Differences in Na and Ca Spikes As Examined by Application of Tetrodotoxin, Procaine, and Manganese Ions

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ABSTRACT The effects of tetrodotoxin, procaine, and manganese ions were examined on the Ca spike of the barnacle muscle fiber injected with Ca-binding agent as well as on the action potential of the ventricular muscle fiber of the frog heart. Although tetrodotoxin and procaine very effectively suppress the "Na spike" of other tissues, no suppressing effects are found on "Ca spike" of the barnacle fiber, while the initiation of the Ca spike is competitively inhibited by manganese ions. The initial rate of rise of the ventricular action potential is suppressed by tetrodotoxin and procaine but the plateau phase of the action potential is little affected. In contrast the suppressing effect of manganese ions is mainly on the plateau phase. The results suggest that the plateau phase of the ventricular action potential is related to the conductance increase in the membrane to Ca ions even though Na conductance change may also contribute to the plateau.

Spike potentials of most excitable tissues such as the squid giant axon (Hodgkin and Katz, 1949) and the skeletal muscle fiber of the frog (Nastuk and Hodgkin, 1950) are believed to occur as a result of an increase in Na ion conductance of the membrane followed by an increase in K conductance. In contrast to this, the spike potential of some crustacean muscle fibers is produced by an increase in the membrane conductance to Ca ions; this is also followed by an increase of K conductance. The latter type of spikes can, therefore, be called the "Ca spike" and the former the "Na spike." The Ca spike was demonstrated in a crayfish muscle fiber by Fatt and Ginsborg (1958), Parnas and Abbott (1964), and in barnacle muscle fiber with low internal Ca^{++} concentration by Hagiwara and Naka (1964). In the action potential of the frog heart ventricle, the rapid depolarization seems to be due to an increase in Na conductance,

while the overshoot and the following plateau seem to be determined to an appreciable extent by an increased permeability to Ca^{++} (Orkand and Niedergerke, 1964).

There are a number of pharmacological agents known to suppress the Na spike. Tetrodotoxin at a concentration of about 10^{-8} g/ml of the external solution abolishes the Na spike in various tissues (Narahashi et al., 1960; Nakajima et al., 1962; Loewenstein et al., 1963; Narahashi, Moore, and Scott, 1964; Nakamura, Nakajima, and Grundfest, 1964 *a*, *b*), by suppressing the Na conductance change without affecting the K conductance. Procaine also suppresses the Na spike at a relatively low concentration, but it affects both the Na^+ and K^+ conductance changes (Shanes et al., 1959; Taylor, 1959).

The present paper is concerned with the manner in which these suppressing agents for the Na spike affect the membrane changes related to Ca ions in the barnacle muscle fiber and the frog heart ventricle. The paper also deals with the effect of Mn ions which are considered to be a suppressing agent of the Ca spike in the crayfish muscle fiber (Fatt and Ginsborg, 1958). A preliminary note has been published (Hagiwara and Nakajima, 1965).

MATERIALS AND METHODS

Barnacle Fibers

Giant muscle fibers were obtained from a species of barnacle, *Balanus nubilus*, collected on the Pacific coast of the United States. The muscle fibers were prepared and injected with solutions as described previously (Hagiwara and Naka, 1964). The potential changes of the membrane were recorded with 3 M KCl-filled glass micropipettes while the current was applied through the injection pipette. The rate of rise of the spike potential was recorded by differentiating the potential change. The time constant of the differential circuit was 100 μsec.

The normal barnacle saline had the following composition (Hoyle and Smyth, 1963) NaCl, 466 mM; KCl, 8 mM; $CaCl_2$, 20 mM; $MgCl_2$, 12 mM; Tris-HCl buffer (pH 7.8), 10 mM. Ca saline was obtained by replacing NaCl of the normal saline with an osmotically equivalent amount of $CaCl_2$. Ca-free saline was obtained by replacing $CaCl_2$ with NaCl. Ba saline was obtained by replacing $CaCl_2$ of the Ca saline with $BaCl_2$. The saline of a desired concentration of Ca^{++} or Ba^{++} was obtained by mixing appropriate amounts of the Ca- or Ba- and Ca-free salines. Tetrodotoxin and procaine were dissolved in the saline. The saline of a desired concentration of Mn^{++} was obtained by replacing an appropriate amount of NaCl in the saline with an osmotically equivalent amount of $MnCl_2$.

