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EXCITATION–CONTRACTION COUPLING IN FROG VENTRICLE: EVIDENCE FROM VOLTAGE CLAMP STUDIES

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SUMMARY

1. Membrane potential, tension and membrane current were simultaneously recorded from frog ventricular strips in a modified sucrose-gap which enabled control of membrane potential by voltage clamp.

2. Shortening the frog ventricular action potential by repolarizing the membrane to the resting potential terminates contraction.

3. Depolarization to the level of the normal action potential plateau for longer than about 80–100 msec (up to 30 sec) produces and maintains tension for the duration of the depolarization.

4. Depolarizations less than about 80 msec in duration generate no tension but can facilitate the tension response to subsequent depolarizations. The facilitating effect of a short depolarizing pulse persists for no longer than 0·5 sec.

5. The mechanical threshold is about -50 mV; the relation between membrane potential and tension is fairly linear from about $+5$ to $+80$ mV.

6. Variation of holding potential, below the mechanical threshold, has no effect on the tension–voltage relation. The absolute membrane potential rather than pulse amplitude determines the developed tension.

7. Increasing external calcium increases the slope of the voltage–tension relation.

8. Contraction of the frog ventricle is directly controlled by the electrical activity of the surface membrane.

INTRODUCTION

It has been established that the membrane action potential triggers contraction in both skeletal and cardiac muscle. The possibility that in cardiac muscle the action potential serves not only to trigger but also to

determine the duration of contraction is suggested by a variety of studies. Under conditions where the action potential duration is varied by changes in heart rate (Kraft & Wiegmann, 1957), temperature (Brady, 1964), ionic environment, or drugs (Antoni & Rotmann, 1968), corresponding changes in the duration of contraction are observed. Additional evidence of the continuous relation between membrane events and contraction is derived from studies of graded depolarization of the membrane by extracellular potassium (Niedergerke, 1956; Lamb & McGuigan, 1966).

With the development of a technique for voltage clamping ventricular muscle (Morad & Trautwein, 1968; Beeler & Reuter, 1970), it became possible to control the membrane potential directly and to avoid the complications inherent in the non-specific manipulations of the action potential by changes in heart rate or environment.

In this report, we have used a voltage–clamp technique to study quantitatively the role of membrane and action potential in controlling the development of tension in frog ventricle. The development of tension in this tissue, which is devoid of a transverse tubular system and with a paucity of sarcoplasmic reticulum (Niedergerke, 1963*a*; Staley & Benson, 1968) appears to be directly related to the electrical activity of the surface membrane.

A preliminary report of this work has been presented (Morad, Orkand & Brady, 1970).

METHODS

Preparation. Quiescent strips 0·3–0·5 mm in diameter and 4–6 mm in length were dissected from a circular portion of the ventricle of frogs (*Rana pipiens*) maintained under ultra-violet light at room temperature 23–25° C. Thinner strips could be prepared but were found to be unsatisfactory, presumably because the ratio of injured to intact tissue was high, and this led to low resting potentials, shorter action potentials, and high current leak across the sucrose-gap (see below). The experiments were conducted at 20–23° C.

Solutions. Three solutions were used with the following compositions in mM: (1) normal Ringer: NaCl 116, $NaHCO_3$ 2, KCl 3, $CaCl_2$ 0·2–1·0; (2) KCl-Ringer: KCl 120, $KHCO_3$ 2; (3) isosmotic sucrose: sucrose 210 (special enzyme grade, Mann Research Laboratories) in deionized distilled water. Dimethylsulfoxide, 210 mM, replaced sucrose in a few experiments and gave similar results. In some experiments, tetrodotoxin, 5×10^{-8} to 2×10^{-5} g/ml. (Sankyo) was added to the normal Ringer (pH 7·4–7·6).

Experimental set-up. A diagram of the experimental arrangement is shown in Fig. 1. Ventricular strips were mounted in a three-compartment chamber. Thin rubber membranes (Trojan) separate the central chamber (1·5 mm wide) from the larger outer chambers. The muscle was fastened with silk suture to an isometric tension transducer (natural frequency 2 kc) in the outer chamber which contained Ringer solution. The other end of the muscle was then pulled through the adjustable holes in the rubber membranes and left loose in the other outer chamber containing KCl-Ringer. Isosmotic sucrose solution flowed at a rapid rate (2–6 ml./min) through

the middle compartment. The rubber membranes formed a snug collar around the muscle and effectively separated the three solutions. The resistance across the sucrose gap was 1–4 MΩ without the muscle (muscle replaced with glass rod), and 100–150 kΩ with the muscle in place. Typically, 0·3–0·5 mm of the strip protruded into the Ringer solution.

