Mechanism of Activation of Contraction in Frog Ventricular Muscle

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SUMMARY The force of contraction and transmembrane potential were recorded from extremely short segments of frog ventricle strips, in which extracellular ionic composition could be changed rapidly, and a condition in which there was a relatively uniform space clamp. Large and abrupt increases in contractile force (and, presumably, in intracellular free calcium concentration) had no detectable effect on subsequent contractions. In contrast, an increase in extracellular calcium concentration was associated with a progressive increase in contraction extending over several beats. Displacement of transmembrane potential during an action potential affected contraction in a manner opposite to that to be expected if a calcium current were to play a significant role in activation. The activation process could be saturated by sufficient displacement of transmembrane potential to high inside positive levels. The voltage displacement necessary for saturation was less the higher the extracellular calcium concentration. Force development occurring after displacement of transmembrane potential to levels above the equilibrium potential for calcium was rapidly sensitive to alterations in extracellular composition, including addition of 10 mM Mn²⁺, an increase in calcium concentration, and a decrease in sodium concentration. These observations provide further evidence that the source of calcium for activation of contraction in frog ventricular muscle consists exclusively, or almost exclusively, of a rapid entry of this ion into the cell. This process contributes throughout the range of voltage dependence of activation: i.e., from threshold to saturation. The observations suggest that such transsarcolemmal calcium movement is brought about by driving forces which add to those of electrodiffusion.

FROG VENTRICLE does not show a variety of positive and negative inotropic effects of one beat on subsequent ones, such as postextrasystolic potentiation and the weakness of the premature beat. This is in marked contrast to mammalian cardiac muscle, in which such delayed inotropic effects are of great magnitude and have been linked, on several grounds, to the function of intracellular calcium stores in the activation of contraction. Intracellular bound calcium, which occurs in large amounts in frog ventricular muscle, thus may not play an important role in activation, a view also in accord with several reports that sarcoplasmic reticulum is sparse and T tubules absent in this tissue.

Other aspects of the behavior of frog ventricle appear, in contrast, to be most easily understood in terms of a significant contribution of calcium storage sites to contraction. These include staircase and the frequency-force relationship and a prolonged, progressive effect on contraction following alteration of extracellular calcium concentration and occurring over many beats. In addition, the positive inotropic action of caffeine has been interpreted in this way and especially the observation of caffeine-induced force development by the frog atrial trabecula in an essentially calcium-free medium.

The contribution of calcium entry, during the action potential, to the activation of contraction (in turtle ventricle) is most strongly indicated by the fact that an increase in extracellular calcium concentration, initiated after the onset of a contraction, increases the force of the same beat. In addition, in frog ventricle, premature relaxation rapidly follows a prematurely induced repolarization or a sudden reduction in extracellular calcium concentration induced during a prolonged voltage clamp pulse.
In the experiments to be reported, the combined techniques of voltage clamp and of rapidly induced alterations in extracellular composition were used to assess further the relative contributions of transmembrane calcium entry and of intracellular calcium release to the activation of contraction in frog ventricular muscle. The observations reported offer further evidence that the source of calcium which mediates activation consists exclusively, or almost exclusively, of an influx into the cell and give support to a suggestion\(^2\) that such an influx may arise from other than purely electrodiffusional driving forces. That the calcium sink (intracellular binding or extrusion) may be an important additional determinant of activation is not excluded by these findings (see Figure 9 and Discussion).

**Methods**

**Preparation**

Strips of frog ventricle (\textit{Rana pipiens}) were dissected from muscle running parallel to the atrioventricular groove. The experimental arrangement is shown in Figure 1. Each strip, 0.8-1.0 mm wide, was mounted by drawing its slightly narrowed, conical end through a hole in a rubber partition until the Rt was fairly snug. The hole was then reduced in size by partially releasing the tension on the rubber sheet. The muscle was firmly snared by a silk thread which connected it, through a rigid glass tube, to an RCA 5734 mechanoelectric transducer, and the end of the muscle was cut off close to the snare. A short segment of the strip thus extended forward, from the rubber partition to the snare, for a distance of 0.10-0.14 mm (at unstretched length). A much longer segment (over 4.0 mm) extended backward behind the partition and floated free in a Ringer-perfused cylindrical channel. A jet of Ringer's solution played vigorously on the short segment, surrounding it with a cuff of fluid which ran off continuously to the floor of the chamber. Flow over each of the segments was at a rate of 10 ml/min., or rapid enough to replace the fluid around the segments more than 10 times each second. The short segment was adjusted to a length just under that optimal for contraction. In satisfactory preparations, the contraction recorded was not affected by changes in calcium concentration in the solution superfusing the long segment, and sodium-lack contracture in this portion of the strip was not transmitted to the force transducer.

