Effects of the Intracellular Ca Ion Concentration upon the Excitability of the Muscle Fiber Membrane of a Barnacle

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ABSTRACT The membrane excitability and contraction were examined in single barnacle muscle fibers with different internal Ca++ concentrations by using buffer solutions made up with EGTA and Ca-gluconate in various proportions. During the passage of dc currents the membrane shows all-or-none spike potentials for internal Ca++ concentrations below about $8 \times 10^{-8}$ M, oscillatory potential changes in the range between $8 \times 10^{-8}$ to $5 \times 10^{-7}$ M, but neither oscillatory nor spike potentials were seen for concentrations above $5 \times 10^{-7}$ M. All-or-none spike potentials were suppressed when the internal Mg++ concentration exceeded 5 mM. The suppression threshold of the internal Ca++ concentration for the Sr spike is much higher than that for the Ca spike. The threshold concentration of internal Ca++ for contraction was about $8 \times 10^{-7}$ M.

Giant muscle fibers 0.5 to 2.0 mm in diameter are found in the barnacle, Balanus nubilus (Hoyle and Smyth, 1963). These muscle fibers do not usually produce spike potentials even when the fiber membrane is depolarized much above the threshold for the contraction. However, the membrane becomes capable of producing all-or-none spike potentials when the intracellular Ca++ concentration is reduced by injecting Ca-binding agents such as EDTA (ethylenediaminetetraacetic acid), EGTA (ethylene glycol bis[β-aminoethyl ether]-N,N'-tetraacetic acid), or K-citrate (Hagiwara and Naka, 1964; Hagiwara, Chichibu, and Naka, 1964). In contrast to the spike potentials found in other excitable tissues the amplitude of the spike potentials depends on the external Ca++ concentration while Na+ is found to be inert in this respect. In these earlier experiments no attempt was made to control the...
internal Ca++ concentration. Therefore, it was not possible to correlate the electrical behavior of the membrane with the intracellular Ca++ concentration. In the present experiments the intracellular Ca++ concentration was controlled by injecting Ca++ buffers containing EGTA and Ca salt in various proportions. This paper deals with the influence of internal Ca++ concentration on membrane excitability and tension development.

MATERIALS AND METHODS

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

Figure 1. Experimental arrangement. See text.

The preparation of the single muscle fiber and general experimental procedure were described previously (Hagiwara and Naka, 1964). A single muscle fiber was isolated from a muscle bundle with a fine tendon on one end, and the other end was cut after it had been tied with a thread. The tendon was tied with another thread. After placing the fiber on a glass mounting plate, the thread to the tendon was connected to the stylus of a mechanoelectrical transducer tube RCA 5734 in order to record the tension change of the fiber due to the injection as well as to current applied to the membrane (Fig. 1). The injection pipette was composed of two concentrically arranged glass tubes. The outer tube had a diameter of about 0.5 mm, and that of the inner was about 0.1 mm. The tip of the inner tube extended about 3 mm beyond the opening of the outer tube, which was connected to a micromanipulator device. The inner tubing was filled with 0.5 M KCl and used for recording the membrane potential. In each experiment the injection pipette was introduced longitudinally into the fiber until the tip of the inner tubing reached a region about 5 mm from the tendinous end of the fiber, at which point a small amount of test solution (4 to 8 µl) was injected. Changes in the fiber tension and in membrane potential were recorded simultaneously during a period of about 5 min starting 30 sec before the injection. Following this, the test solution was injected over the whole length of the fiber until the fiber diameter reached about 1.5 times its original diameter. A conventional 3 M KCl-filled glass micropipette was then inserted into the membrane at the region where the opening of the outer tubing was located and the membrane potential was recorded when current pulses were applied from the outer injection tubing (Fig. 1). When recording
with the micropipette was disturbed by movement, the inner tubing was used to record the membrane potential changes during the application of currents through the outer tubing. In the latter case, the resting potential was measured at least once during the experiment with a 3 M KCl-filled micropipette. The coupling impedance between the inner and outer tubing was reduced by inserting a silver wire close to the opening of each tubing.