Stimulating or clamping current was applied between the ends of the strip across the sucrose gap with Ag/AgCl electrodes close to the muscle. The current passes from one end to the other mostly through the myoplasm due to the high resistance of

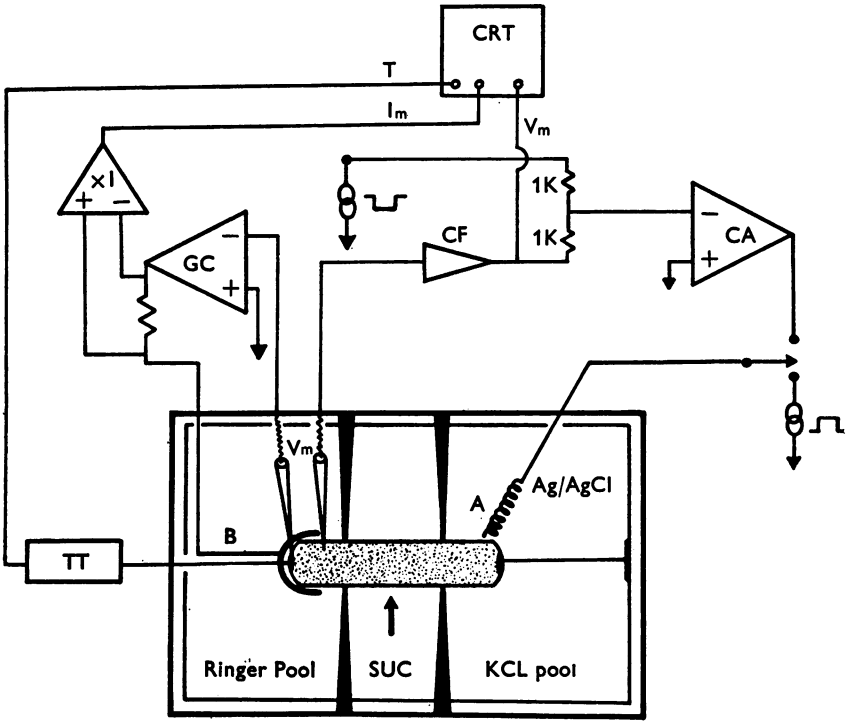

Fig. 1. Schematic of experimental arrangement. See text. SUC, sucrose; TT, tension transducer; GC, ground clamp amplifier; CF, cathode follower; CA, clamp amplifier; CRT, cathode ray tube; I_m, membrane current; T, tension; V_m, membrane potential. The heavy black lines abutting to the muscle (stippled cylinder) at the sucrose-Ringer and sucrose-KCl interface represent the thin rubber membranes. A and B refer to Ag/AgCl extracellular electrodes.

the sucrose solution irrigating the segment of muscle in the middle compartment. The current distribution is reasonably uniform along the muscle bathed in Ringer, if the protruding segment of the muscle is below 0·5 mm in length.

The potential of the surface of the muscle in the Ringer bath is held to the ground potential by a separate circuit. Current necessary to stimulate or clamp the muscle was measured by recording the potential drop across a 1 kΩ resistor incorporated into the 'ground-clamp' circuit (see Fig. 1). Transmembrane potential was measured relative to virtual ground with conventional 3 M-KCl intracellular electrodes con-

nected to the input of a solid-state negative-capacitance preamplifier (Analog Devices 149B). The output of the preamplifier is compared with a signal function generator and the difference voltage fed into the 'summing point' of a clamp amplifier (capable of 300 V output) which supplies the necessary current to the KCl pool. The spatial uniformity of the clamp was tested with another roving intracellular micro-electrode entirely independent of the clamp circuit (see below).

The current flows in an axial direction through the myoplasm in the sucrose-gap. At the sucrose-Ringer interface the current density is uniform over the entire cross-section. Thus, the core and surface fibres of the muscle are all depolarized to the same potential. This is important as tension is measured from the entire cross-section (see Morad & Trautwein, 1968, for an analysis of the distribution of potential through the cross-section and the reliability of tension measurement).

With this method many preparations had normal resting and action potentials for over 6 hr.

