

$$T_{\max}/T = 1 + k_{Ca}^*/[Ca^{++}]_{out} \quad (3)$$

Equation (3) predicts the linearity between $1/T$ and $1/[Ca^{++}]_{out}$. The experimental results are shown in Fig. 4 (filled circles) in a plot of $1/T$ against $1/[Ca^{++}]_{out}$, which approximates a single straight line reasonably well. Sometimes the observed rate of rise at 10 mM Ca was significantly smaller than that expected from the linear relation. This may reflect the fact that sometimes the threshold membrane potential level for the spike becomes more positive at 10 mM Ca than at higher Ca concentrations in the presence of 100 mM $MgCl_2$ (Fig. 3). The dissociation constant, k_{Ca}^* was estimated in six different fibers and it ranged between 25 and 40 mM.

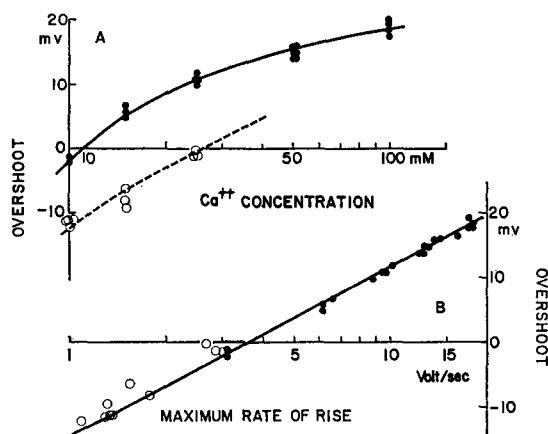


FIGURE 5. Relationship between the overshoot of the spike and the external Ca concentration (above) and between the overshoot and the maximum rate of rise of the spike potential (below). Open and filled circles were obtained from the same fiber with and without 3 mM $CoCl_2$, respectively.

The plots of the spike overshoot vs. $\log [Ca^{++}]_{out}$ obtained in 100 mM Mg media are shown in Figs. 3 and 5A. The relationship deviates significantly from the straight line expected in a Ca electrode. If the overshoot is determined by the density of Ca ions on the membrane surface rather than the concentration in the external solution itself the plots of the overshoot vs. $\log T$ should give the straight line expected in a Ca electrode. Fig. 5B shows that this is the case. The slope for the experiment illustrated in Fig. 5 is 27 mv for a tenfold increase in T . Similar results were obtained with four other fibers and the slope ranged between 26 and 33 mv. These values of the slope agree with the theoretical value of 29 mv within the range of experimental error.

2. Competitive Inhibition of Ca Spike by Divalent Transition Metal Ions

As was shown in a previous paper (Hagiwara and Nakajima, 1966 *b*) the Ca spike is suppressed by a small amount of Mn^{++} applied externally. In the

present study this was extended to other divalent transition metal ions such as Co^{++} and Ni^{++} , and it was found that they all *reversibly* suppress the Ca spike. In the preceding paper it was suggested that these transition metal ions suppress the Ca spike by competitively occupying the membrane sites which are normally occupied by Ca ions. If this is the case, it can be shown that the equation becomes

$$T_{\max}/T = 1 + (1 + [M^{++}]_{\text{out}}/k_M^*)k_{\text{Ca}}^*/[Ca^{++}]_{\text{out}} \quad (4)$$

k_M^* being the dissociation constant of the transition metal ion, M^{++} , to the membrane site in the presence of 100 mM Mg. Filled circles in Fig. 4 show the relation between $1/T$ and $1/[Ca^{++}]_{\text{out}}$ obtained without inhibitor. As described before this gives a straight line. Open circles in the same figure were obtained from the same muscle fiber with 3 mM CoCl_2 . The latter plot also approximates a straight line and the two straight lines obtained with and without inhibitor intersect the Y axis approximately at the same point. In other words, the experimental result satisfies equation (4), and therefore the transition metal ions can be considered as inhibitors of the competitive type.

