The Ionic Requirements for the Initiation of Action Potentials in Insect Muscle Fibers

HIROSHI WASHIO

From the Department of Pharmacology, University of British Columbia, Vancouver 8, British Columbia, Canada

ABSTRACT Electrical properties of locust leg muscle fibers were studied by means of intracellular electrodes. In most fibers, a depolarizing current pulse initiated a local response. A delayed decrease in membrane resistance appeared with more than about 10 mV depolarization. In some fibers a regenerative response also was found. Membrane constants were measured, applying the short cable model. The value of the space constant $\lambda$ was 1.6 mm and the calculated value of $R_m$ was about 1750 ohm cm$^2$. Action potentials could be elicited when the bathing fluid contained more than 2-5 mM Ba or Sr. Similar responses were seen with 2 mM Ca in the presence of tetraethylammonium (TEA). The overshoot of these action potentials increased with increasing $[\text{Ca}^{++}]_o$, $[\text{Sr}^{++}]_o$, or $[\text{Ba}^{++}]_o$, the increment for a 10-fold increase being about 29 mV for Ca and Sr and between 40 and 50 mV for Ba. These action potentials were inhibited by Mn ions but were not affected by tetrodotoxin or procaine. In solutions containing Ba or Sr, action potentials generated were suppressed by addition of Ca. The removal of Na ions did not change the configuration of the action potential. The results suggest that an increase in permeability to Ca, Ba, or Sr ions makes a major contribution to the initiation of action potentials in this tissue.

INTRODUCTION

Arthropod muscle fibers generally respond with graded depolarization to application of outward current pulses. In crustacean muscle it has been shown that such graded responses are converted to all-or-none spike responses by certain divalent cations, by some quaternary ammonium ions, and by drugs (Fatt and Katz, 1953; Fatt and Ginsborg, 1958; Werman and Grundfest, 1961; Hagiwara and Naka, 1964). All-or-none responses also can be evoked in insect muscle by intracellularly applied depolarizing current pulses in solutions containing Ba or Sr ions (Werman et al., 1961) or the alkaloid ryanodine (Usherwood, 1962), an effect which has been attributed to dimin-
ished K conductance. On the other hand, Fatt and Ginsborg (1958) and Hagiwara and Naka (1964) have demonstrated that the regenerative response in crustacean muscle results from an increase in the permeability of the membrane to Ca, Ba, or Sr ions.

In the present work, the effect of these ions on the initiation of action potentials in insect leg muscle fibers has been studied. This report also includes a description of the electrical properties of these fibers as well as the effect of some drugs on the action potentials. A preliminary note has appeared (Washio, 1971).

METHODS

The anterior coxal adductor muscle (1.7–2.3 mm in length) was completely isolated from the metathoracic legs of the African locust *S. gregaria* (Hoyle, 1966) and mounted in a Lucite chamber which had a volume of about 1.5 ml. The preparation was illuminated from below, using a dark-field condenser. Measurements of fiber length and diameter were made with an ocular micrometer.

Membrane potentials were measured with microelectrodes filled with 3 M KCl having a resistance of 5–10 MΩ. In most of the experiments currents were injected through the recording electrode by means of a precision electrometer (W-P Instruments, Inc., Hamden, Conn., M-4) without affecting the input resistance of the amplifier (Fein, 1966). In other experiments designed for the measurement of membrane constants, two microelectrodes were used, one being a current electrode through which hyperpolarizing currents were applied and the other a recording electrode.

The standard locust saline had the following composition: NaCl, 140 mM; KCl, 10 mM; CaCl₂, 2 mM; MgCl₂, 2 mM; Na₂HPO₄, 6 mM; NaHCO₃, 4 mM (Hoyle, 1953). The composition of the stock saline solution of divalent cations was CaCl₂, BaCl₂, or SrCl₂, 100 mM; KCl, 10 mM; MgCl₂, 2 mM. Salines of the desired concentration of Ca, Ba, or Sr were then obtained by mixing appropriate amounts of stock Ca, Ba, or Sr saline with Ca-free saline. The Ca-free saline was obtained by replacing CaCl₂, Na₂HPO₄, and NaHCO₃ of the standard saline with NaCl. Also Ca-free tetraethylammonium (TEA) solutions were prepared by replacing all of the NaCl of the above Ca-free saline with TEA-Cl. All the solutions were buffered to pH 6.8 with tri(hydroxymethyl)amino methane–Cl (Tris-Cl) except the standard salines. Bathing fluid was exchanged through plastic tubes connected to syringes (capacity 10 cc). This arrangement allowed the surface level of fluid to be kept constant. In this way the electrode tip was kept in the fiber while the solution was being changed. The experiments were done at room temperature (20°–24°C).