The normal barnacle saline had the following composition: NaCl, 467 mM; KCl, 8 mM; CaCl$_2$, 20 mM; MgCl$_2$, 12 mM; NaHCO$_3$, 10 mM (Hoyle and Smyth, 1963). The composition of the Ca or Sr saline was CaCl$_2$ or SrCl$_2$, 338 mM; KCl, 8 mM; MgCl$_2$, 12 mM. The Ca-free saline was obtained by replacing the CaCl$_2$ and NaHCO$_3$ in the normal saline with 40 mM NaCl. Solutions of desired Ca$^{++}$ or Sr$^{++}$ concentrations were made by mixing the Ca or Sr saline and the Ca-free saline in appropriate proportions. The method for controlling the Ca$^{++}$ concentration was similar to that employed by Portzehl, Caldwell, and Ruegg (1964). First the two solutions A and B were prepared (Table I). To obtain a desired ratio between concentrations of EGTA and Ca, solutions A and B were mixed in appropriate proportions and the pH was adjusted to 7.0 with small amounts of methanesulfonic acid. The Ca$^{++}$ concentration of the solutions was estimated by the following formula.

$$\frac{[\text{Ca-EGTA}]}{[\text{Ca}^{++}] [\text{free EGTA}]} = 4.83 \times 10^6 \quad (\text{Bjerrum et al., 1957})$$

Since the stability constant is very large, this equation becomes:

$$\frac{[\text{Total Ca}]}{[\text{Ca}^{++}] [\text{total EGTA-total Ca}]} = 4.83 \times 10^6$$

This indicates that the Ca$^{++}$ concentration depends only on the ratio between the concentrations of Ca salt and EGTA and is independent of their absolute values. Thus, the dilution of the solution does not alter the Ca$^{++}$ concentration if this ratio is unchanged.

In order to control internal Mg$^{++}$ concentration, the Mg$^{++}$ buffer solutions were prepared by the same procedure as that for Ca$^{++}$ buffer except that MgSO$_4$ was used instead of Ca gluconate, and EGTA concentration was maintained at 20 mM instead of 100 mM. Since the stability constant of EGTA for Mg is small compared with that for Ca, the Mg$^{++}$ concentration inside the fiber can be maintained at a relatively high level while the Ca$^{++}$ concentration is kept very low. The binding constant of EGTA

| TABLE I |
|---|---|---|---|---|---|
| Ca$^{++}$ BUFFER SOLUTIONS | EGTA | Ca gluconate | KOH | Tris-maleate | KOH | Methanesulfonic acid |
| Solution A, mM | 100 | -- | 400 | 20 | 349 | 180 |
| Solution B, mM | 100 | 100 | 430 | 20 | 287 | -- |
to Mg at pH 7.0 was taken to be 40.5 (Portzehl, Caldwell, and Rüegg, 1964). Because of this low stability constant the Mg$^{++}$ concentration would be altered by dilution. Therefore, the internal Mg$^{++}$ concentration was calculated from the amount of injected solution relative to the original volume of the injected fiber. EGTA used in the present experiment was obtained from Lamont Laboratories, Dallas, Texas.

All the experiments were performed at room temperature (22–25°C).

![Figure 2](image_url)

**Figure 2.** Potential changes (upper trace) of the membrane produced by constant current pulses (lower trace) under the effect of various internal Ca$^{++}$ concentrations. Records 1 and 2 of each pair were taken from the same fiber but with different external Ca$^{++}$ concentrations, 20 mM in 1 and 169 mM in 2. The Ca$^{++}$ concentration of the internal solution was 0 in A, 2.3 × 10$^{-4}$ M in B, 5.2 × 10$^{-4}$ M in C, 1.0 × 10$^{-3}$ M in D, and 2.1 × 10$^{-3}$ M in E. Upward deflection of the potential traces corresponds to depolarization in this and all subsequent figures.

**RESULTS**

1. **Internal Ca$^{++}$ Concentration and the Membrane Excitability**

After injection of each test solution the properties of the fiber membrane were examined by applying constant current pulses of about 200 msec duration. Record A1 of Fig. 2 shows the response obtained with the fiber immersed in normal saline containing 20 mM Ca when the injecting solution contained
only EGTA without Ca. This control recording shows that the membrane is capable of responding with a series of all-or-none spikes to applied constant current pulses when the \([Ca^{++}]_i\) is very small. When the external Ca concentration was increased from 20 mM to 169 mM the overshoot of the spike potential increased (record A2). On increasing \([Ca^{++}]_i\) the membrane continued to show similar all-or-none spike potentials until the \([Ca^{++}]_i\) reached 1.0 × 10^{-7} M (record D). As \([Ca^{++}]_i\) increased the response of the membrane became oscillatory in nature, without all-or-none spike potentials. During the passage of outward current, the amplitude of oscillation increased gradually up to a certain limit.