**Electrical Recording and Stimulation**

Transmembrane potential was led off from intra- and extracellular, KCl-filled, glass microelectrodes, whose tips were very close to one another at about 1/10 of the distance, along the short segment, from the rubber partition to the silk snare. This potential difference, together with an appropriate bias voltage and command signals, was applied to the summing junction of an operational amplifier. Through a pushbutton switch, either the feedback amplifier output or a 5-msec stimulus pulse could be applied to a chlorided silver electrode close to the long segment. Current flow across the rubber partition was recorded through a similar electrode close to the short segment and connected to an operational amplifier in virtual ground configuration. Stimulation threshold for the preparation varied between 1 and 5 \(\mu\)A.

**Solutions and Solution Changes**

The control Ringer's solution applied to both segments had the following (millimolar) composition: sodium chloride, 110; sodium bicarbonate, 2.4; potassium chloride, 2.5; calcium chloride, 0.7; dextrose, 7.5. Fine bubbles of a 99% O\(_2\), 1% CO\(_2\) mixture were passed through the solution, giving a pH of 7.2. Before voltage clamp procedures, the long segment was equilibrated with a Ringer's solution in which 98% of the sodium was isosmotically replaced by choline as the chloride. The freezing points of both solutions were equal. In the experimental maneuvers described below, the calcium concentration was changed (0.1-2.0 mm) or manganous chloride added (to 1.0 and 10.0 mm), as indicated, in the Ringer perfusing the short segment. The Ringer bicarbonate concentration used in these experiments was kept extremely low in order to maximize the attainable concentration of manganous ion and to achieve large and rapid negative inotropic effects. Thus the true chemical activity of the calcium ion may be higher than occurs in the presence of large amounts of bicarbonate buffer. This might amount to an increase in true calcium activity of as much as 25\%, as compared with a medium buffered with 24 mm bicarbonate, for example, according to Schaer.\(^2\) Experiments were conducted at room temperature, which varied between 23°C and 26°C; but the Ringer temperature did not vary by more than 0.5°C in any one experiment.

The solution perfusing the short segment could be changed rapidly by turning a stopcock located close to the jet. Since the force of the jet against the muscle could
contribute significantly to its resting tension, it was necessary to ensure that changes in muscle length due to slight differences in the forcefulness or position of the jet were not responsible for observed alterations in contraction after switching solution. Equal hydrostatic heads of pressure and equal flows for all solutions (as judged from rotometer flowmeters in the lines) helped to minimize such effects, as did holding the short segment near its length-tension plateau, where contraction is relatively insensitive to length. In addition, a delay between the stopcock and the tip of the jet of about 2 seconds was maintained, so that the force record could be seen to return to its original time course after the switching artifact, but before the new solution reached the muscle.

Maximal rate of rise of the action potential upstroke, measured close to the rubber partition, was unchanged after perfusing the long segment with the low sodium Ringer’s solution. It thus seems likely that significant extracellular sodium gradients did not extend into the short segment. Action potential duration was, however, shortened to varying degrees, depending on the extracellular leakage conductance, and this was accompanied by a reduction in time-to-peak and developed force of the associated contraction. Leakage was sometimes very high, but, most frequently, action potentials measured across the rubber partition ranged between 40 and 70 mV in amplitude. This was found with fair regularity when the hole in the partition was made very small, constricting the muscle from a diameter of 0.9 mm to one of 0.35 mm. Such a profound constriction of the muscle interfered seriously, however, with rapid penetration of its extracellular compartment by the perfusing medium on changing solutions. For most of the experiments reported here, for which rapid access of the Ringer medium to the interstitial spaces was important, the hole in the rubber partition was not as constricting, being reduced only to 0.65 mm in diameter.