The open circles in Fig. 5 A show the relation between the overshoot of the spike and the external Ca concentration in the presence of 3 mM Co while the filled circles represent the overshoot of the same fiber obtained in the absence of cobalt. With the presence of the inhibitor a smaller overshoot and a lower value for the maximum rate of rise were found at any given Ca concentration. These overshoots were replotted against the logarithm of the maximum rate of rise in Fig. 5 B, in which it can be seen that the overshoots obtained without (filled circles) and with inhibitor (open circles) fall on the same straight line. This evidence can be interpreted to mean that the suppression is exclusively due to the competitive occupation of the same membrane sites by Ca and inhibitor ions, resulting in a reduction of the Ca^{++} density at the surface.

3. *Suppression of Ca Spike by Mg^{++}*

In the experiments described above the external solution always contained 100 mM MgCl_2 . If Mg^{++} has a suppressing effect on Ca spike the dissociation constant k_{Ca}^* in 100 mM Mg media should be different from the k_{Ca}^* to be found in Mg-free media. In order to examine the effect of Mg^{++} , the maximum rate of rise of the spike was measured during the increase of Mg concentration. The Ca concentration was kept at 42 mM throughout this experiment. A decrease in the maximum rate of rise was always associated with increasing Mg concentrations. In Fig. 6 (lower) $1/T$ was plotted against $[\text{Mg}^{++}]_{\text{out}}$ and this gives a reasonably linear relationship which is predicted from the competitive type inhibition (equation 4). Further evidence of the competitive inhibition of Mg is shown on the Sr spike, as will be described below. The result, therefore,

shows that Mg^{++} also behaves like transition metal ions although it has a much lower affinity for the membrane sites. The k_{Ca}^* obtained in 100 mM Mg media should be given by

$$k_{Ca}^* = (1 + 100 \text{ mM}/k_{Mg})k_{Ca} \quad (5)$$

As will be described later, the ratio between $k_{Ca}:k_{Mg}$ is found to be 1:3 ~ 4. If this ratio is taken k_{Ca}^* of 25 ~ 40 mM indicates that k_{Ca} is smaller than 15 mM.

As mentioned above the overshoot of the spike decreases with increasing external Mg concentration. If this is the result of reduced density of Ca ions on the membrane surface due to the competitive inhibition of Mg, the plots of

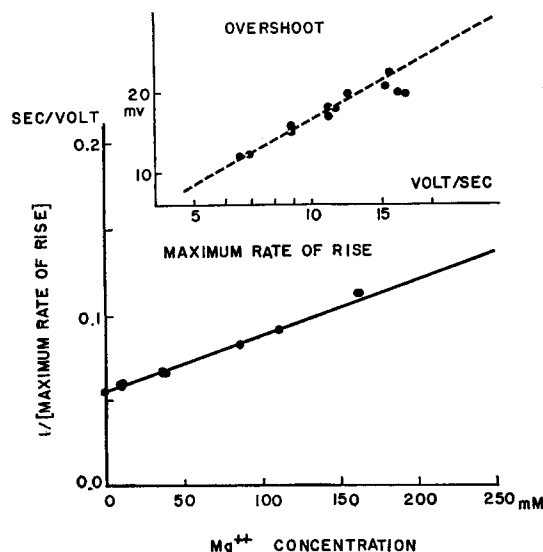


FIGURE 6. Relationship between the overshoot of the spike and the maximum rate of rise of the spike (above) and between the reciprocal of the maximum rate of rise and the Mg concentration (below). The two sets of relationships were obtained from the same experiment in which the Mg concentration was altered by keeping the Ca concentration at 42 mM throughout.

the overshoot vs. $\log T$ should give a straight line with a slope of about 29 mv for a tenfold change in T . Fig. 6 (upper) shows that this is the case.

4. Comparison of Effects among Different Metal Ions

Since Mg^{++} also occupies the site competitively equation (4) becomes

$$T_{\max}/T = 1 + (1 + [Mg^{++}]_{\text{out}}/k_{Mg} + [M^{++}]_{\text{out}}/k_M)k_{Ca}/[Ca^{++}]_{\text{out}}$$

or

$$T'/T = 1 + [M^{++}]_{\text{out}}/k'_M \quad (6)$$

Here T' is the maximum rate of rise in the absence of inhibitor ion, M^{++} , and

$$k'_M = k_M([Ca^{++}]_{\text{out}}/k_{Ca} + [Mg^{++}]_{\text{out}}/k_{Mg} + 1)$$

