Membrane Calcium Activation in Excitation-Contraction Coupling

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ABSTRACT   Depolarization thresholds for eliciting tension and Ca electrogenesis have been compared in isolated crayfish muscle fibers. Just-detectable tensions and Ca spikes induced after treatment with procaine were elicited with intracellularly applied depolarizing currents of fixed duration. Both thresholds were found to increase in a similar manner in fibers exposed to increased concentrations of Ca in the bathing solution or addition of other divalent cations (Mg, Mn, Ni). However, antagonistic effects between divalent cations were also demonstrated. Substitution of increasing amounts of NaSCN for NaCl in the standard saline produced a progressive decrease in both thresholds. The correlation in the change in thresholds for the two processes supports the hypothesis that a change in membrane Ca conductance is an integral step in excitation-contraction coupling.

INTRODUCTION

There is evidence that a membrane conductance change is an essential step in excitation-contraction coupling (ECC) of skeletal muscle fibers (Reuben et al., 1967). The data of the present report demonstrate a correlation between ECC and a process which is known to involve an increase in membrane conductance for Ca ions (Ca-spike electrogenesis). Preliminary reports of some of this work have appeared (Suarez-Kurtz et al., 1970; Reuben et al., 1971).

METHODS

Preparation and Recording

Single muscle fibers were prepared from the flexor muscle within the meropodite portion of the walking limb of the crayfish *Orconectes virilis*, as described by Girardier et al. (1963). The method of mounting the single fiber for recording tension has also

been described (Chiarandini et al., 1970 *a*). In some experiments a Grass Instrument force-displacement transducer model FT 03B (Grass Instrument Co., Quincy, Mass.) was used in conjunction with a Grass polygraph with curvilinear registration. In others, a Bionix F250 isometric transducer (Biological Electronics, Inc., Elcerrito, Calif.) was employed and rectilinear registration was done with a Beckman type R dynograph (Beckman Instruments, Inc., Fullerton, Calif.). The membrane potential was measured with an intracellular micropipette filled with 3 M KCl. A second intracellular micropipette also filled with 3 M KCl was inserted within 200 μ from the recording pipette for applying current. The electrophysiological equipment was standard for the laboratory.

Solutions

The composition of the various stock solutions is given in Table I. During the dissection of the single fibers the preparation was bathed in control saline (solution A).

TABLE I
COMPOSITION OF STOCK SOLUTIONS

Solution	Na	K	Ca	Mg	Me	Cl	SCN
	mM						
A	200	5	13.5	—	—	232	—
B	—	5	80.5	—	—	166	—
C	—	5	13.5	67	—	166	—
D	—	5	—	80.5	—	166	—
E	—	5	13.5	—	67	166	—
F	200	5	13.5	—	—	—	232

Me represents "foreign" divalent cations (Mn, Ni). All of the solutions contained 4 mM Tris maleate (pH 7.4), and the ionic strength was fixed (0.245–0.246). Sucrose was added where necessary to keep the osmotic pressure fixed at 220 mosmol above that contributed by the buffer.

The desired concentrations of Mg and Ca were obtained by appropriate dilution of solutions A, B, C, and D. To obtain various concentrations of other divalent cations (*Me*) solution E was mixed with solution A. For making solutions of SCN in different concentrations appropriate amounts of solutions A and F were used.

RESULTS

Induction of Ca-Spike Electrogenesis

The muscle fibers, which normally respond to depolarization with graded electrogenesis (Grundfest, 1966), can be treated with one of a number of agents to convert the graded responses to all-or-none Ca spikes (Fatt and Ginsborg, 1958; Takeda, 1967; Chiarandini et al., 1970 *a*). In terms of their actions on biological membranes these agents appear to fall into two groups. Tetraethylammonium ion (TEA), Ba, and procaine which reduce or abolish the depolarizing K activation are examples of group 1. Ba and TEA may

also contribute to the inward cation current (Werman and Grundfest, 1961). Group 2 is comprised of the xanthines, of which caffeine has been studied in some detail (Chiarandini et al., 1970 a, b). Their action appears to be that of enhancing the divalent cation permeability and/or modifying the distribution of the divalent cations across the cell membrane.