Tests and precautions. To obtain reproducible data, we found the preparations had to meet a number of experimental requirements. (1) Resting potentials of -75 to -90 mV; action potential duration 0·6–1 sec with a well maintained plateau. (2) No visible contraction in the segment of the muscle in the sucrose gap. To prevent transmission of contraction from the part of the muscle in sucrose to the transducer, the muscle was made slack in the sucrose-gap. This was necessary because frog ventricles contract in very low concentrations of calcium if sodium concentration has been drastically lowered (Lüttgau & Niedergerke, 1958). When sucrose was initially perfused, the muscle in the gap region contracted vigorously in response to electrical stimulation. Rather long and large tension responses were recorded under such conditions. After about 1 hr of perfusion with sucrose, the 'tail' in the tension record disappeared and there was no visible shortening of the muscle in the gap even with currents which strongly depolarize the portion of the muscle protruding into Ringer. (3) The action potential across the gap in viable preparations, monitored by a separate circuit with low impedance Ag/AgCl electrodes, had an amplitude of 80–100 mV. This indicated a good electrical seal between the two Ringer pools and made more reliable the measurement of steady-state membrane currents. (4) The current record provided a good check on the homogeneity of the preparation and activation of contraction. If with a step clamp the recorded current broke into a series of negative deflexions accompanied by bumps in the tension record (Fig. 2), it suggested inhomogeneous distribution of potential and aberrant action potentials in the strip. In support of the above, tetrodotoxin abolishes such oscillations, but such preparations were discarded because of uncertainty about the geometrical homogeneity and therefore about the measurements of tension. Since current records serve as a good control for the homogeneity of clamp potential, they are included in all of the original records of this report, whether they are pertinent to the illustrated point or not. (5) Clamping the membrane potential at any level between -60 and -100 mV for a few seconds produces no tension. These potentials are below the mechanical threshold and should produce no tension. In preparations where hyperpolarization from -80 to -100 mV produced apparent relaxation or contraction, it was considered that either a portion of the strip had been in a contracture which was relaxed by the hyperpolarization, or there was inhomogeneous distribution of current with current exiting at some unknown locus. Such preparations were discarded. (6) A second micro-electrode not in the clamp circuit was often used to check on the homogeneous distribution of clamped potential in a preparation where a critical experimental datum was being established. Deviations in potentials above 5% at various locations of a preparation were not acceptable for our experiments.

As a result of the above enumerated tests, about 50% of the preparations could not be used.

Tension recording. Since it was difficult to assess the number of injured fibres and the multi-directional fibre arrangement of the dissected muscle strips, tension measurements are not represented in terms of surface area or weight.

Current measurement. Outward transmembrane current is indicated by an upward deflexion from the base line and inward current by a downward deflexion in all of the Figures. A fraction of the current which passes from the KCl-pool to the Ringer pool does not flow through the myoplasm. It is, however, included in the current measurements, so it is necessary to estimate the magnitude of this leakage current.

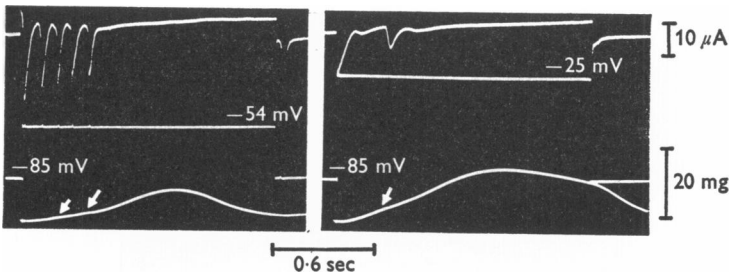

Fig. 2. The tension response of a ventricular strip to an inadequate clamp step to −54 and −25 mV. Under such conditions 'bumps' or oscillations appear in the tension records (arrow). Tension often relaxed before the end of depolarization. Inward current is recorded as a downward deflexion. Around the threshold for the sodium current (left panel), the inadequacy of the clamp is apparent.

A simplified model of the electrical configuration of the gap divides the clamp current between leakage conductance (g_l) and the preparation's myoplasmic conductance (g_p) in proportion to their ratio g_l/g_p. During a non-clamped action potential these conductances form a voltage divider which has the intracellular potential (V_m) as the input and the transgap potential (V_t) as the output. The ratio of idealized leakage conductance to myoplasmic conductance is given by

$$\frac{g_l}{g_p} = \frac{V_m - V_t}{V_t}.$$

In a typical experiment the intracellular action potential was 110 mV and the action potential recorded across the gap was 90 mV. Substitution of these values into the above formula gives a leakage to myoplasmic conductance ratio of 0·22. The measured clamp current is therefore estimated to be 22 % greater than the actual transmembrane current. A similar analysis can be applied to the resting membrane potential. The error estimates ranged from 15 to 30 % in various preparations. After 4 or 5 hr of perfusion with the sucrose solution, the g_l/g_p ratio increases and the error estimate may reach 50 %.