When the inhibitor concentration is varied at given Ca and Mg concentrations T'/T should change linearly with inhibitor concentration and the slope should be equal to $1/k'_M$. Fig. 7 shows the experimental relations obtained with various transition metal ions with the same fiber at 42 mM Ca and 12 mM Mg. The relationships are satisfactorily linear at inhibitor concentrations below 20 mM. The ratio, $k_{Co}:k_{Mn}:k_{Ni}:k_{Mg}$, estimated from the slopes of these relationships was 1.0:2.5:3.7:46.3. Somewhat different ratios were found in other fibers but the order of k 's was always consistently $k_{Mg} \gg k_{Ni} > k_{Mn} > k_{Co}$. As will be shown later k'_{Ca} defined by equation (9) can be obtained experimentally. By combining observed k'_{Mg} and k'_{Ca} 3 ~ 4 was found for the ratio k'_{Mg}/k'_{Ca} or k_{Mg}/k_{Ca} . This indicates that k_{Ca} is smaller than k_{Mg} but larger than k_{Ni} .

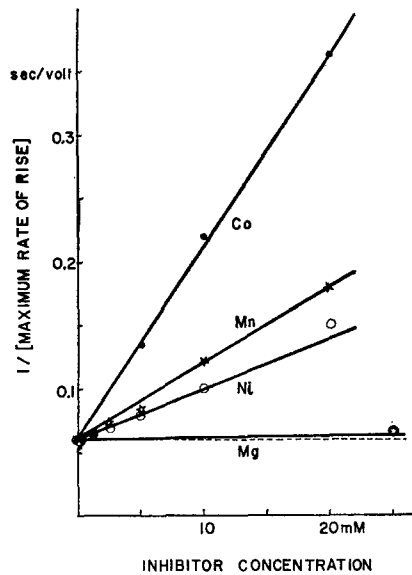


FIGURE 7. Comparison of suppressing effect of different inhibitor species. Ca and Mg concentrations were kept at 42 and 12 mM respectively. For Mg suppression the concentration shown on the abscissa indicates the actual external Mg concentration minus 12 mM.

Suppressions by some other ion species such as Fe^{++} , UO_2^{++} , Zn^{++} , and La^{+++} were also examined although no experiments were performed to determine whether or not they show a competitive type inhibition. UO_2^{++} and La^{+++} suppressed the spike potential completely at concentrations of 2 and 1 mM respectively when the Ca concentration was 42 mM. The effect was often irreversible for these ions. The effect of Fe^{++} and Zn^{++} was always reversible. If these ions are included, the order of binding becomes La^{+++} , $UO_2^{++} > Zn^{++}$, Co^{++} , $Fe^{++} > Mn^{++} > Ni^{++} > Ca^{++} > Mg^{++}$.

5. Sr Spike

The order of binding on the membrane among different divalent cation species is very similar to that found for the binding of the same species to EGTA. This

may indicate the possibility that the observed effect of divalent cations is an artefact of EGTA injection. In order to eliminate this possibility the observation was made with fibers injected with EGTA-free K methanesulfonate internal solution. The purpose of the injection was to raise the internal K concentration to a level comparable to that of the EGTA-treated fiber, and also to reduce the mechanical artefact for recording since the injection usually reduced contraction associated with a depolarization in those fibers. Since the internal solution did not contain any Ca^{++} -binding agent the membrane