The solution injected had the following composition; EGTA, 100 mM; KOH, 400 mM; Tris-maleate buffer, 20 mM; sucrose, 349 mM; methanesulfonic acid, 180 mM. The pH was adjusted to 7.0 with additional small amounts of methanesulfonic acid.

Frog Heart

The ventricular preparation of the heart was obtained from *Rana pipiens*. A strip prepared from the ventricle was placed on a paraffin block in a Petri dish with the

inner surface of the ventricle facing upward. The stimulating electrodes, consisting of a pair of fine silver wires insulated with glass except at the tips, were placed on one end of the strip. The resting and action potentials were recorded with 3 M KCl-filled glass micropipettes. The stimulating frequency was less than 1/min. The rate of change of the action potential was recorded by differentiating the potential change with a time constant of 100 μsec.

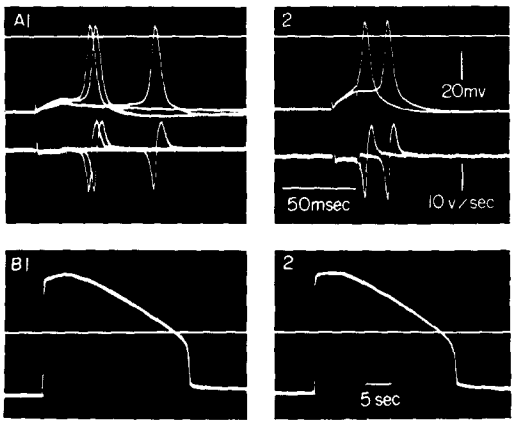

FIGURE 1. Effect of tetrodotoxin on barnacle muscle fiber. *A*, spike potentials (middle trace) in the normal barnacle saline containing 20 mM of Ca^{++}. Two records were taken from the same fiber before (*A*1) and after (*A*2) the application of tetrodotoxin (4×10^{-6} g/ml of the external solution). Upper trace, reference potential level. Lower trace, derivative of the potential change; a downward deflection corresponding to an increase of the rate of rise. A slightly higher firing potential level was seen after the application of tetrodotoxin in this particular example. But this was not a constant phenomenon. *B*, spike potentials in 42 mM Ba-saline before (B_1) and after (B_2) the application of tetrodotoxin (4×10^{-6} g/ml).

The normal Ringer solution used had the following composition: NaCl, 115 mM; KCl, 2.5 mM; $CaCl_2$, 1.8 mM; Tris-HCl buffer (pH 7.2), 10 mM. Preparation of tetrodotoxin, procaine, or manganese solutions was similar to that in the case of barnacle muscle fiber.

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

RESULTS

I. *Barnacle Muscle Fiber*

1. TETRODOTOXIN Following injection of Ca-binding agents such as EGTA into the barnacle muscle fiber, the fiber is capable of initiating all-or-none spikes if the bath solution is the normal barnacle solution described above (Fig. 1*A*1). Record *A*2 of the same figure shows the response of the same fiber after tetrodotoxin was added to the bath at a concentration of 4×10^{-6} g/ml. In each record the lowest trace shows the time course of the

derivative of the membrane potential change and the downward deflection of the trace corresponds to a positive value of the derivative of the action potential and an increase in the magnitude of the deflection corresponds to an increase in the rate of rise. There was no change, either in the spike amplitude or in the rate of rise due to the application of tetrodotoxin at this concentration which is more than 100 times that effective for suppression of the Na spike. The result, therefore, indicates that tetrodotoxin is ineffective for abolishing the Ca spike.