**DEGREE OF SPACE CLAMP**

The uniformity of voltage distribution achieved during clamp is of critical importance for the main conclusions of the present study. This was directly monitored, therefore, with an additional microelectrode impalement at a point on the short segment distal to the feedback electrode. Figure 2 shows one such result, for a two-step clamp: the first beyond threshold for the fast inward current, but short of that for contraction; the second to a very high inside positive potential. Although voltage is poorly controlled at the distal monitoring site with the first clamp step (to $-49 \text{ mV}$ inside), it rises very promptly with the second clamp step, coming close to its final value within less than 1 msec and reaching that value within 10 msec. The falling off in voltage from the site of the feedback electrode to the more distal one of the monitoring electrode amounts to 15%, both for the control Ringer and for that in the presence of 10 mM manganese ion. In three additional preparations, the falloff in voltage from the feedback electrode to a more distal monitoring electrode was found to be of comparable magnitude, amounting to 7-10% over distances of 0.11-0.14 mm, all of which comprised $1/10$ of the total length of the short segment.

**Figure 2** Test of longitudinal uniformity of transmembrane potential during voltage clamp to a markedly inside positive potential. The entire time course of the two-step clamp is shown, in each of the four sets of traces, on a slow sweep (2.4-second calibration); the two depolarizing steps and the two repolarizing steps are drawn in, in each case, as dashed lines. The onset and early time course of the second step is shown, in each set, on a faster trace (48-msec calibration) with the upstroke drawn in as a lighter dashed line and at a greater amplifier gain. When the transmembrane potential at the feedback electrode (traces in left column) is displaced to $+115 \text{ mV}$ inside, the voltage monitored at a more distal site (traces in right column) can be seen to be only $+85 \text{ mV}$ inside. This voltage distribution is the same in the control Ringer (upper row of traces) and in the presence of 10 mM manganese chloride (lower row).
Levels of clamp in these preparations were to +5 mV, +43 mV, and +106 mV, respectively. Finally, the extracellular voltage difference during clamp, for the preparation shown in Figure 2, was small and amounted to 4 mV for the clamp level shown.

Voltage control achieved during clamps in the region between the voltage threshold for the fast inward current and that for contraction was quite variable from one preparation to another. In some it was poor (for example, the first clamp step for the preparation shown in Figures 2, 13, and 14), while, in others, uniform voltage control was rapidly attained and thus no phasic contractions were visible during the first step (preparations shown in Figures 9, 10, and 15). Contraction during the second clamp was not influenced by the introduction of a prior depolarization, as was reported previously. The use of the two-step clamp provides, with the second step, a rapidly attained voltage of fair distribution prior to force development, as documented in Figure 2, and this procedure was used in all maneuvers where contractile responses at high inside positive potentials were studied (results shown in Figures 9-11 and 13-15).

Results

RAPIDITY OF THE EFFECT ON CONTRACTION OF CHANGES IN EXTRACELLULAR CALCIUM CONCENTRATION

If the activation process in frog ventricle were mediated exclusively by a transsarcolemmal influx of calcium, one might expect the contraction to reach a new steady state in the beat immediately following an increase in the calcium concentration of the Ringer’s solution. This might occur if one were able to alter, rapidly and uniformly, the extracellular calcium concentration. Such a result is shown in Figure 3A, where a change in calcium, initiated very early in the 5-second interval between beats, brings the next beat to almost zero force (in 0.1 mM Ca), to 94% of its maximal value (in 2.0 mM Ca) and back to the exact control level (in 0.7 mM Ca). This occurred rarely. A result more typical of these experiments and those of others is shown in Figure 3B, where a new steady state is not quite attained after four beats at 30-second intervals. To a large extent, the beat-by-beat progress to a new steady state could be retarded or speeded up by adjusting the manner in which the jet made contact with the short segment, even after several hours of elapsed experimental time. Rapidity of change in contraction was promoted in general, by not making the silk snare or the hole in the rubber partition any tighter than was necessary to hold the short segment securely. In addition, a small reduction in its length from the optimal one for contraction allowed the segment to arc very slightly in the Ringer jet and to spread apart the fascicles in the spongy structure of the muscle.