The graded electrogenesis observed in control fibers is most likely due to a concomitant, graded increase in permeability of the membrane to both K and Ca ions. This being the case, then either an enhancement of the Ca permeability (group 2) or a reduction of the K permeability (group 1) could convert the graded electrogenesis to an all-or-none spike. Procaine was used in this study for converting the graded response to all-or-none for two reasons:

(a) The effects of procaine can be completely and readily reversed (Takeda, 1967; Reuben et al., 1967).

(b) The twitch tension associated with the procaine-induced Ca spike is comparatively small. Procaine (10^{-3} w/v) probably reduces both the Ca and K currents, but the reduction of the K current must be relatively greater (see Hagiwara et al., 1969).

Spike and Tension Thresholds as a Function of the Divalent Cation Concentration

1. MANGANESE   Fibers exposed to low concentrations of Mn (1–10 mM) contract only when depolarized to considerably greater extents than the nontreated preparation (Fig. 1). The procedure followed in the experiment of Fig. 1 was to record the increment of depolarization for threshold tension (Δt) with each tested concentration of Mn. The fiber was then treated with procaine (10^{-3} w/v) and the increment of depolarization for the spike threshold (Δs) was measured. Then the procaine was washed out, and the Δt again obtained. The control values were readily reproduced, thresholds remaining within a range of ± 1.5 mv after each step exposure to Mn. In the upper half of Fig. 1, the thresholds (in millivolts) for both tension and spikes are evident. In the lower part of the figure, the same parameters are shown in the presence of 3 mM Mn. There was a marked increase in both Δ values upon exposing the fiber to this concentration of Mn. This effect was rapid (within 15 sec) and did not show variation with exposure times up to 1 hr. The resting potential did not change with increasing concentrations of Mn (1–10 mM), nor with increasing time of exposure to Mn. By comparing the current and voltage values, it may be noted that the effective resistance (R_E) also remained constant.

Fig. 2 consists of plots of the increases in Δs and Δt as functions of Mn concentration. They increased linearly with increasing Mn concentrations from 1 to 7 mM. The slope was 55 mv/10 mM Mn for the Δt plot and 25 mv/10

mM Mn for the Δs plot. In other experiments the linear relationship was noted to extend up to 10 mM Mn (see Fig. 9). However, abolition of tension sometimes occurred at concentrations of Mn as low as 8 mM. Four experiments were performed in which both parameters were measured on the same fiber, and in three other experiments Δt was alone determined. The mean slope for Δs was 27.8 mv/10 mM Mn (± 4.2 SEM, number of experiments $N = 4$) and the mean slope for the Δt was 57.6/10 mM mn (± 2.4 SEM, $N = 7$).
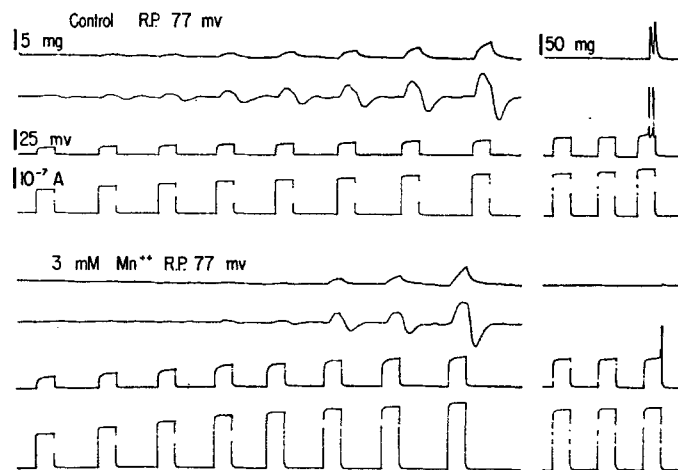
FIGURE 1. Example of experimental procedure; determination of tension and spike thresholds and their modification by 3 mM Mn. *Upper left four rows:* tension, the derivative of tension, membrane potential, and transmembrane current in control saline. The depolarizing current pulses of increasing amplitude each lasted 1 sec except in the experiments with SCN. *Upper right 3 rows:* the corresponding recordings (without the derivative of tension) after treating the fiber with procaine (10^{-3} w/v). *Lower left:* after exposing the fiber to 3 mM Mn a larger depolarization is required to elicit a just-detectable tension. *Lower right:* the fiber was again exposed to procaine, but in the presence of 3 mM Mn. The threshold for evoking Ca spikes was also elevated. Note the large attenuation of the tension during the spike in the presence of Mn. *R.P.* is resting potential in all figures.