RESULTS

Relation between duration of depolarization and contraction

Electrical shortening of action potential. To determine the influence of action potential duration on contraction, the voltage-clamp technique was used to terminate the action potential at various times during the

plateau. The strip was stimulated at a fixed rate (usually 12/min) and at various times after the beginning of the onset of excitation the action potential was terminated by clamping the membrane potential to the rest level or a value slightly more negative. The result of one such experiment is shown in Fig. 3*A*. The time course and amplitude of contraction depend directly on the duration of the action potential. The initial rate of rise of tension for action potentials of different durations is independent of the

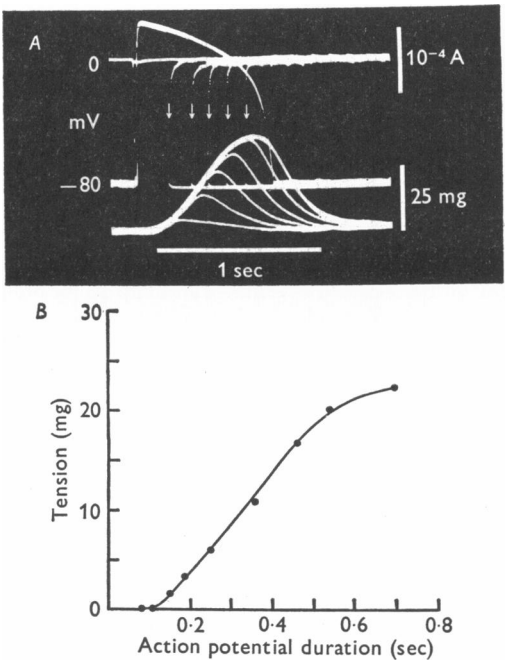

Fig. 3*A*. Superimposed traces of action potential, membrane currents (inward current downward deflexion) flowing at the repolarization of the action potential, and the accompanying contractions. The arrows indicate when the membrane was clamped to the resting potential. Temp. 23° C.

B. The relation between developed tension and duration of action potential (same experiment as Fig. 3*A*). The tendency of the graph to plateau reflects the beginning of rapid repolarization and the maximum tension developed with the action potential.

duration of the depolarization when the frequency of stimulation is constant. The amplitude and time course of the tension generated by the abbreviated action potentials was independent of the membrane potentials to which the plateau was repolarized, viz. from −70 to 110 mV tested. Fig. 3*B* depicts the relation between duration of action potential and maximum developed tension. After the first 100 msec, tension increases with duration in a nearly linear manner. As repolarization progresses

terminating the plateau, the increment of tension developed falls, leading to a plateau in the tension–duration curve. Relaxation begins about 50 msec after the potential is clamped to rest level.

Varying duration of a step-clamp. The effect of duration of depolarization on tension was also studied independent of that dictated by the normal action potential to the level of the plateau for varying durations (Fig. 4*B*). For comparison, contractions were also produced in the same preparation by shortened action potentials (Fig. 4*A*). The results of such experiments consistently showed that the duration of depolarization determines the duration of contraction.

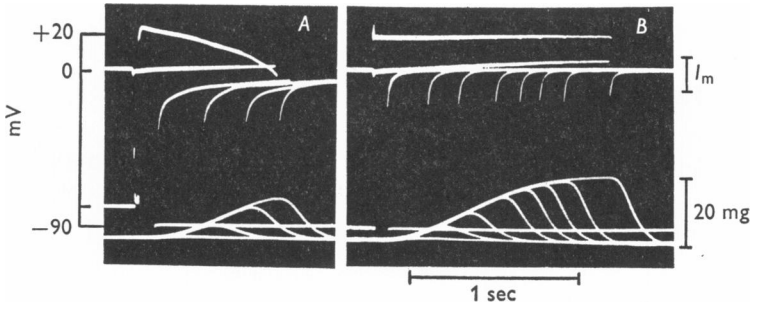

Fig. 4. Contractile responses elicited by depolarizations of varying duration. Panel *A*, superimposed traces of shortened action potentials and accompanying contractions. Panel *B*, superimposed traces of clamp steps of various duration. The delay in onset of activation is the same with either of the procedures. Inward current is recorded as a downward deflexion. Current calibration mark is 10 μA in panel *A* and 20 μA in panel *B*.