Even when such maneuvers were painstakingly carried out, muscles varied widely in the promptness of their contractile responses to a change in Ringer calcium. In Figure 4, the fraction of the steady state change achieved in the first beat in the calcium-rich medium is plotted for 11 preparations. In general, this fraction increases as the period of exposure to the new Ringer (i.e., the cycle length) is prolonged. This result is most easily understood as indicating that the technical problem of access of the altered Ringer’s fluid to the interstitial spaces of the muscle makes a large contribution to the observed rapidity of the change in contraction. Other possibilities are, however, not excluded by this finding, such as a rate-limiting step of binding (or unbinding) of extracellular calcium to some sarcolemmal site. Also, technical limitations on extracellular access may actually obscure an underlying participation of intracellular calcium stores in the beat-by-beat response to a change in Ringer calcium concentration.
These results are in marked contrast to those obtained by Vassort and Rougier\textsuperscript{26} for frog atrial trabeculae. Both found contraction to be changed in the same direction as the electrodiffusional driving force on calcium and, with the effect confined to the early time course of the action potential.

The effect could be graded by varying the duration of the clamp pulses, after which an increase in intracellular calcium storage and subsequent release. Since extracellular calcium concentration cannot, apparently, be controlled at will with any regularity, this alternative possibility was assessed in an indirect manner. A series of regularly repeating stimulus pulses was suddenly replaced by a train of 4-second clamp pulses, at the same repetition rate (Fig. 5). Contraction with the first clamp pulse was more than 10-fold greater than that accompanying the preceding action potential, and more than 6 times as long-lasting. It is thus reasonable to infer that there was a large increase in free intracellular calcium concentration which did not lead to any further increase in contraction with two subsequent clamp pulses, after which an increase in extracellular calcium concentration was followed by the characteristic, beat-by-beat, progressive increase in contraction during three clamp pulses.

**FIGURE 5** Effects on subsequent contractions of an abrupt rise in intracellular calcium concentration (stimulated twitch, followed by three voltage clamp-induced contractions) and those of an increase in extracellular calcium concentration (the last three clamps, in the Ca-rich medium). Traces (force above, transmembrane potential below) are interrupted to show compression of the time axis (slower chart speed) in the period between pulses, which were delivered every 20 seconds.

**LACK OF A DELAYED INOTROPIC EFFECT DUE TO AN INCREASE IN EXTRACELLULAR CALCIUM CONCENTRATION**

The problem of access, then, may account totally for the beat-by-beat response of frog ventricle to a change in Ringer calcium concentration; or it may, alternatively, obscure a significant contribution to the activation process of intracellular calcium storage and subsequent release. Since extracellular composition cannot, apparently, be controlled at will with any regularity, this alternative possibility was assessed in an indirect manner. A series of regularly repeating stimulus pulses was suddenly replaced by a train of 4-second clamp pulses, at the same repetition rate (Fig. 5). Contraction with the first clamp pulse was more than 10-fold greater than that accompanying the preceding action potential, and more than 6 times as long-lasting. It is thus reasonable to infer that there was a large increase in free intracellular calcium concentration which did not lead to any further increase in contraction with two subsequent clamp pulses, after which an increase in extracellular calcium concentration was followed by the characteristic, beat-by-beat, progressive increase in contraction during three clamp pulses.

**FIGURE 6** Inotropic effects of displacement of transmembrane potential (lower traces) during the action potential plateau. Current pulses making the cell interior more positive (1) and more negative (2) by about 5 mV were applied 2–3 msec after completion of the upstroke. Contractile force traces (upper traces, increased tension is in downward direction) are numbered correspondingly. The control, unmodified action potential and contraction, omitted for clarity, had time courses intermediate to 1 and 2.

**ACQUISITION OF CONTRACTION DURING THE ACTION POTENTIAL PLATEAU**

A polarizing pulse, applied after completion of the action potential upstroke, displaced the plateau to a more inside positive or inside negative value for a period of about 300 msec (Fig. 6). These maneuvers would correspond to a decrease (traces 1) or an increase (traces 2), respectively, in the driving force for a calcium current. The accompanying contractions were, however, oppositely affected, being increased when the plateau was made more inside positive and decreased by the reverse maneuver. The effect could be graded by varying the duration of the polarizing pulse from 30 msec to that of the entire plateau. These results are in marked contrast to those obtained by Sumbera\textsuperscript{26} for trabeculae from sheep and calf ventricles and by Vassort and Rougier\textsuperscript{26} for frog atrial trabeculae. Both found contraction to be changed in the same direction as the electrodiffusional driving force on calcium and with the effect confined to the early time course of the action potential.