At \([Ca^{++}]_i\) of 1.0 × 10^{-7} M (record D) the response often changed from an oscillatory potential to repetitive all-or-none spikes when the external Ca concentration was increased from 20 mM to 169 mM. However, the increase of the external Ca concentration no longer restored all-or-none spike potentials when the \([Ca^{++}]_i\) exceeded 2.1 × 10^{-7} M. The three records of Fig. 3 were obtained from the same fiber at \([Ca^{++}]_i\) of 2.1 × 10^{-7} M. Records A and C show responses to current pulses when the membrane was depolarized or hyperpolarized by conditioning dc currents respectively, while record B shows a control obtained at resting potential level. The result indicates that the alteration of the resting potential by either sustained de- or hyperpolarization does not restore the capability of the membrane to produce all-or-none type spike potentials.

As the \([Ca^{++}]_i\) was increased further, the tendency to oscillatory potentials became less marked. At \([Ca^{++}]_i\) of 4.2 × 10^{-7} M (Figs. 4A and B) the membrane often did not show any oscillatory potential change when the external Ca concentration was low (Fig. 4B1). When the \([Ca^{++}]_i\) was higher than 8.3 × 10^{-7} M, oscillatory potential changes were no longer observed even when the external Ca concentration was high (Figs. 4C and D). The relation...
between the membrane behavior and the \([\text{Ca}^{++}]_{\text{in}}\) is shown schematically in Fig. 5.

In the experiment for Fig. 4B2 the \([\text{Ca}^{++}]_{\text{in}}\) and \([\text{Ca}^{++}]_{\text{out}}\) were \(4.2 \times 10^{-7}\) m and 169 mM respectively, hence the ratio \(\frac{[\text{Ca}^{++}]_{\text{out}}}{[\text{Ca}^{++}]_{\text{in}}}\) was about \(4 \times 10^{5}\). Only a slight oscillatory potential change was seen in this case. A similar ratio was found when \([\text{Ca}^{++}]_{\text{in}}\) was \(5.2 \times 10^{-8}\) m and \([\text{Ca}^{++}]_{\text{out}}\) was 20 mM, but this condition gave all-or-none spike potentials, as shown in Fig. 2C1. This observation suggests that the ratio between \([\text{Ca}^{++}]_{\text{out}}\) and \([\text{Ca}^{++}]_{\text{in}}\) is not a determining factor for the behavior of the fiber membrane, and that the absolute concentration of the internal Ca\(^{++}\) is more important, even though some degree of restoration of either all-or-none spikes or oscillatory potentials can be obtained by increasing the external Ca\(^{++}\) concentration.

Records A–C of Fig. 2 show that the overshoot of the spike potential depends only on the \([\text{Ca}^{++}]_{\text{out}}\) and is relatively independent of the \([\text{Ca}^{++}]_{\text{in}}\) when the membrane produces all-or-none spikes. This indicates that the factor which

![Figure 4](image_url)
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determines the overshoot of the spike potential is not the ratio, \([\text{Ca}^{++}]_{\text{out}}/\text{[Ca}^{++}\text{]}_{\text{in}}\), but \([\text{Ca}^{++}]_{\text{out}}\) only. The ratio \([\text{Ca}^{++}]_{\text{out}}/\text{[Ca}^{++}\text{]}_{\text{in}}\) was always higher than \(4 \times 10^5\) for these spikes. If the overshoot represents the concentration potential of \(\text{Ca}^{++}\) across the membrane, it should be larger than 150 mv, which is not the case; this is further evidence that the spike overshoot is not determined by the ratio \([\text{Ca}^{++}]_{\text{out}}/\text{[Ca}^{++}\text{]}_{\text{in}}\). As shown by the previous work (Hagiwara, Chichibu, and Naka, 1964) the overshoot of the spike is determined by \([\text{Ca}^{++}]_{\text{out}}/\text{[K}^{+}\text{]}_{\text{in}}\).

From the foregoing results the \(\text{Ca}^{++}\) concentration inside the noninjected normal muscle fiber can be estimated. Since, as will be described later, the threshold \(\text{Ca}^{++}\) concentration for contraction is about \(8 \times 10^{-7}\) M, \([\text{Ca}^{++}]_{\text{in}}\) in the resting fiber should be smaller than this value. The fact that the normal muscle fiber does not usually produce all-or-none spike potentials indicates that the normal \([\text{Ca}^{++}]_{\text{in}}\) must be above \(8 \times 10^{-8}\) M. Two pairs of records, \(A\) and \(C\), in Fig. 6 show responses of two different normal fibers to current pulses. In each pair, record 1 was obtained in 20 mm and record 2 in 169 mm external \(\text{Ca}^{++}\). Most of the uninjected fibers show oscillatory potential changes such as those of record \(A\) and in a few cases small spike potentials were seen (records \(C\)). The potential changes observed from the normal uninjected muscle fiber are, therefore, similar to those obtained with \([\text{Ca}^{++}]_{\text{in}}\) of \(2 \times 10^{-7}\) to \(8 \times 10^{-7}\) M. Records \(B\) of Fig. 6 show responses of a fiber injected with a simple K methanesulfonate and sucrose solution; i.e., a solution in which 100 mm of EGTA in solution A in Table I had been replaced with methanesulfonic acid sufficient to neutralize the solution. The injection of this solution did not alter the electrical behavior of the membrane. This shows that the behavior of the membrane described above is simply due to the effect of EGTA and Ca salt.