RESULTS

1. Electrical Properties of Muscle Fibers in the Standard Locust Saline

The resting potential of fibers immersed in standard saline was −57.7 ± 9.4 mv (mean ± SD, determined in 66 fibers of six muscle preparations). When
outward current pulses were applied through an intracellular electrode, the muscle fiber responded with graded depolarizations as shown in Fig. 1 A. These responses were accompanied by a small oscillatory potential change during their initial parts. The relation between the steady displacement of potential and the applied current is shown in Fig. 2. There was a deviation in the direction of the abscissa at about 10 mv depolarization (curve A). However, a regenerative response also was found with a strong outward current pulse in some fibers, as shown in Fig. 1 B. Overshoot during action potentials was not found in standard saline with these fibers and their current-voltage relation was different from that of other fibers (Fig. 2, curve B). Also, the

resting potentials and time constants of the fibers in Fig. 1 A and B were different, the fiber in B having a higher resting potential and longer time constant. The fibers which exhibited spikes, such as the example illustrated in Fig. 1 B, always had high resting potentials (about 70 mv). However, there was no apparent correlation between the time constant length and the evoking of a regenerative response.

To measure membrane constants two microelectrodes were used. The current electrode was inserted at the middle of the fiber. The recording electrode was inserted first as close as possible to the current electrode, then near the end of the fiber. The theoretical equations for determining membrane constants in short fibers with infinite resistance at both ends have been described as follows (Weidmann, 1952):

\[ \frac{V_x}{V_0} = \frac{\cos \frac{h(L - X)}{\lambda}}{\cos \frac{h(L)}{\lambda}} \]  

(1)
FIGURE 2. Two types of current-voltage relationship in standard saline. The dashed portion in curve B shows the relation at the peak of the regenerative response. Positive ordinate represents depolarization; positive abscissa represents outward current. Data obtained from Fig. 1 A and B. See text.

\[ \frac{V_o}{I_o} = \frac{1}{2} r_i \lambda \cot h \left( \frac{L}{\lambda} \right) \]  
\[ R_i = \frac{1}{2} \pi d^2 r_i; R_m = \pi d \rho_m \]  

where:
- \( V_o \) is the electrotonic potential produced by current \( I_o \) at \( X = 0 \),
- \( V_x \) is the potential at the distance \( X \) from the current electrode,
- \( L \) is the half length of the fiber,
- \( \lambda \) is the space constant equal to \( \sqrt{\frac{r_m}{r_i}} \),
- \( r_m \) is the membrane resistance times unit length,
- \( r_i \) is the internal resistance per unit length,
- \( R_i \) is the specific membrane resistance,
- \( R_m \) is the specific resistance of the myoplasm,
- \( d \) is the fiber diameter.

At \( X = L \), equation 1 is reduced to

\[ \frac{V_L}{V_o} = \frac{1}{\cos h(L/\lambda)}. \]

Fig. 3 shows an example of an experiment in which \( \lambda \) was obtained from the above relation. In this fiber (1.8 mm in length) the electrotonic potential recorded at the end of the fiber (record B) was 10% less than that recorded at the center (record A). The fiber diameter was known roughly from visual measurement. However, it was important to have a more precise estimate of fiber diameter, because of its influence on the calculated value of \( R_m \). As-
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Figure 3. Electrotonic potentials (upper trace in each part of figure) recorded at the center (A) and at the end (B) of a fiber (1.8 mm in length) in standard saline. A current electrode was kept inserted at the center of a fiber. The lower traces represent the hyperpolarizing current applied to the membrane. See text.