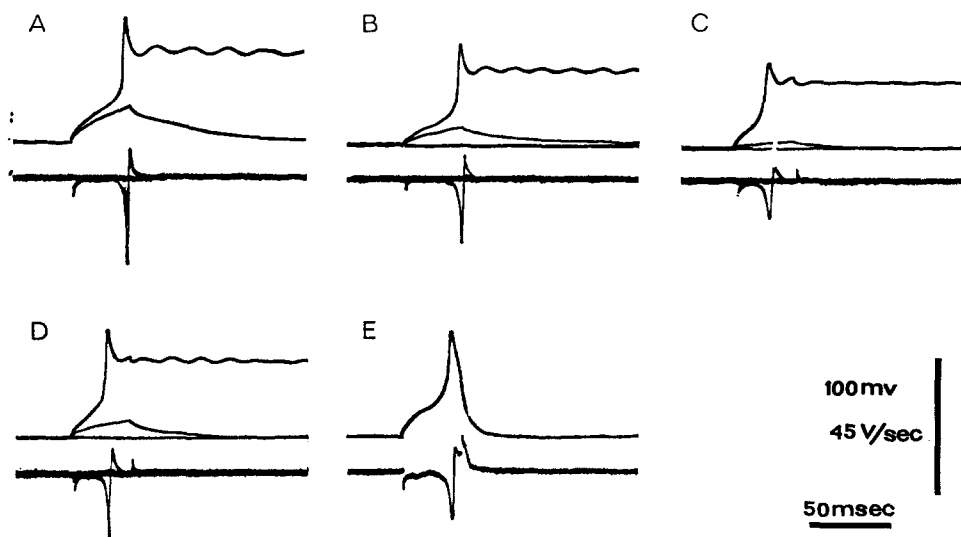


FIGURE 8. Spike potentials in Sr media. The fiber was injected with EGTA-free K methanesulfonate solution. The Sr concentration was 100 mM for A; 25 mM, B; 10 mM, C, and 50 mM, D and E. The lower trace of each record shows the derivative of the potential change. The external solution contained neither calcium nor magnesium in A-D. D and E were obtained before and after adding 10 mM of calcium to the external solution. The calcium reversibly suppressed the maximum rate of rise of the spike and the time course of the spike became shorter. All records were taken from the same fiber.

of the fiber was no longer capable of producing all-or-none Ca spikes. However, as described in a previous paper (Hagiwara and Naka, 1964) the membrane became capable of initiating all-or-none spikes in Sr media and therefore the analyses were done with Sr spikes instead of Ca spikes.

The spike potentials shown in Fig. 8 A, B, C were obtained from a fiber when the external Sr concentration was 100, 25, and 10 mM respectively and Sr was the only divalent cation in the solution. The reciprocal of the maximum rate of rise of the Sr spike, $1/T$, was plotted against $1/[\text{Sr}^{++}]_{\text{out}}$ in Fig. 9 (filled circles). The plot approximates a straight line and k_{Sr} estimated from this relationship is about 20 mM. In two other cases examined k_{Sr} was 25 and 30 mM

respectively. When a similar experiment was performed in the presence of Mg ions a competitive type of suppression was seen. Competitive suppression was also found with divalent transition metal ions such as Mn^{++} . The ratio among dissociation constants of different inhibitor species obtained in this experiment was similar to that found for the Ca spike of the EGTA-treated fiber. This indicates that the EGTA treatment is not essential to the binding of divalent cations to the membrane.

When the fiber is not injected with EGTA, the membrane is no longer permeable to Ca ions but they may occupy the membrane sites in such a fiber equally well. If this is the case, Ca should act as a competitive inhibitor for the Sr spike. This was, in fact, found when Ca ions were added to Sr media. Spike potentials in Fig. 8 D and E were obtained with the same fiber before and after adding 10 mM $CaCl_2$ in 50 mM Sr saline. Ca ions reversibly reduced the maximum rate of rise. This was associated with a change of time course of the

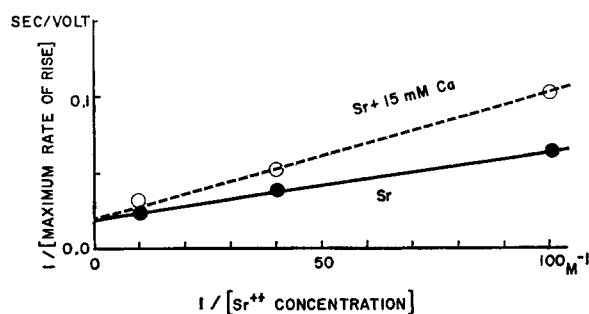


FIGURE 9. Demonstration of competitive type of suppression of calcium on Sr spike. Two sets of plots were obtained from the same fiber with (open circles) and without (filled circles) 15 mM Ca.

spike potential; i.e., the plateau phase of the Sr spike became much shorter or disappeared in the presence of 10 mM Ca ions. A similar change of time course was observed during the suppression by other inhibitor ions. The open circles in Fig. 9 show the relationship obtained when the solution contained 15 mM $CaCl_2$, and the filled circles, the results from the same fiber without $CaCl_2$. In this case the result fulfills the requirements for competitive inhibition. However, there were also cases in which the $1/T - 1/[Sr^{++}]_{out}$ relation in the presence of Ca^{++} showed a slight deviation from linearity. Although at the present stage no clear explanation has been found for this deviation, it may be due to the fact that in some fibers, even when they have not been treated with EGTA, the membrane is slightly permeable to Ca ions.