All-or-none spike potentials are also obtained from the injected fibers in

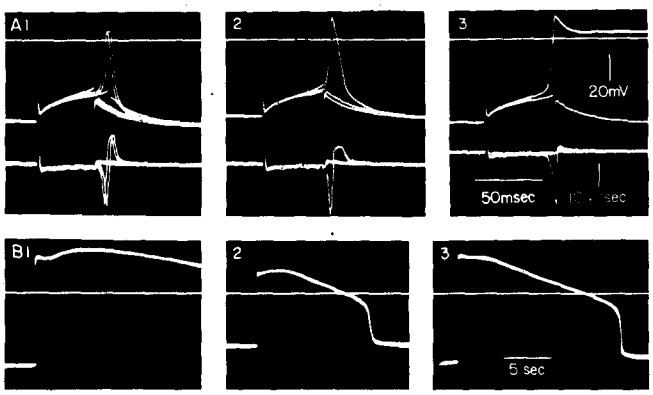

FIGURE 2. Effect of procaine on barnacle muscle fiber. *A*, spike potentials in the normal saline containing 20 mM Ca^{++}. 1, no procaine; 2, 5×10^{-4} g/ml procaine; and 3, 2×10^{-3} g/ml procaine. *B*, spike potentials in 42 mM Ba saline without (1) and with (2 and 3) 1×10^{-3} g/ml procaine. A hyperpolarizing current was applied to the membrane in 3.

Ba media (Hagiwara and Naka, 1964). Records *B*1 and *B*2 show Ba spikes obtained in 42 mM Ba solution before and after the application of 4×10^{-6} g tetrodotoxin per ml of the external solution. It is seen that tetrodotoxin is also without effect on the Ba spike.

2. PROCAINE The effects of two different concentrations of procaine on the spike potential of a muscle fiber are shown in Fig. 2*A*. In low concentration (5×10^{-4} g/ml) there was an increase of the overshoot of the spike as well as of the rate of rise, and the time course became slightly prolonged (Fig. 2*A*2). At higher concentrations (Fig. 2*A*3, 2×10^{-3} g/ml) the overshoot and the rate of rise did not show any further increase but the time course became very much prolonged and the initial peak of the spike was followed by a plateau which often lasted for several seconds. The results show that procaine has no appreciable suppressing effect on the Ca conductance change. In the squid giant axon procaine reduces the K conductance increase (Shanes et al., 1959; Taylor, 1959). The slight increase of the spike overshoot and the

prolongation of the spike with procaine in the barnacle muscle can be explained if procaine also reduces the K conductance increase.

The effect of procaine on the Ba spike (Fig. 2*B*1) was complicated. When procaine was added to the external medium (1×10^{-3} g/ml) the overshoot of the spike potential became smaller (Fig. 2). The decrease was always associated with a substantial depolarization (up to 20 mv). When the resting potential was restored in the procaine media by applying a dc hyperpolarizing current, the overshoot of the spike potential recovered to the original value (Fig. 2*B*3). The results, therefore, indicate that the decrease of spike overshoot in procaine is a secondary effect due to depolarization. At present we have no adequate explanation for the depolarization by procaine in Ba media. The

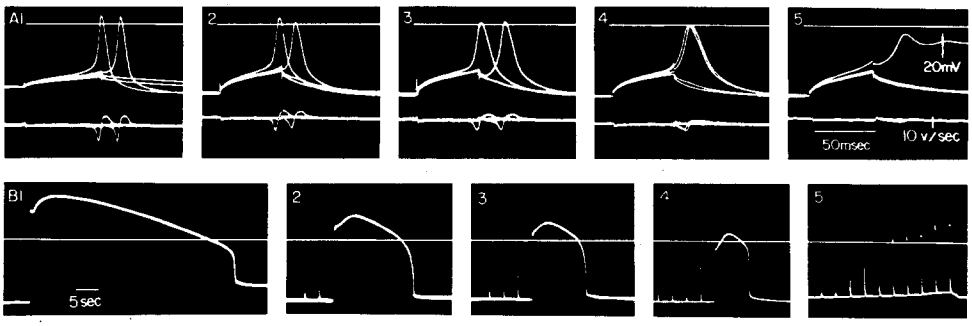

FIGURE 3. Effect of Mn^{++} on barnacle muscle fiber. *A*, spike potentials in normal saline containing 20 mM of Ca^{++}. 1, no Mn^{++}; 2, 2 mM Mn^{++}; 3, 4 mM Mn^{++}; 4, 8 mM Mn^{++}; and 5, 16 mM Mn^{++}. *B*, spike potentials in 42 mM Ba saline. 1, no Mn^{++}; 2, 2 mM Mn^{++}; 3, 4 mM Mn^{++}; 4, 16 mM Mn^{++}; and 5, 40 mM Mn^{++}.

results, nevertheless, indicate that procaine has no appreciable suppressing effect on the membrane conductance change to Ba ions.