2. Effect of Internal \(\text{Mg}^{++}\)

Since the binding constant of EGTA to Mg is much smaller than that to Ca, it is possible to maintain the \([\text{Mg}^{++}]_{\text{in}}\) at a relatively high level while keeping the \([\text{Ca}^{++}]_{\text{in}}\) below \(8 \times 10^{-8}\) M; i.e., in the range of internal \(\text{Ca}^{++}\) concen-
tration in which the membrane is capable of producing all-or-none spike potentials. A series of records in Fig. 7 were obtained in the normal external saline under different internal Mg\(^{++}\) concentrations. The injecting solution did not contain Mg for record A, which, therefore, constitutes a control record. The estimated Mg concentrations were 0.7, 3.7 and 12 mM for B, C, and D respectively. The results indicate that the suppressing effect of the internal Mg\(^{++}\) on the spike potential starts when the \([\text{Mg}^{++}]_{\text{in}}\) reaches about 5 mM.

![Figure 6](image)

**Figure 6.** Membrane potential changes (upper trace) produced by constant current pulses (lower trace) in normal uninjected fibers (A, C) and in a fiber in which K methane-sulfonate with sucrose was injected (B). Records 1 and 2 in each pair were obtained from the same fiber, but the external Ca\(^{++}\) concentration was 20 mM in 1 and 169 mM in 2.

Since the corresponding figure for Ca\(^{++}\) is about \(8 \times 10^{-8}\) M, the sensitivity of the membrane to Mg\(^{++}\) is about \(10^5\) times less than to Ca.

3. **Effect of Internal Ca\(^{++}\) Concentration on Sr Spike**

As described in the previous paper (Hagiwara and Naka, 1964), the spike potential of the muscle fiber treated with Ca-binding agents is retained after the replacement of the Ca ions in the external solution with an equivalent concentration of Sr ions. The three pairs of records in Fig. 8 were obtained in Sr media with different internal Ca\(^{++}\) concentrations. The \([\text{Ca}^{++}]_{\text{in}}\) was \(2.3 \times 10^{-8}\) M in A, \(2.1 \times 10^{-7}\) M in B, and \(1.9 \times 10^{-6}\) M in C. These spikes are essentially the same as those obtained following injection of Ca-free EGTA solution. These results indicate that the Sr spike is not suppressed even when the internal Ca\(^{++}\) concentration is more than 10 times higher than that sufficient to suppress the Ca spike.
4. Threshold of Internal Ca++ Concentration for Contraction

The records of Fig. 9 show membrane potential and tension development recorded simultaneously when a small amount (4 to 8 μl) of Ca++ buffer solution was introduced inside the muscle fiber. A bar at the bottom of each record indicates the period during which the injection was made. The injection often resulted in a slight shift of the membrane potential in the negative direction. This occurred when the injection solution covered the tip of the inner tubing through which the potential was recorded. Therefore, this seems to be attributable to some sort of junction potential rather than to true hyperpolarization. This presumed artifact did not depend on the Ca++ concentration of the injecting solution. When the final Ca++ concentration was less than

Figure 7. Membrane potential changes (upper trace) produced by constant current pulses (lower trace) under different internal Mg++ concentration. [Ca++]_{out}, 20 mM; [Mg++]_{in}, 0 in A, 0.7 mM in B, 3.7 mM in C, and 12 mM in D.

Figure 8. Effect of internal Ca++ concentration on Sr spike. Records 1 and 2 of each pair were obtained from the same fiber but the external Sr++ concentration was 43 mM in 1 and 169 mM in 2. The Ca++ concentration of the injecting solution was 2.3 × 10^{-4} M in A, 2.1 × 10^{-4} M in B, and 1.9 × 10^{-4} M in C.
or equal to $4.2 \times 10^{-7}$ M, no observable tension increase occurred following the injection (Fig. 9A). This was true of all 12 fibers examined. At $8.3 \times 10^{-7}$ M, however, a marked tension development occurred, as is shown in record B. At this concentration the tension started to rise after a delay of 10 to 15 sec. These findings were consistent in all 7 fibers examined. When the concentration was raised to $1.9 \times 10^{-6}$ M the tension developed with a much shorter delay although the experimental conditions were otherwise un-

![Figure 9](image_url)

**Figure 9.** Changes in membrane potential (upper trace) and in tension (lower trace) due to microinjection. The injection was made during the period indicated by a bar at the bottom of each record. Ca ++ concentration was $4.2 \times 10^{-7}$ M in A, $8.3 \times 10^{-7}$ M in B, and $1.9 \times 10^{-6}$ M in C.

changed (Fig. 9C). Thirteen fibers were examined at this concentration and all showed the same pattern of tension development.