6. Threshold Membrane Potential

The foregoing results suggest that various divalent cations are adsorbed at the outer edge of the membrane with different binding constants. The threshold

membrane potential is assumed to be linearly related to the total density (in moles cm^{-2}) of divalent cations adsorbed at the membrane surface. If V represents the threshold membrane potential measured from the level found in the absence of any divalent cation species, V will be proportional to the density. However, V cannot be observed experimentally since no spike potential is obtained in the absence of divalent cations. In the present analysis the threshold membrane potential obtained at 42 mM Ca, 12 mM Mg, and no other divalent cation species was taken as the standard and the difference of the threshold potential, ΔV , from this potential level was observed when the concentration of various species of inhibitor ions (M^{++}) was altered without changing the Ca and Mg concentrations. This experimental condition is similar to the one used for comparing the effects of different inhibitor species (Fig. 7). Then ΔV is given by

$$\begin{aligned} \frac{\Delta V}{\Delta V_{\max}} &= \frac{\frac{[M^{++}]_{\text{out}}}{k_M}}{\frac{[M^{++}]_{\text{out}}}{k_M} + \left(\frac{[Ca^{++}]_{\text{out}}}{k_{Ca}} + \frac{[Mg^{++}]_{\text{out}}}{k_{Mg}} + 1 \right)} \\ &= \frac{\frac{[M^{++}]_{\text{out}}}{k'_M}}{\frac{[M^{++}]_{\text{out}}}{k'_M} + 1} \end{aligned} \quad (7)$$

Here $k'_M = k_M \left(\frac{42 \text{ mM}}{k_{Ca}} + \frac{12 \text{ mM}}{k_{Mg}} + 1 \right)$; i.e., k'_M represents the dissociation constant of M^{++} in the presence of 42 mM Ca and 12 mM Mg. Equation (7) indicates that the relationship between ΔV and $[M^{++}]_{\text{out}}/k'_M$ is independent of inhibitor ion species. In Fig. 10 the observed ΔV is plotted against $[M^{++}]_{\text{out}}/k'_M$ for Co^{++} , Mn^{++} , Ni^{++} , and Mg^{++} . All measurements were done with the same fiber and the dissociation constants, k'_M 's, were obtained from the measurement of the maximum rate of rise as described previously. For the effect of Mg^{++} , $[Mg^{++}]_{\text{out}}$ represents the actual external Mg concentration minus 12 mM. Fig. 10 shows that the relationships for Co^{++} , Mn^{++} , and Ni^{++} are practically identical. The common curve illustrated by a solid line was drawn to obtain the best fit of equation (7). The experimental results follow equation (7) reasonably well. The above evidence strongly suggests that the shift of the threshold membrane potential is linearly related to the total density of divalent cations adsorbed at the membrane surface.

The relationship obtained with Mg shows a significant deviation from the common solid curve drawn for transition metal ions. The threshold membrane potential in Mg media was more positive than that found for other inhibitors even when the binding was the same. One of the factors which may relate to

this discrepancy is the ionic strength of the external solution. Since the dissociation constant, k'_M , is small for Co^{++} , Mn^{++} , and Ni^{++} the ionic strength does not increase appreciably during the increase of binding found in the present experiment. In contrast to this, the dissociation constant of Mg^{++} is much larger than those of other inhibitor species and therefore, the ionic strength increases very rapidly with the increase of binding. At $[\text{M}^{++}]_{\text{out}}/k'_M = 1$ the ionic strength of the Mg solution was 0.877M while the corresponding figures for Co , Mn , and Ni solutions were 0.620, 0.627, and 0.634 M, respectively. At the present stage however, there is no evidence that the discrepancy is exclusively due to high ionic strength. The possible effect of the ionic strength upon the threshold membrane potential will be discussed later.