In some experiments the effect of long depolarization clamps (30–40 sec) was tested on the time course and maintenance of contraction. Large tensions which tended to reach a 'plateau' within 2–3 sec were generated with such depolarizations. The level of plateau tension as well as the time required to reach these plateau tensions were dependent on extracellular calcium concentrations $[Ca]_o$. Tension never decreased or relaxed with maintained depolarization. A comparison between the time-to-speak of tension and the duration of depolarization suggests a direct and linear relation between these two phenomena for clamp duration greater than 100 msec. This relation holds at all amplitudes of depolarization tested (-40 to $+80$ mV). Fig. 5*A* shows the effect of the duration of depolarization on the time-to-peak of tension at two selected depolarization steps ($+2$ mV and $+25$ mV). Although the final level of tension increased with larger depolarizations (Fig. 5*B*), the length of time until the onset of relaxation is only determined by the duration of depolarization (Fig. 5*A*). Similar results were obtained from preparations treated with tetrodotoxin.

Onset of activation. When ventricular strips are clamped to the level of the plateau for less than 100 msec, no tension response is recorded (see Figs. 3 and 4). The delay in activation of contraction is seen whether the membrane potential is step clamped for less than 100 msec or the action potential is terminated within the first 100 msec. In some instances, however, where the clamp is not uniform, when the plateau is terminated soon after the upstroke of the action potential, a slow and small tension is generated. These contractions generally accompany an inward 'hump' in the current record. Fig. 6*A* shows that the action potential terminated at the vertical arrow (well within the first 100 msec) produces a mechanical

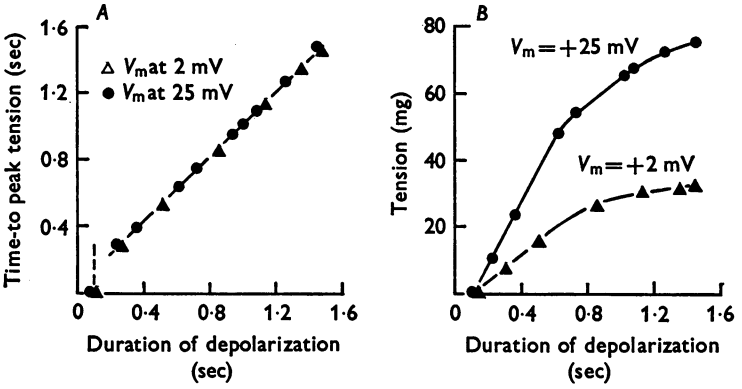

Fig. 5. Correlation of the duration of depolarization with the time-to-peak of tension (*A*) and the magnitude of tension (*B*) at two membrane potentials (+2 mV triangles and +25 mV circles).

response lasting long after repolarization. The 'hump' of the inward current (heavy arrow) occurring with premature repolarizations of the action potential generally corresponds to the peak tension. Fig. 6*B* shows a similar abbrevation of the action potential in the same preparation after the segment of the muscle in Ringer has been shortened in length from 0·8 mm to 0·3 mm. Note that 100 msec depolarization (first arrow) produces no tension and that the repolarizing current is smooth and without any inward current 'hump'.

Large or small depolarizations for less than 80–100 msec fail to produce any measurable tension when the tissue is under uniform clamp control. Fig. 7 shows that step depolarization to −25 or +30 mV for about 100 msec produces no tension, while a longer depolarization elicits a mechanical response. Large transient inward currents (downward deflexions) are recorded with the smaller depolarization (−25 mV) and net outward currents (upward deflexions) with the larger depolarization (+30 mV).

In preparations treated with tetrodotoxin (employed to suppress the

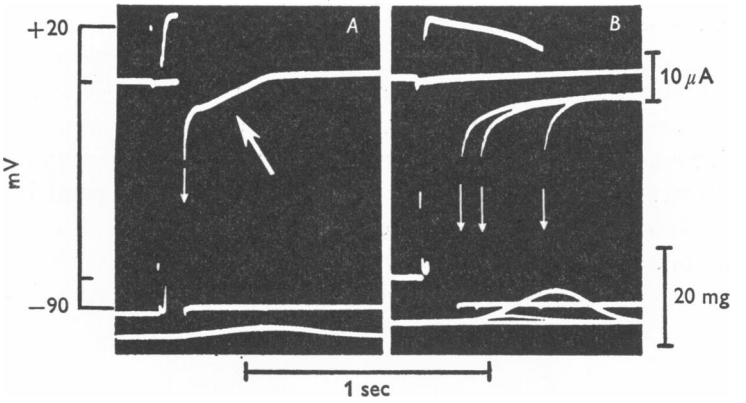

Fig. 6. Experiment of shortening of action potential in a preparation where the length of the ventricular strip was reduced from 0·8 mm (panel *A*) to 0·3 mm (panel *B*). The 'hump' of the inward current (heavy arrow, panel *A*) represents an aberrant action potential in a segment of the preparation not under adequate clamp control. As the preparation is shortened (panel *B*), short depolarization produces neither tension nor long inward 'humps' in the current record.