Assuming \( R_t = 100 \text{ ohm cm} \) (Henček et al., 1968) the calculated fiber diameter was between 75 and 40 \( \mu \text{m} \). The histological study on the muscle indicated that the fiber diameter ranged between 90 and 35 \( \mu \text{m} \). The time constant (\( \tau_m \)) was taken as the time required for the voltage change to reach the appropriate percentage fraction of its final value, which lay between 84\% for the infinite cable model and 63\% for a spherical core conductor. The theoretical value for this percentage at various values of \( L/\lambda \) has been given by Stefani and Steinbach (1969). From the time constant the membrane capacity per unit area (\( C_m \)) was calculated. Table I summarizes the results obtained from 10 fibers in five preparations. The calculated values of \( \lambda \) and \( \tau_m \) ranged from 1.4 to 1.9 mm and from 14 to 23 msec, respectively.

2. Initiation of Action Potentials in the Presence of Ca Ions

Increasing the Ca concentration of the standard saline (10–20 mm) failed to initiate action potentials with overshoots in response to outward current pulses, but small spikes with regenerative features appeared. When \([\text{Ca}^{++}]_o\) was raised to higher levels, about 40 mm or more, these regenerative responses were consistently suppressed. In contrast, when all of the Na of the standard saline was replaced with TEA ions, action potentials with appreciable overshoots and long durations were elicited in response to depolarization. Under these conditions an increase of Ca concentration produced an increase in the amplitude and duration of the action potential as shown in Fig. 4, although the total duration of the action potential was not checked in the solutions.
TABLE I
MEMBRANE CONSTANTS OF ANTERIOR COXAL ADDUCTOR MUSCLE FIBERS OF THE LOCUST IN THE STANDARD SALINE

Diameters were calculated assuming $R_i = 100 \text{ ohm cm}$. Two microelectrodes were used; see text.

| Effective membrane resistance | Half length of the fiber | Fiber diameter | Space constant | Time constant | Specific membrane resistance | Specific membrane capacitance |
|-------------------------------|--------------------------|----------------|----------------|---------------|-------------------------------|------------------------------|
| $3.3 \times 10^6$             | 0.88                     | 70             | 1.4            | 17            | 1121                          | 15.1                         |
| $4.3 \times 10^6$             | 0.88                     | 75             | 1.9            | 15            | 1674                          | 8.9                          |
| $8.6 \times 10^6$             | 1.12                     | 40             | 1.4            | 22            | 1957                          | 11.0                         |
| $8.6 \times 10^6$             | 1.00                     | 52             | 1.8            | 23            | 2537                          | 9.0                          |
| $9.3 \times 10^6$             | 0.94                     | 44             | 1.5            | 22            | 2166                          | 10.1                         |
| $4.8 \times 10^6$             | 1.00                     | 62             | 1.6            | 23            | 1649                          | 13.9                         |
| $5.7 \times 10^6$             | 0.95                     | 52             | 1.4            | 17            | 1551                          | 10.9                         |
| $5.3 \times 10^6$             | 0.90                     | 65             | 1.7            | 18            | 1776                          | 10.1                         |
| $4.2 \times 10^6$             | 1.00                     | 62             | 1.5            | 14            | 1432                          | 9.6                          |
| $4.0 \times 10^6$             | 1.00                     | 68             | 1.6            | 16            | 1620                          | 9.9                          |
| Mean                          | $5.8 \times 10^6$        | 0.97           | 59             | 1.98          | 18.7                         | 1754                         | 10.9                         |
| ±SD                           | $2.1 \times 10^6$        | 0.07           | 11             | 0.17          | 3.3                          | 376                          | 1.8                          |

FIGURE 4. Action potentials recorded from muscle fibers in different concentrations of Ca in solutions in which all of the Na was replaced with TEA. (A) 0 mm; (B) 5 mm; (C) 10 mm; (D) 40 mm Ca. Records A and B were obtained from the same fiber. The upper traces depict current intensity and the reference potential level; the lower traces record the transmembrane potentials. Scales: $5 \times 10^{-8}$ A, 50 mv, and 100 msec.