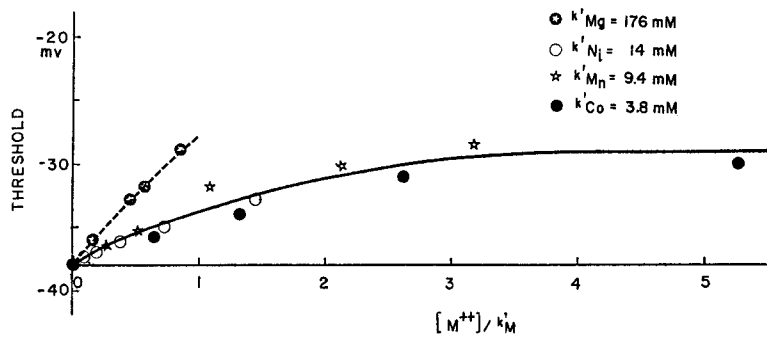


FIGURE 10. Threshold membrane potential for the spike potential at different concentrations of various inhibitor ions. All data were obtained from the same fiber and the Ca and Mg concentrations were kept at 42 and 12 mM throughout. Mg concentration used for the abscissa represents the actual external Mg concentration minus 12 mM. The dissociation constants, k'_M , for each inhibitor species were obtained from the experiments on maximum rate of rise.

In the above experiment the shift of the threshold membrane potential was analyzed when the inhibitor concentration was increased without altering the Ca^{++} concentration. As already described a similar shift was also observed when the Ca^{++} concentration was increased without altering the concentrations of other divalent cation species. In this experiment the threshold membrane potential at 42 mM Ca and 12 mM Mg was also taken as the standard and the difference of the threshold potential, ΔV from this potential level was observed during the increase of Ca^{++} concentration above 42 mM, the Mg^{++} concentration being kept at 12 mM throughout. In this case ΔV should be given by an equation similar to (7), i.e.

$$\frac{\Delta V}{\Delta V_{\text{max}}} = \frac{\frac{\Delta[\text{Ca}^{++}]}{k'_{\text{Ca}}}}{\frac{\Delta[\text{Ca}^{++}]}{k'_{\text{Ca}}} + 1} \quad (8)$$

Here,

$$\begin{aligned} \Delta[\text{Ca}^{++}] &= [\text{Ca}^{++}]_{\text{out}} - 42 \text{ mM} \quad \text{and} \\ k'_{\text{Ca}} &= k_{\text{Ca}}(42 \text{ mM}/k_{\text{Ca}} + 12 \text{ mM}/k_{\text{Mg}} + 1) \end{aligned} \quad (9)$$

First k'_{Ca} was obtained from the maximum rate of rise observed with the same fiber and then ΔV was plotted against $\Delta[\text{Ca}^{++}]/k'_{\text{Ca}}$ in Fig. 11. The points fit reasonably well the solid curve drawn to obtain the best fit of equation (8). The dissociation constant k'_{Ca} was 60 mM for the case shown in Fig. 11 and similar values were obtained in the few other cases observed. Since k'_{Ca} is not small compared with the divalent cation concentration (64 mM)

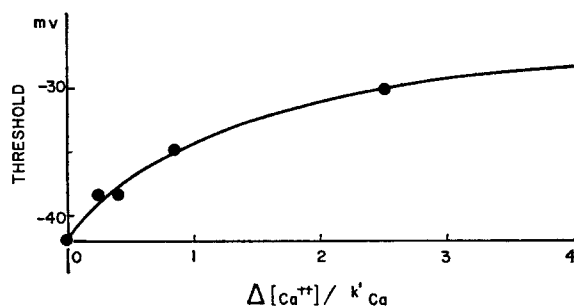


FIGURE 11. Threshold membrane potential for the spike and the external Ca concentration. $\Delta[\text{Ca}^{++}] = [\text{Ca}^{++}]_{\text{out}} - 42 \text{ mM}$. The Mg concentration was kept at 12 mM throughout.

under the standard condition, the increase in the ionic strength associated with the increase in Ca^{++} concentration may not be negligible. The curve for Ca shown in Fig. 11, however, does not seem to be very different from the curve for the transition metal ions shown in Fig. 10. In the former the positive shift of the threshold potential is associated with the increasing surface density of Ca ions whereas it is associated with the decrease of the Ca^{++} density in the latter. This indicates that the threshold membrane potential is related to the total surface density of the divalent cations and that Ca ions are not distinguished from other divalent cations. In contrast to this the overshoot of the spike depends exclusively on the surface density of Ca ions.