3. MANGANESE IONS The series of records in Fig. 3*A* was obtained in normal barnacle saline during stepwise increases of $MnCl_2$ concentration. The concentrations were 0, 2, 4, 8, and 16 mM in records 1 to 5, respectively. The rate of rise of the spike potential decreased as the Mn^{++} concentration increased and at 16 mM the spike became very small (Fig. 3*A*5). At relatively high concentrations of Mn^{++} the spike was sometimes followed by a long lasting depolarization (Fig. 3*A*5). The depolarization, however, did not seem to be specific for Mn since it occurred under various other conditions. In addition, the magnitude of the long lasting depolarization did not increase with an increase of the Mn^{++} concentration, but, on the contrary, decreased. A similar suppression by Mn^{++} was also seen for the Ba spike (Fig. 3*B*). With increasing levels of Mn^{++} the firing level of the membrane potential for the spike rose and the overshoot decreased, and the membrane became inexcitable at 40 mM Mn^{++} when the Ba concentration was 42 mM.

The suppressing effect of Mn^{++} on the Ca spike became less marked as the external Ca concentration was increased. In Fig. 4 the records in column 1 show the responses in 10 mM Ca^{++} and those in column 2 show the responses in 270 mM Ca^{++}. In the latter situation the overshoot as well as the rate of rise is larger as expected (*A*1 and *A*2). The effect of the addition of 8 mM Mn^{++} is shown in *B*1 and *B*2. The rate of rise and the overshoot were both

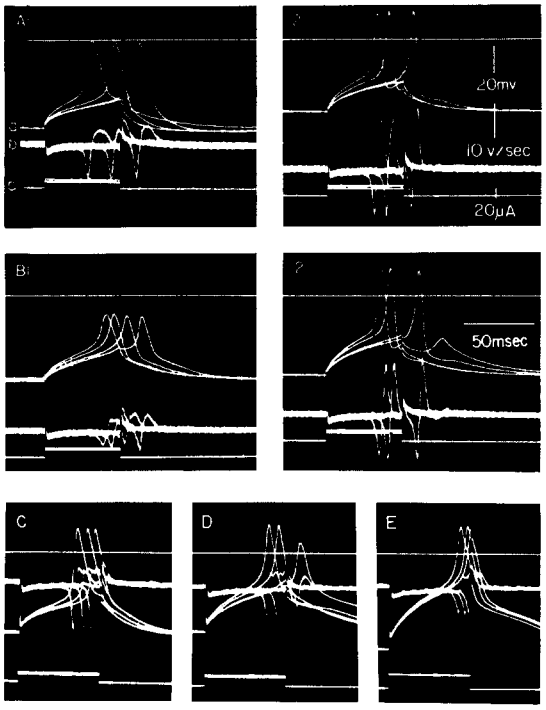

FIGURE 4. Effect of Ca^{++} concentration on Mn inhibition. Records *A* and *B* were obtained from the same fiber. Mn^{++} concentration, 0 in *A* and 8 mM in *B*. Ca^{++} concentration, 10 mM in 1 and 270 mM in 2. Records *C*, *D*, and *E* were taken from a different fiber and the external Ca^{++} concentration was 12 mM. *D* and *E* were obtained with 8 mM Mn^{++}. A hyperpolarizing current was applied in *E*. Trace *a*, potential. Trace *b*, derivative of potential. Trace *c*, current.

markedly suppressed in 10 mM Ca, but the suppression in 270 mM Ca was not as striking. It is known that lowering the external Ca concentration shifts the inactivation curve for spike initiation towards a more negative membrane potential in the squid axon (Frankenhaeuser and Hodgkin, 1957). If Mn^{++} caused a similar shift of the inactivation curve in the negative direction, the spike potential would be suppressed and this suppressing effect of Mn^{++} would be especially apparent at lower Ca concentrations. However, if this were the case, the suppression by Mn^{++} at lower Ca concentrations should be partially reversed with membrane hyperpolarization. Fig. 4 *C*, *D*, and *E* shows records

obtained from the same fiber in 12 mM Ca media; *C* was taken before the application of 8 mM Mn^{++} and the maximum rate of rise was reduced to approximately half after the application (*D*). Hyperpolarization of the membrane did not reverse the effect of Mn^{++} (*E*). Therefore, the more marked suppression by Mn^{++} at lower external Ca concentrations cannot be explained by a shift of the inactivation curve, but is due rather to a direct effect of Mn^{++} on the spike process.