containing more than 10 mm Ca. This finding is in keeping with that of Fatt and Ginsborg (1958) in crayfish muscle. The maximum rate of rise of the action potential also increased with elevated Ca concentrations. Fig. 5 shows the relationship between the action potential and the Ca concentration of the solution in which all of the Na was replaced with TEA ions, determined in several different fibers of a pair of locust leg muscle preparations. The overshoot increased by about 29 mv between 4 and 40 mm $[\text{Ca}^{++}]_o$ (curve A). In addition, increasing the Ca concentration from 4 to 40 mm made the threshold potential for initiation of action potentials less negative (curve B) and increased the resting potential by about 10 mv (curve C).
Another effect of increasing the external Ca concentration was an increase in resting membrane resistance. Table II shows the effective membrane resistance and the time constant in solutions containing 4 and 40 mM Ca with TEA ions. For short muscle fibers the effective membrane resistance is proportional to $\lambda \cot h (L/\lambda)$ from equation 2. Assuming $R_i = 100$ ohm cm, $d = 60 \mu$, and $L = 1.0$ mm, calculated values of $\lambda$ were 2.4 and 3.2 mm for 4 and 40 mM Ca, respectively, in the presence of TEA. About a 65% increase in the effective membrane resistance was brought about by the high external Ca. If the fibers were considered to be an infinite cable, this would cause a 2.7-fold increase in the specific membrane resistance (Fatt and Katz, 1951). However, as the specific membrane resistance of a short cable increases, the electrical characteristics of the fiber approach those of a sphere (see Stefani and Steinbach, 1969). Thus, the calculated specific membrane resistance of

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| Solution           | Effective membrane resistance | Time constant | Space constant | Specific membrane resistance |
|--------------------|-------------------------------|---------------|----------------|-----------------------------|
| 4 mM Ca with TEA   | $11.2 \pm 1.7$ (6)            | 38.1$\pm$8.1 (6) | 2.4            | 3843                        |
| 40 mM Ca with TEA  | $18.5 \pm 2.5$ (5)            | 51.2$\pm$5.2 (5) | 3.2            | 6830                        |
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Figure 5. Effects of the external Ca concentration on the overshoot (A), the threshold potential for initiation of action potentials (B), and the resting membrane potential (C). All of the Na was replaced with TEA ions. Vertical bars represent the standard deviation of the mean.
these muscle fibers increased at almost the same rate as the effective membrane resistance.

An increase in membrane conductance during the action potential was always observed. This membrane conductance increased with increasing Ca concentration. The change in membrane potential during activity in 10 mM Ca-TEA saline was measured by applying constant hyperpolarizing current pulses of 200 msec as shown in Fig. 6. At the beginning of the plateau, this potential was much diminished and even at the end of the plateau it was much smaller than at rest. The apparent conductance increase of the plateau was always found to outlast the action potential by more than 10 sec. During this time, an action potential could not be elicited. By analogy with the squid giant axon (Hodgkin and Huxley, 1952), one might expect a persistent increase in K ion permeability with a gradual return to normal. In the present experiments the membrane continued to be depolarized; thus, it is presumed that an increased permeability to Ca ions may also persist during this period. Since TEA probably abolishes the increased K conductance (see Discussion), it is now more likely that only a persistent elevation in Ca conductance is involved. It remains possible, however, that Cl conductance may be increased.

When Ca ions were removed from the TEA saline, only abortive action potentials were observed (Fig. 4 A). The addition of 1.5 mM ethylenediaminetetraacetic acid (EDTA) invariably suppressed regenerative responses while the fiber suffered a marked decline of the resting potential. Omission of Mg had no effect on the action potentials in the Ca-TEA saline.