The dissociation constant, k'_{Ca} , was obtained in the following way. The maximum rate of rise, T , was observed at each concentration of Ca and then $\Delta T = T - T_s$ was obtained, T_s being T in the standard condition. Then ΔT will be given by an equation similar to equation (8)

$$\frac{\Delta T_{\text{max}}}{\Delta T} = 1 + \frac{k'_{\text{Ca}}}{\Delta[\text{Ca}^{++}]} \quad (10)$$

This indicates a linear relation between $1/\Delta T$ and $1/\Delta[\text{Ca}^{++}]$. The experimental data usually show a slight tendency to deviate from a straight line. This is very likely due to the shift of the membrane potential at which the rate becomes maximum. Therefore, k'_{Ca} was estimated in the range of smaller $\Delta[\text{Ca}^{++}]$.

DISCUSSION

The results obtained for the maximum rate of rise suggest that divalent cations are adsorbed at the outer surface of the membrane. Different species of cations are bound competitively to the same membrane sites with different binding constants. The Ca ions bound at the membrane surface seem to be in an equilibrium with those in the external solution. If this equilibrium condition is maintained during the increase of membrane permeability to Ca, the membrane potential should approach the Ca equilibrium potential which is determined by the Ca ion concentration in the solution. This potential can be independent of the Ca ion density at the membrane surface. The behavior of the overshoot of the Ca spike, however, shows a significant deviation from the equilibrium potential calculated from the Ca ion concentration in the solution. The experimental results always show that the overshoot is related to the Ca^{++} concentration at the membrane surface in the manner which is found in a Ca electrode. In the present paper, however, the discussion will be based only upon this experimental fact and further theoretical interpretations of the fact will be left for the future. When the surface Ca^{++} density is the determining factor, the overshoot of the Ca spike, V_a , will be given by,

$$V_a = \frac{RT}{2F} \ln \left(\frac{[\text{Ca}^{++}]_{\text{out}}}{k + [\text{Ca}^{++}]_{\text{out}}} \right) + \text{constant} \quad (11)$$

Where $[\text{Ca}^{++}]_{\text{out}}$ is the in bulk Ca ion concentration of the solution and k does not always represent k_{Ca} since it varies in different conditions; e.g., it becomes $(1 + 100 \text{ mM}/k_{\text{Mg}})k_{\text{Ca}}$ if 100 mM Mg is present. For $[\text{Ca}^{++}]_{\text{out}} \ll k$, the equation becomes

$$V_a = \frac{RT}{2F} \ln [\text{Ca}^{++}]_{\text{out}} + \text{constant} \quad (12)$$

Under this condition V_a varies linearly with the logarithm of $[\text{Ca}^{++}]_{\text{out}}$. The conditions for equation (12) can be found when the Ca spike is suppressed by inhibitor ions. When the inhibitor ions are added in the Ca solution, k increases from k_{Ca} to $(1 + [\text{M}^{++}]_{\text{out}}/k_{\text{M}})k_{\text{Ca}}$ and therefore, it may become large compared with $[\text{Ca}^{++}]_{\text{out}}$ in a wider range of Ca^{++} concentrations (see Fig. 5 A).