2. MAGNESIUM Magnesium is much less effective than manganese in increasing Δ for both tension and the Ca spike. Fig. 3 shows the two Δ curves with Mg concentrations varying from 0 to 67 mM. The concentration of Mg was increased stepwise, and after the last determination, the control values were reproduced to within ± 1.5 mv. The shapes of the two curves for the two parameters are similar, but the initial curvature is greater for Δt. Both Δt and Δs were obtained on the same fiber in three experiments, and Δt was determined alone on two additional preparations. The mean initial slope for the five Δt curves was 16.3 mv/10 mM Mg (± 1.8 SEM, $N = 5$). The mean initial slope for the Δs curves was 5.5 mv/10 mM Mg (± 0.29

SEM, $N = 3$). In the range of 35–67 mM Mg, however, the thresholds increased by only a few millivolts per 10 mM Mg. The membrane resistance increased slightly when the fiber was exposed to high concentrations of Mg. The effective resistance (R_E) was about 9% higher in the presence of 20 mM Mg and about 18% higher in 67 mM Mg. Concentrations higher than 67 mM could not be tested without increasing the ionic strength of the medium above that of the control (see Methods).
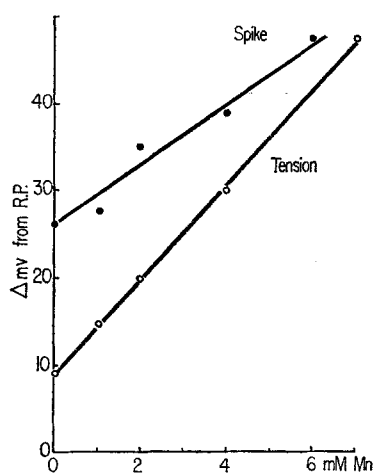

FIGURE 2   FIGURE 3

FIGURE 2. The elevation of tension and spike thresholds (ordinate) with increasing concentration of Mn. o, the depolarizations from the resting potential (-81 mv) for eliciting just-detectable tension. ●, depolarization for inducing the Ca spikes. Lines were drawn by inspection; average slopes ±SE are given in the text for each experimental condition. Statistical analysis of the accumulated data is presented in Fig. 6 and in corresponding text.
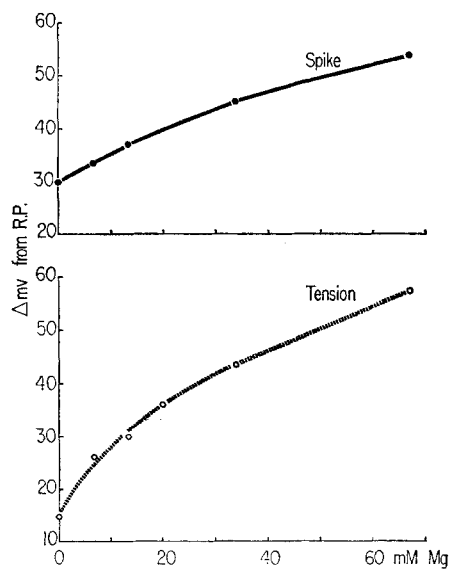
FIGURE 3. The increase in thresholds (ordinate) as a function of the Mg concentration. Upper graph, the depolarization required to elicit Ca spikes. Lower graph, the corresponding data for tension thresholds. Resting potential was 83 mv.

3. CALCIUM   Increasing Ca_o to 40.5 mM (three times the control level) increased both Δ's by about 10 mv (Fig. 4). In seven experiments Δt increased with a mean initial value of 3.5 mv/10 mM Ca_o (± 0.39 SEM, $N = 7$). The slope decreased by about 50% as the concentration of Ca_o was increased above 40 mM in all but one experiment (see Fig. 9). In two of these experiments Δs was also measured and increased by about the same amount as Δt (Fig. 4), 4.6 mv/10 mM Ca_o (± 0.57 SEM, $N = 2$). The resting potential increased by a few millivolts in fibers exposed to high Ca and R_E also increased 1.25 times in 47 mM Ca and 1.5 times in 87 mM Ca.