That the effect shown in Figure 6 cannot be attributed to a calcium conductance, which is maximally activated at an unusually large inside positive potential, is shown by the experiment illustrated in Figure 7. Here the plateau is displaced, by a 100-msec pulse, to a level (+107 mV) which should be well beyond the equilibrium potential for calcium and, subsequently, to a level still more positive (+140 mV). Whether the pulse is applied during the upstroke (left traces) or 100 msec after the upstroke (right traces), the associated contraction is augmented, relative to the control, and the augmentation is greater, the more positive the value to which the transmembrane potential is displaced. The graph in Figure 8 gives all of the results obtained in an experiment of this type, for displacements over the range from the naturally occurring plateau to the

**FIGURE 7** Inotropic effects of extreme displacement of the action potential plateau by 100-msec polarizing pulses. Superimposed traces of the unmodified (+ 30 mV) and modified (to +107 and +140 mV) action potentials are shown for pulses starting during the upstroke (left) and 100 msec after the upstroke (right). The associated contractions are shown in the superimposed traces below, where peak tension can be seen to increase with increasing inside positive levels of the displaced action potential plateau.
relationship between the degree of displacement of the action potential plateau by a 100-msec pulse and the augmentation of the accompanying contraction. The overshoot of the unmodified action potential amounted to +25 mV. Each plotted symbol gives the peak force attained, for a given displaced level of the action potential plateau (abscissa) as a multiple (ordinate) of that attained in control beats bracketing the experimental maneuver. Solid lines connect the mean values for each group of points, which represent results for application of the polarizing pulse during (closed circles), 100 msec after (crosses), and 200 msec after (open circles), the upstroke of the action potential.

**Figure 9** Contractile response (upper trace) to increasing depolarizations (lower trace) in 0.7 mM Ca Ringer. Interrupted portions of the traces show a slowing of chart speed during the period between two-step clamp pulses, which were applied every 20 seconds. Total duration of each clamp is indicated. The second clamp step followed the first by 1 sec and was 1.6 seconds in duration.

Large inside positive levels shown. Each experimental point was bracketed by control contractions during which no polarizing current was passed during the plateau. Without exception, displacement of the plateau toward a more inside positive value enhances contraction, and the degree of augmentation is monotonically related to the extent of voltage displacement.

**Saturation of the Activation Process**

In 0.7 mM Ca Ringer, long clamp pulses elicit a maximum or near maximum of force development at voltage levels between +33 and +50 mV (Fig. 9), while, as reported previously, contraction in 0.2 mM Ca Ringer increases monotonically over a considerably greater range of inside positive potentials (Fig. 10). In addition, the voltage-tension relationship can be changed from one type to the other by altering the extracellular calcium concentration (Fig. 11).

In Figure 9 it can also be seen that, while a transmembrane potential of −46 mV is the approximate mechanical threshold before the second clamp step, it is no longer so after the second step and its accompanying large contraction. This effect was seen frequently at clamp levels which produced saturation, or near saturation, of force development.

**Nature of Activation at Large Inside Positive Potentials**

Whether force development induced by extreme levels of intracellular electropositivity is also strongly and rapidly dependent on extracellular composition is of special interest, since such voltage levels appear to lie beyond the equilibrium potential for calcium and thus bring about a net outward electrodiffusional driving force on this ion. That the entire extent of the intracellular compartment had been clamped to such voltage levels was supported, in a few instances, by an additional impalement to monitor voltage distribution (see Figure 2 and related text: Methods, Degree of Space Clamp). That contractile force developed at such highly positive intracellular potentials is mediated by calcium influx into the cells was tested by abrupt changes in Ringer composition which are known to affect calcium influx, i.e., addition of manganous ion, increase in calcium concentration, and decrease in sodium concentration.
FIGURE 12 Rapid effects on contraction due to manganous ion (upper traces) and to a decrease in extracellular sodium concentration (lower traces). Contractions were induced by voltage clamp pulses (middle trace) delivered at a frequency of 3/min. Numbers indicate consecutive contractile responses, the indicated change in Ringer composition having occurred just after the preceding clamp pulse.

FIGURE 13 Rapid and promptly reversible effect of extracellular manganous ion on force (lower trace) developed at a high inside positive potential (upper trace). T, a test of resting potential recording on switching off feedback; ad, repositioning of baseline; C, reimposition of clamp feedback. Chart speed is constant. The first two clamps are the same ones shown in Figure 2, where voltage monitored at a more distal point is also displayed. Clamp durations are as in Figure 2: total duration of each clamp pulse was 3.8 seconds; interval between onset of first and second clamp steps was 1.0 seconds; duration of second step was 2.4 seconds.