The shift of the threshold membrane potential with increasing external

divalent cation concentration is a part of the general shift of the parametric membrane potentials in the current-voltage relation (stabilizing action, Frankenhaeuser and Hodgkin, 1957). This is found for the Ca spike as well as for the Na spike. The surface of the membrane probably has a negative fixed charge coming from the phospholipid or protein molecules (Ling and Ochsenfeld, 1965). Divalent cations are adsorbed at this surface and form a compact electric double layer. When the densities of the fixed charge and the charge of adsorbed cations are δ_1 and δ_2 (sign included) respectively, there is a diffuse double layer of net charge density $\delta_3 = \delta_1 + \delta_2$. This results in a potential difference, ψ , from the interior of the solution to the surface of the membrane (more precisely, just outside the layer of adsorbed ions). Since the resting membrane is permeable to monovalent ions such as K^+ and Cl^- , these charges do not contribute to the over-all resting potential observed between a pair of electrodes, one inside and the other outside the fiber. If the spike potential is initiated when the potential difference across the membrane itself attains a certain fixed value, V_o (reference; outside), regardless of the concentration of external divalent cations the threshold membrane potential observed with this pair of electrodes should become $V_o + \psi$. The change of threshold membrane potential is, therefore, linearly related to the density of the adsorbed divalent cations if ψ is proportional to δ_3 . The experimental results for threshold values approximately follow the prediction given by the above idea. However, ψ is expected to be proportional to δ_3 only when it is smaller than 12.5 mv and the ionic strength of the solution is constant. In the present work the analysis was made for cases in which ψ does not seem to be much larger than 12.5 mv and the change in the ionic strength is almost negligible.

Theoretically ψ is not a simple function of δ_3 even when calculated under certain simplified conditions, i.e.

$$\delta_3^2 = DRT \sum_i C_i \left(\exp \frac{-z_i F \psi}{RT} - 1 \right) \quad (13) \text{ (Grahame, 1947)}$$

Here R , T , and F have the usual meanings, C_i , the concentration of i th species of ions in the interior of the solution; z_i its valency, therefore in the present case, $+1$, -1 or $+2$ and D , a constant relating to the dielectric constant of water. When ψ is smaller than 12.5 mv, ψ will be approximately given by

$$\psi = \frac{R^{1/2} T^{1/2} \delta_3}{D^{1/2} F I^{1/2}} \quad (14)$$

I being the ionic strength of the solution.

The experimental results have been discussed in terms of adsorption of divalent cations to limited membrane sites. However, it must be mentioned

that the experimental results obtained with the maximum rate of rise can be explained equally well by assuming competitive binding between divalent cations and Ca^{++} (or Sr^{++} , Ba^{++}) carrier molecules at the membrane. The idea of adsorption was adopted in this paper since it seems to explain the shift of the threshold membrane potential on the same basis.

The order of stabilities of different divalent or trivalent cations to the membrane site in the barnacle fiber is La^{+++} , $\text{UO}_2^{++} \gg \text{Zn}^{++}$, Co^{++} , $\text{Fe}^{++} > \text{Mn}^{++} > \text{Ni}^{++} > \text{Ca}^{++} > \text{Mg}^{++}$, Sr^{++} . This order is very similar to that found with many chelating agents such as oxalate EGTA, etc. Hafemann and Miller (1966) and Blaustein and Goldman (1966) studied the stabilizing action of different cations in the membrane of a lobster giant axon and found a similar order of binding among different ion species.

It is known that the amount of transmitter released at the neuromuscular junction by a nerve impulse varies directly with the Ca concentration and inversely with the Mg concentration in the external medium (del Castillo and Stark, 1952; del Castillo and Engbaek, 1954; Boyd and Martin, 1956). Hubbard (1961) has suggested that the antagonism between Ca and Mg on the end plate is due to a competitive occupation of the same sites. Sr and Ba can restore the Ca spike of the barnacle muscle fiber in Ca-free media while other divalent cations act as inhibitors of the spike potential. A similar restoring action of Sr and Ba has been found for the release of transmitter in the neuromuscular junction (Elmqvist and Feldman, 1965). As shown by Ozeki and Grundfest (1965) and Hagiwara and Nakajima (1966 *b*) tetrodotoxin is ineffective on the Ca spike even at a concentration much higher than that effective for the Na spike. Recent experiment by Katz and Miledi (1965) indicates that the depolarization of the nerve terminal membrane at the muscle end plate can initiate transmitter release in the presence of tetrodotoxin even after the nerve impulse has failed to conduct through the motor nerve to the terminal. These similarities between the initiation of the Ca spike in the barnacle muscle fiber membrane and the transmitter release from the presynaptic membrane at the neuromuscular junction may suggest that some common mechanism could be found in these two phenomena.

The authors express their indebtedness to Drs. S. L. Miller and A. Grinnell for their comments on the manuscript.

This work was supported by grants from the National Institutes of Health, PRL/USPHS NB03536, and from the United States Air Force PRL-AF-AFSOR 933-65.

Received for publication 7 April 1966.

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