Variation of Δt and Δs as a Function of SCN Concentration

Thiocyanate was selected as a test agent because it potentiates tension in crayfish (unpublished observations) as well as in vertebrate striated muscle (see Sandow, 1965). The procedure for testing the effects of SCN on the Δ's was similar to that previously described for the divalent cations. In order to determine more accurately the Δ's in SCN, pulses of 200 msec duration rather than 1 sec duration were used, since the threshold depolarization for evoking tension increases as the duration of the stimulus is shortened (Edwards et al., 1964; Reuben et al., 1967; Adrian et al., 1969). Re-
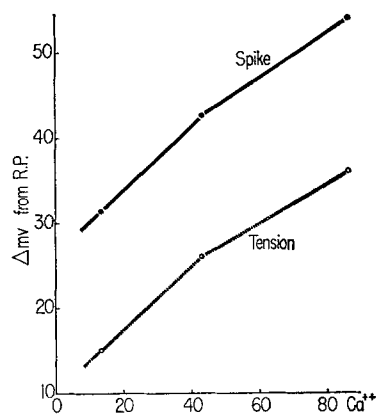
FIGURE 4   FIGURE 5

FIGURE 4. Tension and spike thresholds (ordinate) as a function of extracellular Ca_o (given as millimolar concentration). ●, spike thresholds; ○, tension thresholds under the same conditions.
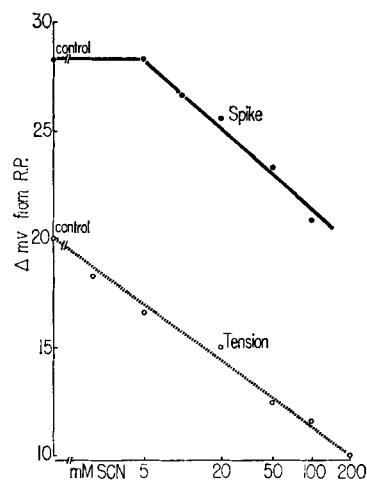
FIGURE 5. Reduction of the tension and Ca spike thresholds with increasing concentrations of SCN. ●, thresholds for the spike; ○, tension thresholds. The initial resting potential was −80 mv and the thresholds were determined with 200-msec pulses. For further description see text.

duction of Δt and Δs occurred rapidly (within 30 sec) after first exposing the fiber to SCN and with each subsequent step elevation of the SCN concentration. There was no detectable change in the effective resistance as determined with depolarizing current pulses. The membrane potential usually hyperpolarized by a small amount (1–3 mv) in fibers exposed to the higher concentrations of SCN. The effects of SCN were rapidly reversible except when concentrations greater than 100 mM were used. Both Δ's decreased linearly when plotted against the log of the SCN concentration with a slope of −4.3 mv/decade SCN (Fig. 5). In some experiments like those of Fig. 5 Δs did not change until the concentration of SCN was increased above 5 mM, after which the slope of this semilog plot became −5.3

mv/decade SCN. Three other experiments were done in which both Δ's were measured on the same fiber exposed to various concentrations of SCN. In two additional experiments only the variation of Δt was determined. The mean slopes of the Δs vs. log SCN concentration were -9 mv/decade SCN (± 1.45 SEM, $N = 4$) and -7.43 mv/decade SCN (± 1.42 SEM, $N = 6$) for Δt.

Correlation between the Δt and Δs

The points composing the graph of Fig. 6 represent only measurements from fibers in which both Δ's were determined when the fibers were exposed to the same concentration of divalent cations or SCN. Two points are also included from an experiment on a fiber exposed to 2 and 4 mM Ni. The regression equation is

$$\Delta t = 1.23 \, \Delta s + 2.96$$
r (correlation coefficient) $= 0.95$
$P > 0.95$ for the significance of the correlation
Δt = incremental change in tension threshold
Δs = incremental change in Ca-spike threshold.