3. Initiation of Action Potentials in the Presence of Ba or Sr Ions

As already reported by Werman et al. (1961), Ba and Sr ions converted the graded responses of insect leg muscle fibers to all-or-none responses. Action potentials were elicited in response to outward current pulses almost immediately after the introduction of Ba or Sr at concentrations in the range of 2–5 mM without any replacement of Na with TEA. In Ba or Sr concentrations up to approximately 20 mM, spontaneous discharge of the action potential appeared. The amplitude of the action potentials varied from fiber to fiber, but...
always increased with increasing \([\text{Ba}^{++}]_o\) or \([\text{Sr}^{++}]_o\). Fig. 7 (records A and B) shows the action potentials produced in solutions containing 10 mM, 30 mM, and 100 mM \([\text{Ba}^{++}]_o\) or \([\text{Sr}^{++}]_o\). In solutions containing 100 mM \([\text{Ba}^{++}]_o\) or \([\text{Sr}^{++}]_o\) all of the Na was removed. The other solutions contained Na to isomolarity. Fig. 8 shows examples of the relation between the overshoot and the external Ba and Sr concentrations. For Ba, the change in overshoot per 10-fold increase in \([\text{Ba}^{++}]_o\) was 40 and 47 mv in the examples shown. The increment ranged between 40 and 50 mv in another five fibers. The maximum rate of rise of the action potential appeared to increase with increasing \([\text{Ba}^{++}]_o\) (Fig. 7 A). The spike was followed by a persistent plateau which lasted for several seconds, in some fibers for about 1 min. In addition, the resting membrane resistance increased with increasing \([\text{Ba}^{++}]_o\). Estimation of membrane resistance during the action potential produced in 20 mM Ba saline by applying constant hyperpolarizing current pulses showed an initial decrease followed by a gradual increase as shown in Fig. 9. As termination of the plateau approached, membrane resistance increased, becoming as large as that at
rest, then even greater. The mechanism underlying this apparent increase in membrane resistance to more than the resting level remains unknown. However, the shape of the response suggests an active response of K conductance to hyperpolarization with regenerative characteristics which would account for synchronization of the termination of the action potential with the hyperpolarizing current pulse. Fatt and Katz (Fig. 15, 1953) observed similar changes in membrane resistance during the action potential in crab muscle in the presence of tetrabutylammonium (TBA).

![Graph](image)

**Figure 8.** Relationship between the overshoot and external concentration of Ba (open circle with continuous line) and Sr (filled circle with dashed line) on a logarithmic scale. Data were obtained from four different fibers.

![Graph](image)

**Figure 9.** Changes of membrane resistance during a spontaneous discharge of the prolonged action potential produced in 20 mM Ba saline. The upper trace represents current intensity and the reference potential level. The lower trace represents membrane potential changes. Currents were injected through the recording electrode.

For Sr, the change in overshoot per 10-fold increase in $[\text{Sr}^{++}]_o$ was about 29 mv (Fig. 8). The increment for a 10-fold increase in $[\text{Sr}^{++}]_o$ ranged between 26 and 32 mv in another four fibers. Increasing $[\text{Sr}^{++}]_o$ also increased the maximum rate of rise and prolonged the duration of the action potential. However, the duration was always much shorter than that in corresponding concentrations of Ba. Replacement of all of the Na with TEA in the presence of Sr ions caused a marked prolongation of action potentials without affecting the overshoot and the rate of rise of the action potential. This may be seen in Fig. 7 C.
4. The Effect of Ca and Mn Ions on the Action Potential

When \([Ca^{++}]_o\) was increased in solutions containing Ba or Sr ions while keeping osmolarity constant by reduction of \([Na^+]_o\), the threshold potential for the initiation of action potentials was raised and the duration of the action potential was reduced. Finally, the action potential was completely suppressed. Fig. 10 A shows an experiment in which \([Ca^{++}]_o\) was increased to 20 and 40 mM in 20 mM Ba saline. The duration of the action potential was much shortened in 20 mM \([Ca^{++}]_o\) (record A 2) and the action potential was suppressed in 40 mM \([Ca^{++}]_o\) (record A 3).

Mn ions inhibited the action potentials produced in Ba, Sr, or Ca-TEA saline. In the experiment shown in Fig. 10, B 1, the action potential was elicited in 20 mM Ba saline. Addition of 20 mM Mn decreased the overshoot and the rate of rise of action potential (record B 2). However, the spike was followed by a long plateau, even after the application of the Mn ions. Increase in the concentration of Mn to 40 mM abolished the regenerative response (record B 3). A similar result was obtained in another fiber examined in solutions containing 20 and 40 mM Mn with both 10 mM Ca and TEA ions.

5. The Effect of Na Deprivation

The possible role of Na as a carrier of membrane current during action potentials produced in the presence of Ba ions was studied in Na-free solutions.
in which all of the NaCl was replaced by sucrose, Tris-Cl, or choline-Cl. When sucrose was used, fibers suffered a decrease in resting potential which was not observed in Tris-Cl or choline-Cl salines. Only small regenerative responses were obtained in the presence of 20 mM Ba in these fibers, perhaps because of the low resting membrane potentials. However, action potentials with overshoot and long plateaus were found in some fibers having resting potentials of about 40 mV. In Tris-Cl or choline-Cl saline, action potentials elicited in the presence of 20 mM Ba appeared to be the same as in NaCl saline. Thus the action potential occurring in these solutions was independent of external Na ions.