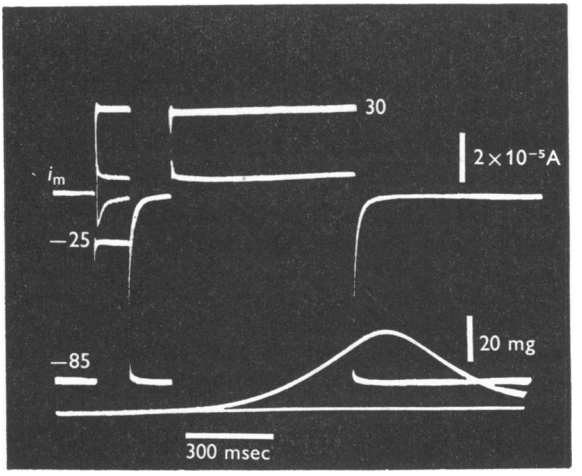

Fig. 7. Superimposed traces of three clamp steps to −25 mV and +30 mV for 100 msec and to +30 mV for approximately 600 msec. No tension is generated with either one of the short steps. The −25 mV depolarization triggers a transient inward current (downward deflexion), while outward current seems to flow with depolarization of +30 mV. Longer depolarization (to +30 mV) produces and maintains tension. The continuation of voltage base line for the short depolarization has been removed in order to enhance the clarity of the tension recording.

sodium spike, Hagiwara & Nakajima, 1966), a slow inward current (duration, 50–80 msec) still persists in the range of −40 to +10 mV, which is sensitive to variations in extracellular calcium concentration. In these muscles, however, varying the clamp level from −30 to +60 mV for less than 100 msec or increasing the extracellular calcium concentration did not significantly affect the time necessary for activation of tension. Lowering the temperature of bathing solution from 23 to 13° C increased the delay for activation of contraction by only a factor of 1·2–1·4 times (four experiments).

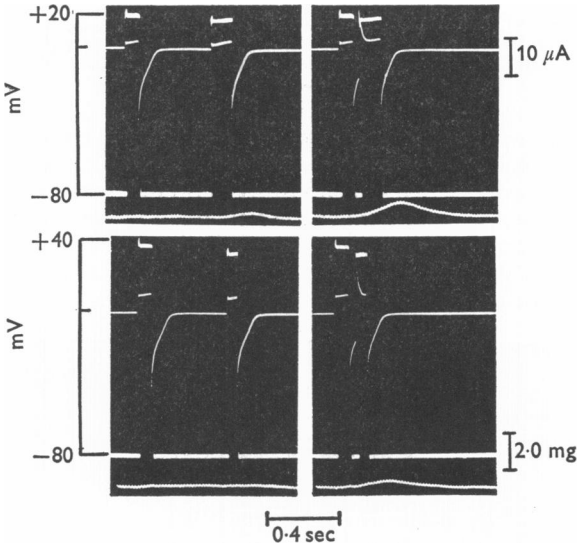

Fig. 8. Facilitation of tension by a depolarization which is ineffective by itself in producing tension. Upper two panels show that the tension generated by a second depolarization is potentiated when the second pulse follows shortly after the first pulse. The lower two panels show that two pulses, although each by itself ineffective in producing tension, when given at close proximity do produce tension. Outward current is indicated as upward deflexion in the current records.

Facilitation of tension. Depolarizations of less than 100 msec, although ineffective in producing any measurable tension, often facilitated the tension produced by a subsequent depolarization. If the second pulse is capable of producing tension, the preceding ineffective pulse facilitates the tension accompanying the second pulse (Fig. 8, top panels). Two pulses which would be ineffective alone also produce tension (Fig. 8, bottom panels) when given in rapid succession. The facilitating effect of a depolarization step on a subsequent depolarization was not limited to mechanically ineffective depolarizations. In fact, marked facilitation of tension could often be observed when two mechanically effective pulses were given

within a short interval of each other. The inset of Fig. 9 shows that when the test pulse is given close to the conditioning depolarization, facilitation is most prominent and that it is less so as the test pulse is moved away from the initial depolarization step. A plot of total tension with respect to the duration of the interval between the test and conditioning pulse (Fig. 9) indicates that the facilitation effect is almost completely dissipated within 500 msec of the conditioning pulse.