Antagonistic Effects on Δt between Ca and Mg or Mn

It seemed desirable to determine whether Mg and Ca acted synergistically or antagonistically on the process initiating tension in view of the well-known antagonistic effect of these ions on excitation-secretion coupling (see del Castillo and Engbaek, 1954). The increase in tension threshold with increasing concentrations of Mg (Fig. 3) is considerably reduced if the Ca_o concentration is raised from 13.5 mM (control value) to 40 mM (Fig. 7). In the latter condition the concentration of Mg could not be increased above 34 mM without increasing the ionic strength above the control level. Whereas in solutions containing 13.5 mM Ca_o the initial slope of Mg vs. Δt was 13 mv/10 mM Mg, it decreased to 4.5 mv/10 mM Mg in the presence of 40 mM Ca_o. In another experiment Δt with increasing concentrations of Mg was determined first in a fiber equilibrated in 3 mM Ca_o and then in the control concentration of 13.5 mM Ca_o. The initial slope in 3 mM Ca_o of the Δt vs. Mg curve was 25 mv/10 mM Mg and 11 mv/10 mM Mg in 13.5 mM Ca_o. A more dramatic demonstration of the antagonism between Ca and Mg on the tension threshold is shown in Fig. 8. The threshold for tension could not be determined when the fiber was exposed to 36.5 mM Mg and 2 mM Ca even though the membrane potential was reduced to a value that was inside positive by 25 mv at the site of the current electrode impalement. Increasing Ca_o reversed this effect of high Mg and the threshold decreased as Ca_o increased. A lower background concentration of Mg (18.5 mM) was

less effective in terms of increasing Δt in low Ca_o (Fig. 8), but again Δt decreased as Ca_o was increased above the initial 2 mM level. A control curve of Δt vs. Ca_o (zero Mg) is also included in Fig. 8. The antagonism between Ca_o and Mg_o is evident from the experiments of Figs. 7 and 8.

The marked increase in Δt of fibers exposed to 5–10 mM Mn was also reduced when the concentration of Ca_o was elevated to 47 and 67 mM (Fig. 9). In the absence of Mn, Δt was increased from 9 to 20 mv in the presence of 47 mM Ca_o and from 11 to 30 mv in 67 mM Ca_o. Mn in concentrations ranging from 1 to 5 mM had essentially no effect on the Δt in fibers exposed
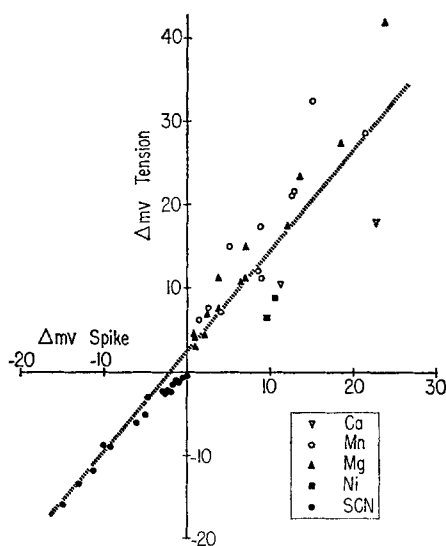

FIGURE 6   FIGURE 7

FIGURE 6. Correlation between the depolarization required to elicit Ca spikes (abscissa) and tension (ordinate). Symbols are identified in the inset box. Further description is given in text.
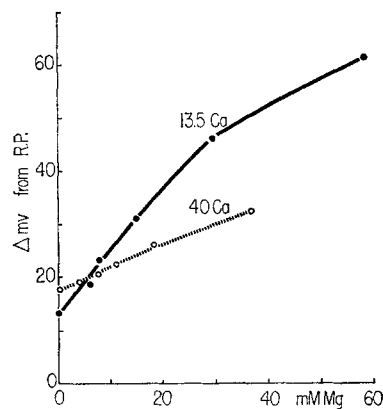
FIGURE 7. Reduction of the effect of increasing Mg on the tension threshold by high Ca_o. ●, control threshold curve (13.5 mM Ca_o); ○, tension thresholds in the presence of 40 mM Ca_o. The resting potential was −80 mv in 13.5 mM Ca_o and −83 mv in 40 mM Ca_o.

to 67 mM Ca_o (upper graph of Fig. 9). However, in the lower concentration of Ca_o (47 mM), Δt increased approximately 8 mv when the Mn concentration was increased to 5 mM (lower graph of Fig. 9).