6. The Effect of Tetrodotoxin and Procaine

The action potentials elicited by depolarizing current pulses in the presence of Ba or Sr or in TEA solutions containing Ca were not affected by the application of 10^-5 g/ml tetrodotoxin (TTX). Also, no significant change in the configuration of the action potentials was found in Ba or Ca-TEA media after treatment with 10^-3 g/ml procaine. However, application of the same concentration of procaine prolonged the action potentials in Sr saline. The action of procaine in the Sr saline was similar to the effect of replacing Na with TEA in the presence of Sr. Addition of procaine alone in the standard saline did not give rise to full-scale action potentials, although regenerative oscillatory potentials were observed. This effect of procaine was different from that on the crustacean muscle fibers, which is to make those fibers capable of all-or-none electrogenesis (Fatt and Katz, 1953; Girardier et al., 1961; Ozeki et al., 1966).

DISCUSSION

The experimental results showed that the relation between overshoot of the action potential and the logarithm of the [Ca++] or [Sr++] gave the slopes expected for Ca and Sr electrodes. Also, the rate of rise of the action potential increased with increasing [Ca++] and [Sr++]. Furthermore, membrane conductance during action potentials increased with increasing Ca concentration, while the conductance of the resting membrane decreased. These results support the concept that the membrane permeability to Ca or Sr becomes high during the action potential. Similar ionic requirements for the initiation of the action potential have been observed in crayfish muscle (Fatt and Ginsborg, 1958) and barnacle muscle (Hagiwara and Naka, 1964; Hagiwara et al., 1964). Recently Hagiwara et al. (1969) have shown that, when barnacle muscle fibers are subjected to voltage-clamp conditions and perfused with internal solutions containing a calcium chelating buffer, the early increase in membrane conductance varies with the concentration of Ca ions. They also showed that the peak potential of the spike is determined not only by the early
inward current but also by the late outward current which is carried by K ions. If this also is the case with locust muscle, it may explain why the relation between the overshoot of the action potential and the logarithm of the $[\text{Ba}^{++}]$, gave a slope much larger than that expected for a Ba electrode, and why the spike was followed by a very long plateau in Ba saline, since Ba has been shown to depress K permeability in *Romalea* muscle (Werman et al., 1961) and in lobster muscle (Werman and Grundfest, 1961).

Further support for this concept was provided by the observation that the application of $10^{-6}$ g/ml TTX, which is more than 100 times the concentration sufficient to block the Na-dependent action potential in frog muscle fibers (Narahashi et al., 1960), has no suppressant effect on the action potentials in locust fibers. Procaine also has been shown to abolish the Na spike, but it suppresses both Na and K conductance changes (Shanes et al., 1959; Taylor, 1959). In Ba or Ca-TEA salines, no significant change in the configuration of the action potential was produced by the introduction of $10^{-5}$ g/ml procaine. However, addition of the same amount of procaine to Sr saline prolonged the duration of the action potential. This prolongation may be explained by the effect of the drug on the K conductance.

For the initiation of action potentials in the presence of Ca, it was necessary to replace Na with TEA. Many investigators have shown that TEA depresses K conductance changes in excitable tissues. Replacement of Na with TEA in Sr saline prolonged the duration of the action potential without significant change in the overshoot and the maximum rate of rise of the action potential (Fig. 7 C). This effect also may be explained by a TEA-induced decrease in K conductance. Another possible mechanism which might explain the action of TEA was advanced by Fatt and Ginsborg (1958), who suggested that TEA facilitates the increased Ca permeability of the active membrane of crayfish muscle fibers. Beaulieu and Frank (1967) reported that 40–50 mM TEA partially restored K-contracture capacity of frog skeletal muscle in calcium-free Ringer’s solution and concluded that TEA can release Ca from membrane sites. However, no action potentials with overshoot were observed in Ca-free TEA saline in locust muscle fibers. This finding suggests that the effect of TEA ions in insect muscle, in addition to the reduction of K conductance, may be due to some interaction with Ca on the surface of the muscle membrane rather than to the internal release of Ca ions resulting in the facilitation of the Ca permeability of the membrane.

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