In a few cases the effects of 8 mM Mn on the rate of rise of the spike potentials were examined in the same muscle fiber in various external Ca concentrations. The relation between the maximum rate of rise (*Y*-axis) and the Ca^{++}

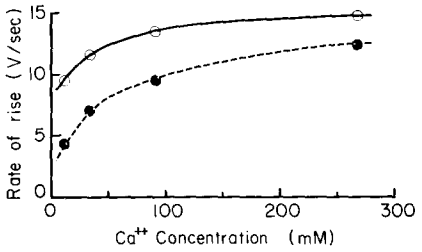

FIGURE 5. Relationship between the rate of rise of the spike potential and the external Ca^{++} concentration with (filled circles) and without (open circles) 8 mM of Mn^{++}. All data were obtained from the same fiber.

concentration (*X*-axis) obtained in one of these experiments is shown in Fig. 5. The percentage reduction of the maximum rate of rise by Mn^{++} was small at 270 mM Ca^{++} while the maximum rate of rise was reduced to less than half at 8 mM Ca^{++}. The result suggests that Mn may compete with Ca for occupancy of the sites necessary for spike initiation.

II. *Ventricular Muscle Fiber of Frog Heart*

1. TETRODOTOXIN The action potential of a ventricular fiber of frog heart in normal Ringer's solution is shown in Fig. 6*A*. Trace *a*, taken with a slow sweep speed, shows the total time course of the action potential; trace *b*, with a faster sweep speed the rising phase of the action potential; and trace *c*, the reference potential level. Trace *c* also shows the derivative (recorded simultaneously with trace *b*) of the potential change with respect to time and its downward deflection corresponds to a positive value of the derivative of the action potential. The action potential initially rose rapidly to a given potential level and then slowly to the maximum potential level; this was followed by a slow fall, forming a plateau. The maximum potential level was, therefore, reached during the plateau phase sometime after the initial rapid rise. The overshoot of the fiber immersed in the normal Ringer solution measured 44 ± 6 mv (SD).

The effect of tetrodotoxin is shown in Fig. 6 *B*, *C*, and *D*. The records were taken from the same ventricular strip preparation as *A*, but with different impalements. In 10^{-8} g/ml tetrodotoxin the maximum rate of rise usually

decreased to approximately ½ of the normal value (Fig. 6*B*). When the concentration was raised to 2.5 × 10^{-8} g/ml the maximum rate of rise was sharply reduced (Fig. 6*C*). In contrast to this, no significant decrease of the overshoot of the action potential was found. When the preparation was kept in the solution of 2.5 × 10^{-8} g/ml for a long time (Fig. 6*D*, more than 30

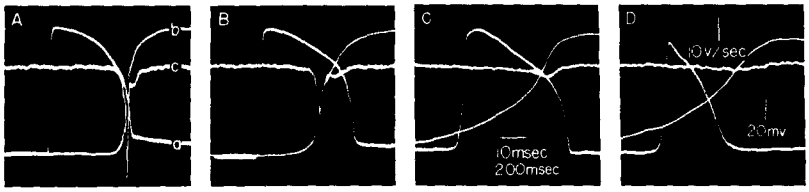

FIGURE 6. Effect of tetrodotoxin on frog cardiac muscle fiber. Traces *a*, *b*, and *c*: a slow sweep potential recording, a fast sweep potential recording showing the rising phase of the action potential, and the reference potential level respectively. Trace *c* also includes differentiated record of trace *b*. *A*, in normal saline, *B*, 1 × 10^{-8} g/ml of tetrodotoxin. *C* and *D*, 2.5 × 10^{-8} g/ml of tetrodotoxin. *D* was obtained after long immersion (more than 30 min) of the preparation in the test solution. The records were obtained from different impalements in the same strip.