The experiments of Figs. 7–9 suggest a dual effect of Ca_o on the processes of initiating tension. On the one hand increasing Ca_o elevates Δt as do the other tested divalent cations. On the other hand, when Mg or Mn is ≥ 5 mM increasing Ca_o lowers Δt (Figs. 7 and 9). Therefore, an increase in Ca_o can lower Δt as well as raise it, depending upon the concentration of other divalent cations. A similar reduction in Δt with increasing Ca_o was also shown
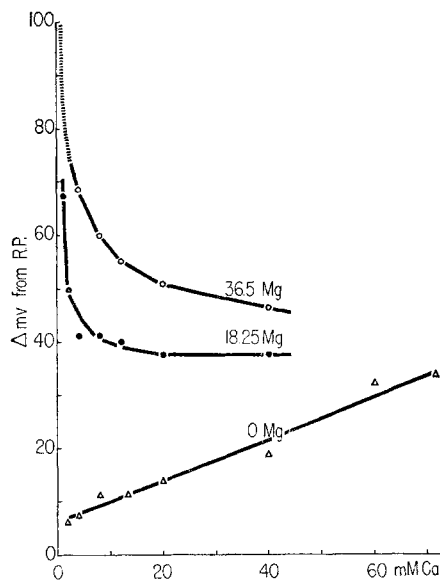
FIGURE 8   FIGURE 9

FIGURE 8. Antagonism between Ca and Mg in altering the tension threshold. △, thresholds in the absence of Mg; ○, decreasing threshold values with a background Mg concentration of 36.5 mM; ●, threshold in the presence of 18.25 mM Mg. Further description is given in the text.
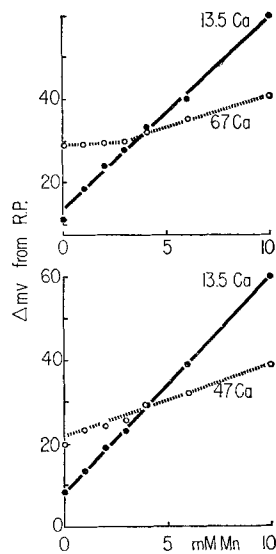
FIGURE 9. Influence of increasing Ca_o on the tension threshold elevation induced by Mn; two experiments. *Upper graph:* the elevation of Δt by increasing Mn was examined first in 13.5 mM Ca_o and then in the presence of 67 mM Ca. *Lower graph:* in this experiment Ca was raised from 13.5 to 47 mM. Further description is given in the text.

for muscle fibers bathed in Cl-free saline (Reuben et al., 1967, Fig. 18). Hagiwara et al. (1968) also concluded that Ca_o has a dual action on Δt.

DISCUSSION

Our earlier studies (Reuben et al., 1967) on excitation-contraction coupling (ECC) in crayfish muscle fibers supported the channeled current proposal (Girardier et al., 1963) in showing that a membrane conductance change and transmembrane current are controlling parameters in the process. Although we suggested (Reuben et al., 1967) that both Cl and Ca ions might transport the current which is critical for ECC, it was evident that further information was necessary. The present work shows that the changes that are induced by several ions in the threshold for initiating tension are similar to the effects of the same agents on the threshold for an electrogenic process, the Ca spike, that is known (Takeda, 1967) to involve an inward movement of Ca across the membrane. In striking contrast were the results of a few preliminary experiments in which muscle fibers were exposed to TEA. Since this ion diminishes or blocks depolarizing K activation (Werman and Grundfest, 1961)

the finding that the threshold for spike electrogenesis is decreased upon increasing TEA was to be expected. Also expected was the finding that the threshold for initiating tension by intracellularly applied currents, which is a Ca-dependent process, remained unchanged even in the presence of 100 mM TEA. Thus, it is likely that a contribution of a potassium current to ECC can be ruled out in crayfish muscle as well as in frog (Adrian et al., 1969; Kao and Stanfield, 1968, 1970).

There are abundant data in the literature which show a correlation between membrane Ca fluxes and contractile activation (see Bianchi, 1969). A transient Ca movement has been noted to occur during a phasic tension response (Bianchi, 1961) and persistent Ca flux occurs during prolonged tension (Shanes, 1961). However, there are numerous divergent proposals regarding the interpretation of the Ca-flux data in ECC. In heart, slow muscle fibers, and smooth muscle, the membrane Ca current is believed to provide Ca ions to the activator sites on the contractile proteins (see Niedergerke, 1956; Lüttgau, 1963; Somlyo and Somlyo, 1969; Bianchi, 1969) and presumably the Ca influx is essential for ECC in these muscles. In striated skeletal muscle, however, the role of Ca movement across the boundary membrane of the fiber in ECC has been deemphasized for several reasons (see Feinstein and Paimre, 1969; Sandow, 1965):

(a) The amount of Ca estimated to enter the fiber for full electrical activation is considerably smaller than that required to saturate the troponin (see Bianchi, 1969).