**DISCUSSION**

As shown by Ebashi (1960, 1961), Ebashi and Lipmann (1962), and Hasselbach and Makinose (1961), the relaxing factor found inside the normal muscle fiber has a strong Ca-binding action. Therefore, some modification might be required in the estimation of the Ca ++ concentration of injected fibers. The determination of the internal Ca ++ concentration required for threshold contraction was made by observing the immediate response to the injection. According to the recent work by Hasselbach (personal communication) the rate of Ca ++ uptake by 1 kg wet weight of barnacle muscle is very small.
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(0.006 mmole/sec) and therefore, the removal of Ca by the relaxing factor is negligible just after the injection. Therefore, no modification seems to be necessary in the estimation for the threshold concentration. Portzehl, Caldwell, and Ruegg (1964) have shown that the threshold concentration of internal Ca++ for contraction in a crab muscle fiber is $3 \times 10^{-7}$ to $1.5 \times 10^{-6}$ m. The threshold concentration of 4.2 to $8.3 \times 10^{-7}$ M obtained in the present experiment is in agreement with their figures.

For the study of membrane excitability the injection is made until the fiber diameter becomes about 1.5 times that of the original one, so that the injected solution is diluted by a factor of approximately two. Since the EGTA concentration of the original solution is 100 mM, the final concentration in the injected fiber should be approximately 50 mM. Membrane potential changes induced by current pulses were usually recorded within 10 min after the injection. As mentioned above, the Ca uptake of the barnacle muscle fiber is very slow and the maximum amount of Ca which could be removed by the relaxing factor within this period seems to be less than 3 mmoles/kg of muscle fiber. If corrections are made according to this figure, the final internal Ca++ concentrations, estimated above to be $5.2 \times 10^{-8}$, $2.1 \times 10^{-7}$, and $1.9 \times 10^{-6}$ M, become instead $3.7 \times 10^{-8}$, $1.8 \times 10^{-7}$, and $1.2 \times 10^{-6}$ M. These constitute conservative estimates of the maximum change in internal Ca++ concentration. We might therefore conclude that the relaxing factor does not significantly alter the internal Ca++ concentration of the injected fiber from that of the injecting solution. In a few cases similar experiments were performed with solutions containing 20 mM EGTA instead of 100 mM EGTA. With 20 mM solutions the membrane usually showed oscillatory responses even when the Ca++ concentration of the injecting solution exceeded $1 \times 10^{-6}$ M. This probably indicates that the Ca-binding action of the relaxing factor does become significant when the capacity of the buffer solution is much reduced. On the other hand the presence of Mg in the injected fiber would give an increase in the free Ca level, but this would probably be small because of the low binding constant of EGTA to Mg.

Tasaki, Watanabe, and Takenaka (1962) have shown that conduction block occurs in the internally perfused squid axon when the Ca++ concentration of the perfusing solution is raised above 5 mM, although no buffer was used in this experiment. This is a concentration 10^6 times greater than that affecting barnacle muscle fibers, and suggests either that the membrane of the barnacle muscle fiber is much more sensitive to internal Ca++ than the squid axon membrane or that the sensitivities of the Ca spike and the Na spike differ. Tasaki et al. (1962) also have shown that the effect of Mg++ is smaller than that of Ca++, as is also observed in barnacle muscle fibers. The present results show that the Sr spike is relatively insensitive to the increase of the [Ca++]_in. The spike is not altered even when [Ca++]_in is raised above
the normal internal concentration. This is consistent with the observation that a spike potential is obtained from normal crustacean muscle fibers in Sr media (Fatt and Ginsborg, 1958).

The authors wish to express their indebtedness to Drs. L. Kruger, C. Edwards, A. Grinnell, and T. H. Bullock for their help in preparing the manuscript. The work was supported by grants from the National Institutes of Health (NB 03536) and the United States Air Force (AFOSR 555) to Drs. Hagiwara and Bullock.

Received for publication 25 June 1965.

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