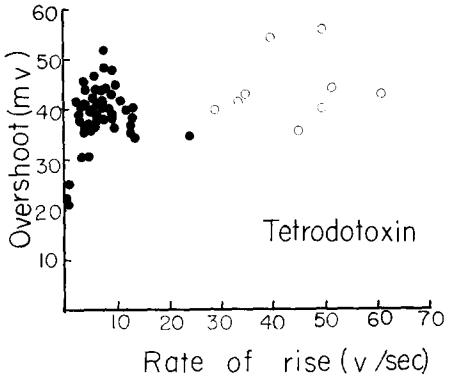

FIGURE 7. Relationship between the rate of rise and the overshoot obtained in tetrodotoxin media. Open circles in normal saline and filled circles, with tetrodotoxin of various concentrations.

min) or exposed to higher concentrations (5 × 10^{-8} g/ml) of tetrodotoxin, the maximum rate of rise was reduced further and at the same time some decrease of the overshoot occurred. This was soon followed by conduction block.

The relation between the maximum rate of rise (*X*-axis) and the overshoot (*Y*-axis) of the action potential is plotted in Fig. 7. Data were obtained from fibers in two strip preparations, open and filled circles representing, respectively, data in normal Ringer's and in media containing various concentrations of tetrodotoxin. The results show that the decrease of the maximum rate of rise in tetrodotoxin is not associated with an appreciable decrease of the overshoot until the rate of rise becomes extremely small. These effects of

tetrodotoxin were reversible as a rule but sometimes the recovery was incomplete following prolonged exposure to high concentrations of tetrodotoxin.

2. PROCAINE The effects of procaine were similar to those found for tetrodotoxin. The maximum rate of rise was reduced to almost 20% of the normal value at a concentration of 5×10^{-4} g/ml (Fig. 8*B*) and at 2×10^{-3} g/ml the rate of rise became markedly reduced (Fig. 8*C*). However, the overshoot was unchanged by procaine. The relation between the maximum rate of rise and the overshoot is shown by Fig. 9. These results show that the over-

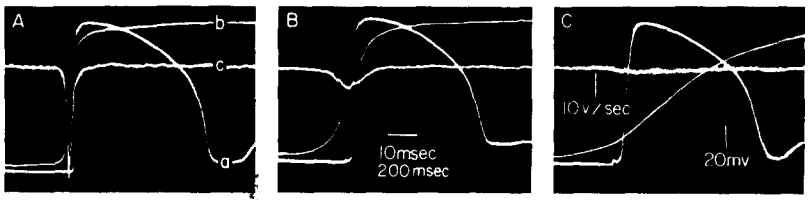

FIGURE 8. Effect of procaine on frog cardiac muscle fiber. *A*, in normal saline. *B*, 5×10^{-4} g/ml procaine. *C*, 2×10^{-3} g/ml procaine. Three records were obtained from different impalements in the same strip.

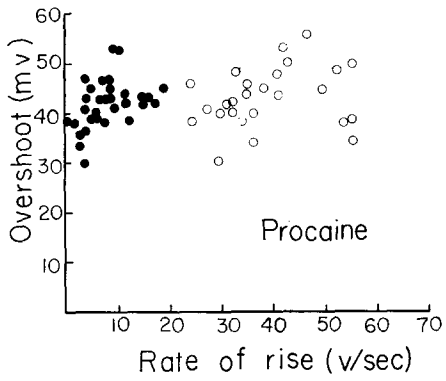

FIGURE 9. Relationship between the rate of rise and the overshoot in procaine media. Open circles, in normal saline and filled circles, with procaine of various concentrations.

shoot is less dependent upon the rate of rise under procaine than under tetrodotoxin. Procaine suppresses the K conductance increase of the membrane in the squid axon (Shanes et al., 1959; Taylor, 1959) while the K conductance change is not affected by tetrodotoxin (Nakamura, Nakajima, and Grundfest, 1964 *b*; Narahashi, Moore, and Scott, 1964). If this is also applicable to cardiac muscle fibers, the suppression of the K conductance increase may partly contribute to maintaining the overshoot at a high level in procaine. The effect of procaine was usually reversible.