(b) In most cases tension and its initiation have not been correlated with the electrical and chemical driving forces for Ca ions across the fiber membrane (see Hagiwara et al., 1968).

(c) The extensive work on the Ca-accumulating properties of the sarcoplasmic reticulum (SR) has led many workers to conclude that the SR is the source of Ca during ECC.

Although the correlation between the thresholds for the initiation of tension and membrane Ca conductance change ($P > 0.95$; Fig. 6) provides the basis for the proposal that a membrane Ca current is a required step in ECC in striated muscle, at present the evidence is rather meager regarding the steps that must intervene between a membrane Ca current and the activation of the contractile proteins. In excitation-secretion coupling (ESC) the membrane Ca current does not appear to release the transmitter by merely supplying Ca ions to the cytoplasm, since injection of Ca into the presynaptic terminal is not an effective stimulus for the release of transmitter (Kusano, 1968; Miledi and Slater, 1966). In view of these data some care should be exercised in evaluating the role of a membrane Ca current in muscle, even though the injection of Ca induces tension.

Mn is perhaps the best experimental agent available for indicating the

existence and functional importance of a membrane Ca current in a given cellular process. It has been shown to block selectively the Ca current which causes the action potential in barnacle muscle fibers (Hagiwara and Nakajima, 1966) and to eliminate the Ca spikes in crayfish fibers (Fatt and Ginsborg, 1958; Takeda, 1967). Mn blocks ESC at the squid synapse (Katz and Miledi, 1969) and also acts presynaptically to block transmission in the lobster neuromuscular system (April and Reuben, 1971). The blockage or reduction of tension in a wide variety of muscle fibers, crayfish (Orkand, 1962; Chiarandini et al., 1970 b), frog (Chiarandini and Stefani, 1971), and heart (Rougier et al., 1969; Ochi, 1970; Kohlhardt et al., 1971) by Mn is consistent with the necessity of a membrane Ca current for ECC, since the Ca blocking action of Mn appears to be restricted to the cell surface (unpublished data). Furthermore, the effect of Mn is reduced by increasing the concentration of Ca ions in the external bathing solution (Fig. 9).

The correlation of the effects of SCN and the "foreign" divalent cations (Me) on Δt and Δs (Fig. 6) supports the view that many known potentiators and depressants of contractile activation act by modifying the Ca conductance of the fiber membrane. However, the view that ECC involves Ca influx appears to be contradicted by the effect of increased Ca_o which also raised Δt and Δs. The discrepancy is resolved by the data of Figs. 7–9 which demonstrate that Ca has a dual action. Like the other divalent cations Ca "stabilizes" cell membranes (Shanes, 1958; Werman and Grundfest, 1961; Hagiwara et al., 1969) and, thus, both Δ's are elevated as Ca_o is increased. Thus, the increased influx of Ca that results from an increased Ca gradient is manifested when the membrane is already "stabilized" by other Me^{++}. The degree of the resulting decrease in Δt must be in competition with the "stabilizing" effect of Ca itself. That the final level of Δt as Ca_o is increased in the presence of Mg (Fig. 8) is about the same as when Ca_o is high might result from a reduction of Ca conductance (g_{Ca}) or from a shift in the Ca activation threshold to greater depolarization.

The data of Fig. 8 are strikingly similar to those of Fig. 18 of Reuben et al. (1967) on the effects of changing Ca_o in the presence or absence of Cl. When the fiber is bathed in the control saline (solution A of the present work) progressive increase in Ca_o from 3 to 13.5 mM causes an increase in Δt of about the same degree as in the present work. However, when Cl is replaced with propionate Δt is very high in low Ca_o, but decreases as Ca_o is raised. The effect of the Cl-free medium is overcome by 13.5 mM Ca_o, but (unpublished experiments) as Ca_o is increased further Δt increases no matter whether Cl is present or absent. Thus, the present data in conjunction with the earlier findings (Reuben et al., 1967) support the proposal of the channeled current hypothesis (Girardier et al., 1963) that the transmembrane current and, specifically, that carried by Ca ions, is a critical parameter in ECC.

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