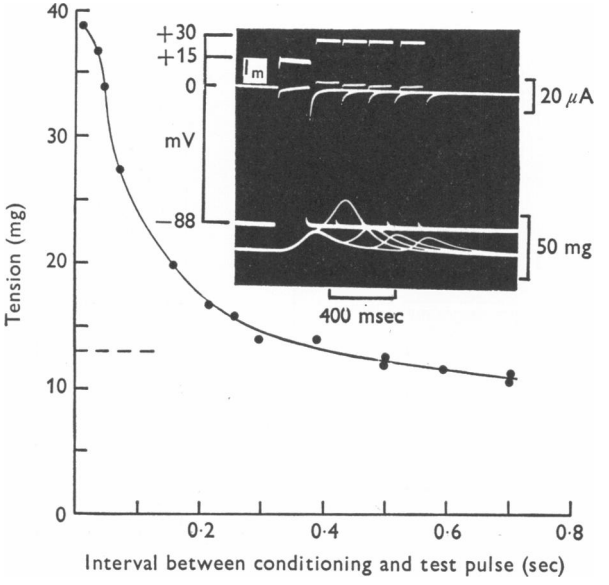

Fig. 9. Time course of decay in facilitation of tension elicited by a second clamp pulse (graph). The inset shows how such an experiment was carried out. The conditioning pulse is repeated at a constant rate of 12/min. The test clamp is then applied progressively closer to the conditioning depolarization. Developed tension is progressively facilitated. More outward current (upward deflexions) flows with the larger tensions. Dashed line shows the amplitude of non-facilitated tension.

Degree of depolarization and contraction

In the previous section it was shown that the action potential or step clamp duration controls the development and maintenance of tension in a direct manner. The next question to consider is how the tension relates to the magnitude of the depolarization. The result of clamping the membrane potential at various levels for a constant duration of 1·2 sec is shown in Fig. 10 (inset panels). As the membrane is progressively depolarized the tension increases. The increase in tension is independent of the magnitude of the initial inward current (recorded as downward deflexions in panels 1–5, Fig. 10) and seems to be directly related to the level of depolarization.

Tension develops at about −50 mV (Fig. 10, graph) and shows no maximum up to +80 mV tested in this preparation. The relation between tension and voltage is fairly linear from +5 to +80 mV. In some experiments the membrane potential was clamped to higher levels, up to +150 mV, and tension still increased with depolarization. In such cases, action potentials following the large experimental step produced normal contractions comparable to those observed before the large steps clamps. This indicates that such large depolarizations did not require currents

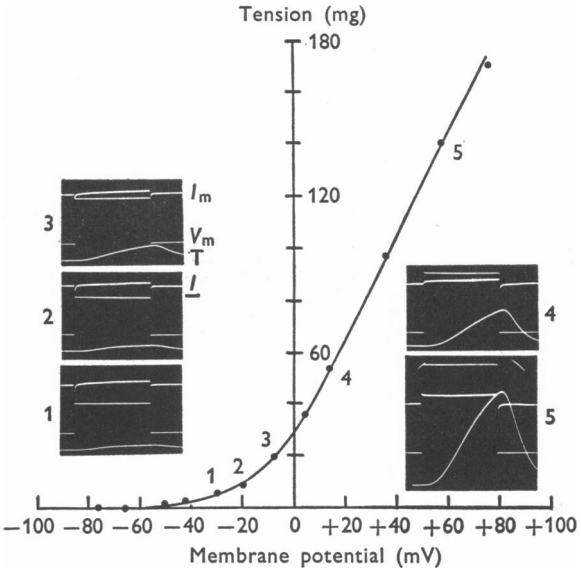

Fig. 10. Relation between tension and membrane potential. The inset records are numbered from 1 to 5 according to the points they represent on the graph. Tension increases in an almost linear manner from +5 to +80 mV. Calibration markings of the inset are horizontal: 200 msec, vertical: V_m 20 mV, and I_m 20 μA. The transient inward current (downward deflexions) is quite prominent (panel 1 and 2) and is completely reversed at +60 mV (panel 5).

which irreversibly damaged the tissue. As it was difficult to maintain the intracellular penetration with strong contractions generated by large depolarizations, such tests were not routinely employed. If the penetration was lost during a large step clamp, the feed-back amplifier was capable of passing sufficient current to damage or 'fry' the tissue. In one experiment where good penetrations were maintained, the membrane was depolarized by 50 mV steps from −70 to +250 mV. Although the tension developed with progressive depolarizations (1·5 sec) was linear in the range of −10 to +120 mV, tension did reach a maximum around +200 mV.