3. MANGANESE IONS In contrast to the effects of tetrodotoxin and procaine, the effect of manganese ions on the cardiac action potential was mainly on the plateau phase of the overshoot. A series of records in Fig. 10

shows action potentials of different fibers in the same ventricular strip following stepwise increases in Mn^{++} concentration. In each pair of records three traces, *a*, *b*, and *c*, were obtained simultaneously. As described above, the action potential rises to the maximum level in two steps, first rapidly to a given level,

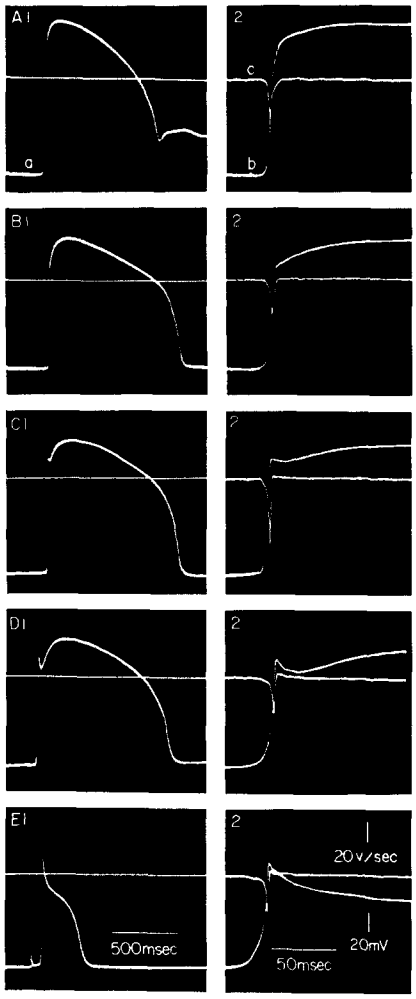

FIGURE 10. Effect of Mn^{++} on frog cardiac muscle fiber. *A*, in the normal saline. *B*, 2 mM Mn^{++}. *C*, 4 mM Mn^{++}. *D*, 8 mM Mn^{++}. *E*, 10 mM. Record 2 of each pair shows the rising phase of the action potential in trace *a* of record 1 with a faster time base (trace *b*) and the rate of rise of the action potential (trace *c*). The records were obtained from different impalements in the same strip.

and then slowly to the maximum. The maximum rate of rise of the action potential represents the rate during the first phase. In normal Ringer's solution, however, these two steps are not very clear. They became clear under the influence of manganese since it affects mainly the second slow phase. The maximum potential level was reached with an increasing delay from the initial rise, and at the same time the maximum potential level itself was reduced; i.e., the overshoot of the action potential became smaller. In this range of Mn^{++} concentrations (2 to 4 mM) no significant changes were

found in the maximum rate of rise of the action potential. In other words the initial rapid phase of the potential rise was unchanged, and the effect was exclusively on the overshoot of the action potential. At 8 mM Mn^{++} (Fig. 10*D*) the delay in the development of the plateau became more marked so that a dip appeared between the initial rapid rise and the following slow rise of the potential. When the concentration reached 10 mM (Fig. 10*E*), the membrane potential failed to rise after the initial rapid rise and therefore, the major part of the plateau potential disappeared. In these concentrations of Mn^{++} (8 to 10 mM) the rate of rise showed some decrease. The relation between the maximum rate of rise and the overshoot for Mn ions is plotted in Fig. 11. An appreciable decrease in the overshoot can occur independently of the decrease

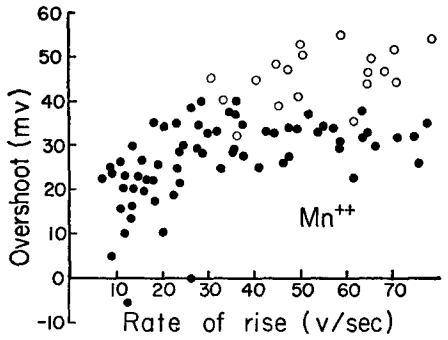

FIGURE 11. Relationship between the rate of rise and the overshoot in Mn^{++} media. Open circles, in normal saline and filled circles, with Mn^{++} of various concentrations.

in the rate of rise. But a further decrease of the overshoot generally occurs together with the decrease of the maximum rate of rise. Since a similar decrease of the maximum rate of rise occurs under tetrodotoxin without any significant decrease of the overshoot, suppression of the overshoot is presumably independent of the effect on the rate of rise. Nothing is known about the effect of Mn^{++} on K permeability in the ventricular fiber. An enhancement of K conductance increase could be a cause of the suppression of the overshoot. Acetycholine suppresses the overshoot in certain cardiac muscle fibers by enhancing the K permeability (Weidmann, 1956). In this condition, however, the decrease of the overshoot was associated with a shortening of the duration of the action potential although this was not the case for the action potential of the fiber in Mn^{++}. In the barnacle muscle fiber, Mn^{++} neither increased the resting conductance nor enhanced the delayed rectification. The results therefore indicate that the suppression of the overshoot by Mn^{++} does not seem to be a secondary effect of the change in K permeability.