The drugs verapamil and D-600 were also tried, but these required several beats to have appreciable effects which were very slowly reversed, on withdrawal of the drug. Changes in sodium concentration and addition of manganous ion, in contrast, had the extremely rapid effects on contraction shown earlier for alterations in calcium level (Fig. 12).

Voltage distribution during clamp was found to be quite variable, especially at large inside positive potentials. It was, therefore, thought advisable to verify longitudinal uniformity of voltage in the same preparation and during the same clamp pulses, from which the most critical observations of contraction were obtained. Thus, the two-step clamps whose voltage uniformity is given in Figure 2 are the first two clamps in Figure 13, which shows that application of 10 mM Mn²⁺ Ringer immediately after the first clamp reduced the developed force of the next contraction almost to its steady state value. Withdrawal of the 10 mM Mn²⁺ was followed by a complete return to the control value with the next contraction. Rate of force development was even more profoundly decreased by the manganous ion than was peak force. In five other preparations, in which voltage distribution was not monitored, more dramatic decreases in contraction were observed. Similarly, the force developed at a large inside positive potential was also rapidly sensitive to a change in extracellular calcium concentration (Fig. 14). Switching to 50% Na Ringer between two clamps at a high level of electropositivity does not affect the peak level of force development (Fig. 15). This is as would be expected from the information given above that saturation of the activation process is present at this voltage level in 0.7 mM Ca²⁺. The rate of force development, however, was markedly speeded up, bringing contractile force to its peak in about half the control time.

FIGURE 14 Effect of an increase in extracellular calcium concentration on force developed at a high inside positive potential. Same preparation, same display, and same clamp durations as in Figure 13.

FIGURE 15 Effect of a decrease in extracellular sodium concentration on force (upper traces, increased tension is in downward direction) developed at a high inside positive potential. Two consecutive clamps are shown, which precede (left) and immediately follow (right) a change in Ringer composition, at a frequency of 3/min. Total duration of each clamp pulse was 3.8 seconds; interval between onsets of first and second clamp steps was 0.7 seconds; duration of second step was 2.2 seconds.
Discussion

These results strongly suggest that activation of contraction in frog ventricular muscle is mediated by an influx of calcium into the cell. They do not totally exclude the participation of intracellular calcium binding sites, but they do indicate that the failure to attain a new steady state in the beat immediately following alteration of extracellular concentration is not attributable to beat-by-beat increases in binding and release of calcium at such sites. An immediate new steady state of contraction was attained in a minority of preparations (Fig. 3A). In the great majority, where this did not occur, an abrupt and large increase in intracellular free calcium concentration, caused by a prolonged voltage clamp pulse, had no residual effect on subsequent contractions. It is, therefore, reasonable to explain the progressive beat-by-beat increase in contraction as being largely due to limitations of technique in terms of the rapidity and uniformity with which extracellular composition can be changed in most preparations.

In addition, the abrupt abolition of tension, during a prolonged contraction, by sudden reduction in Ringer calcium concentration argues against a significant role for calcium-induced release of calcium from intracellular storage sites, since the contraction is more sensitive to the altered extracellular calcium level than it is to the high concentration of intracellular free calcium reflected by the large developed force.

This finding does not exclude the participation of calcium release from such sites earlier on in the development of activation. In addition, the cyclic behavior of contraction which can be a consequence of regenerative release of calcium from storage sites was not observed by Fabiato and Fabiato in preparations of frog ventricle from which the sarcolemma had been removed, in contrast to mammalian preparations from several species. Spontaneous contractions of much slower time course and frequency were observed by Winegrad in a frog ventricle preparation from which the sarcolemma was not removed but rendered highly calcium-permeable by exposure to EDTA.

It does not seem likely that all of the reported observations on the beat-by-beat time course of contraction of frog ventricle can be dismissed as arising from a slowness of extracellular access. The contractile response to changes in Ringer calcium is extremely slow for a considerable period following a prior exposure to a calcium-poor medium, according to Chapman and Niedergerke, and this was consistently observed in the present experiments. In addition, staircase and the frequency-force relationship have been observed countless times in this tissue. In view of the findings presented here, however, it may be as reasonable to attribute such effects to progressive alterations in transsarcolemmal calcium flux as to the kinetics of calcium binding to, and release from, intracellular sites. An explanation of this sort would also be consistent with the very great and almost instant sensitivity of contraction to alterations in extracellular calcium concentration. Also, the results presented here do not argue against an important role of activation of mechanisms which might remove calcium from its site of action, either through intracellular binding or extrusion from the cell.