Although no quantitative measurements were performed, the action potential usually propagated at an almost normal velocity in relatively low concentrations of Mn^{++} even though the overshoot of the action potential was reduced. In contrast to this, the conduction velocity was sharply reduced under tetrodotoxin and procaine even when the overshoot was still normal.

The difference seems to be related to the fact that the rate of rise is not much affected under Mn^{++} while it is sharply reduced by tetrodotoxin and procaine. Another remarkable difference found between fibers treated by tetrodotoxin and procaine, and by Mn^{++}, was in the contraction associated with the action potential. The action potential with tetrodotoxin and procaine was associated with a slight decrease of the twitch owing probably to the asynchronization of activities among different parts of the strip preparation. This was caused by the reduction of the conduction velocity of the action potential. The contraction was very much reduced under the influence of Mn^{++} even when the overshoot was not much reduced. A similar effect of Mn^{++} on the contraction of crayfish muscle fibers has been observed by Orkand (1962).

DISCUSSION

The results on the barnacle muscle fiber show that tetrodotoxin and procaine are both ineffective on the conductance change of the membrane to Ca ions even though these agents have a strong suppressing effect on the Na conductance change. Recently Ozeki and Grundfest (1965) have shown that the spike occurring in a crustacean muscle fiber in the presence of procaine is not suppressed by tetrodotoxin. Since this "procaine spike" is very likely related to the Ca conductance change, this fact also indicates the ineffectiveness of tetrodotoxin on the Ca conductance change. On the other hand, the Ca conductance increase is suppressed by Mn ions. The suppressing effect of manganese on the Ba spike of the crayfish muscle fiber has been described by Fatt and Ginsborg (1958). The experimental results suggest that Mn may compete with Ca for occupancy of the sites of the membrane necessary for spike initiation. Jenden and Reger (1963) have shown that the deterioration of the membrane potential of the frog skeletal muscle fiber in Ca-free media is prevented by adding a small amount of manganese and that an increase in manganese concentration increases the threshold potential level for the spike, just as observed with an increasing Ca concentration. In the barnacle muscle fiber also, the threshold potential level increased with increasing Mn concentration. Lorković and Edwards (unpublished) found a similar shift of threshold by Mn for K contracture in frog muscle fibers. This suggests also that manganese ions occupy the sites of the membrane normally occupied by calcium ions.

The initial rapid rise of the action potential in the frog ventricle is related to the conductance increase of the membrane to Na^+ (Brady and Woodbury, 1960). However, the overshoot of the action potential of a guinea pig ventricle is rather insensitive to the external Na^+ concentration (Coraboeuf and Otsuka, 1956). Recently Orkand and Niedergerke (1964) have shown that the plateau phase of the action potential of the frog ventricle is related to a

Ca^{++} conductance increase, although the permeability increase may not be sufficient to explain completely the potential change, and the Na^{++} permeability may also contribute to the plateau. The present results show that tetrodotoxin and procaine suppress the rate of rise of the action potential at concentrations similar to those effective on the Na spike of other excitable tissues. This finding is in agreement with the conclusion that the initial rise of the action potential is produced by the conductance increase of the membrane to Na ions. In contrast to this, the plateau phase of the action potential is fairly insensitive to both tetrodotoxin and procaine, but is affected by Mn ions which suppress the Ca spike in barnacle muscle fiber. This suggests that the membrane change during the plateau phase of the action potential in the frog ventricle is related to Ca^{++} conductance increase even though Na conductance change may also contribute to the plateau. The above conclusion is, however, based exclusively on the analogy between the Ca spike of the barnacle fiber and the plateau potential of the heart.

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