The calcium influx that mediates activation does not have the characteristics of a calcium current. If, in this tissue, a calcium current does exist (cf. Morad and Orkand), it does not appear to be a major determinant of contractile activation. This can be concluded from the fact that displacements of the action potential plateau, which should alter (and even reverse) the driving force for such a current, affect contraction in a manner opposite to that to be expected, even when they are confined to an early portion of the plateau. The early and later portions of the contractile time course do not differ from one another in terms of the sensitivity of contraction either to displacements of transmembrane potential (Figure 8 and related text) or to changes in extracellular calcium concentration. Thus, if more than one mechanism mediates excitation-concentration coupling in mammalian ventricle and in frog atrium, there is as yet no evidence that this is the case in frog ventricle.

For the contraction accompanying a conventionally stimulated action potential, activation is close to being saturated in 2.0 mM calcium Ringer. The calcium concentration of 0.7 mM was chosen for the control medium since this gives a contractile force close to the foot (20-30% of the way to peak) of the sigmoid calcium-force curve. During prolonged clamp pulses, which elicit a plateau of contractile force, near saturation of activation occurs at +33-+50 mV in 0.7 mM calcium Ringer and at +120 mV in 0.2 mM calcium Ringer. Saturation or near saturation was consistently observed in several experiments at each calcium level. The reason for the contrary report of Morad and Orkand is not clear. It should be noted that space clamp is relatively nonuniform in frog ventricle, and we were at some pains to be sure that poor voltage distribution did not invalidate the observations made at high inside positive potentials. Conceivably, in preparations longer than those used here, a greater voltage difference along the muscle might obscure a saturation effect.

Although the possibility has not been excluded that it is sarcolemmal calcium transport which is being saturated, it is reasonable to assume that this effect occurs at the level of activation. Accordingly, if one takes the 0.35-mm hole size (in the rubber partition) as the diameter of compact frog ventricular muscle, the average developed force, for 10 preparations at high inside positive potentials amounts to 360 mg. This gives a value of 4 g/mm², compared to a "contractile maximum" of about 6 g/mm² for cat ventricular trabeculae.

Thus, at +120 mV, myoplasmic calcium concentration should be close to 10⁻⁴ M, certainly in excess of 10⁻⁸ M. For the latter concentration, the calcium reversal potential in 0.2 mM calcium Ringer amounts to +69 mV. As shown in Figure 2, the transmembrane potential is displaced from below the threshold for contraction to beyond the calcium reversal potential within a few milliseconds. Force development, which occurs following the completion of this voltage displacement (Fig. 13, first two clamps), is extremely sensitive to the application of manganous ion to the muscle, which was only begun in the 16-second period of "diastole" prior to that depolarization. It thus appears unlikely that manganous ion is exerting its negative inotropic effect at an intracellular site. A more plausible
interpretation of this result is that manganous ion is blocking calcium influx into the fibers in a manner analogous to that well demonstrated for calcium currents. Activation of contraction at such high inside positive potentials, therefore, appears also to be mediated, at least in part, by a transmembrane calcium flux, whose driving force includes components in addition to an electrodiffusional one. The nature of such an additional component of the driving force is not clear. One possibility is that of counterflow, the linking of calcium influx to efflux of some other ion through affinity for a common membrane carrier, as formulated for uncharged molecules by Rosenberg and Wilbrandt. Sodium efflux may play such a role at such extremely inside positive potentials, and the result shown in Figure 15 is in accord with this possibility, but this result might, alternatively, be attributed to a decrease in the rate of calcium extrusion from the fibers. The possibility that calcium influx may be linked to potassium efflux, which would provide such an additional driving force for all degrees of depolarization from the potassium equilibrium potential, remains as yet to be evaluated. In its simplest form, at least, it faces two difficulties: first, elevation of extracellular potassium concentration has a positive, voltage-independent inotropic effect; and, second, the addition of potassium chloride to Ringer's solution without a positive extracellular potassium concentration has degree of depolarization from the potassium equilibrium potential, age-independent inotropic effect; and, second, the addition of potassium has been created.

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