Pulses longer than 1·2 sec often produced larger tension; however, the relation between tension and membrane potential was qualitively similar to that already described. A similar voltage–tension relation was found by staircase depolarizations and repolarizations (Fig. 11). Staircase changes in potential were accompanied by changes in tension. Fig. 11 illustrates that step clamping a preparation to + 20 for approximately 3 sec produces large tension (5 to 6 times twitch tension). The muscle is then clamped to a higher membrane potential (+ 45 mV) and even larger tension is recorded. The preparation was then returned to the resting potential in two steps.

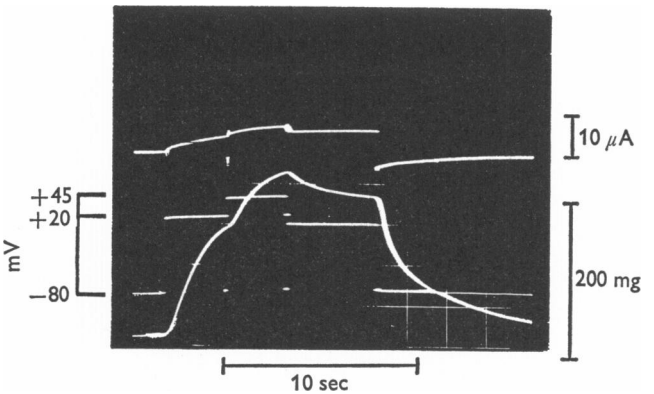

Fig. 11. Staircase clamp experiment. Membrane potential is clamped for 3 sec to + 20 mV. As the tension tends to plateau, a second clamp pulse to + 45 mV is applied for a similar time. More tension accompanies the second pulse. The membrane is then clamped to the resting potential in two steps. Current flow is primarily outward (upward deflexions) after the initial 100 msec of depolarization.

In some preparations progressive 10 mV step clamps between − 50 and 0 mV produced an anomalous, N-shaped voltage–tension curve. The tension increased with small depolarizations, up to about − 30 mV, then decreased or plateaued to about − 10 mV and then again increased. With larger depolarizations the tension continued to increase. Such results appeared to be due to non-uniform clamping as hyperpolarizing step clamps in these preparations frequently caused contraction and the shape of the voltage–tension curve depended greatly on the position of the intracellular feed-back electrode in the strip.

Effect of external calcium. Increasing extracellular calcium concentration increases the strength of contraction. Above the mechanical threshold the tension generated by a step clamp is always greater with increased calcium. The experiment illustrated in Fig. 12 describes the relation between tension and voltage in two calcium concentrations (0·2 and 1·0 mM).

At these concentrations a fivefold increase in calcium did not seem to shift the mechanical threshold. Because of the difficulty of maintaining good penetration in vigorously contracting tissue, it was not feasible to examine the possibility of variation in mechanical threshold at higher calcium concentrations. Attempts to explore a maximum in the voltage–tension relation at higher calcium concentrations were abandoned for the same reason.

Comparison of tension produced by action potential and clamp pulses. The finding that the voltage–tension relation is linearly increasing in the region of the plateau of the action potential indicates that normal excitation may

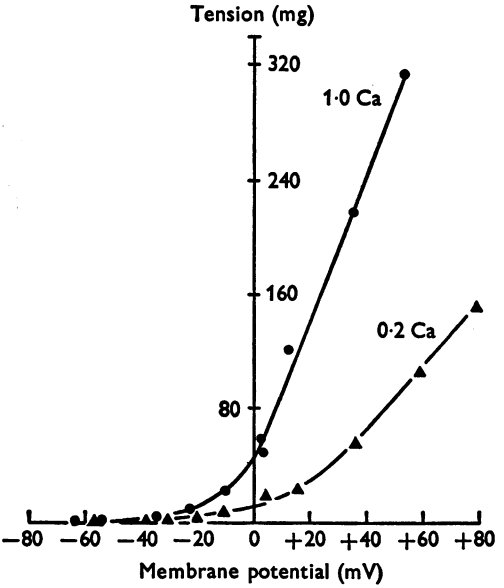

Fig. 12. Voltage–tension relation at two calcium concentrations (0·2 and 1·0 mM). See text.

not maximally activate contraction. In fact, the tension response is highly sensitive to variations in membrane potential at the level of the plateau. Thus, the precise shape of the plateau should determine the form of the tension response. To test this proposition, clamp pulses were used which approximated in duration and magnitude the normal action potential. Fig. 13*A* and *B* show normal action potentials and corresponding clamps which simulate the action potentials (to + 20 and + 30 mV). It is apparent that the initial rate of rise of tension is the same for action potential and the clamp pulse. The tension responses deviate only when the level of the plateau falls below that of the clamp pulse. Repolarization of the clamp pulse is much more rapid than that during the action potential leading to

corresponding differences in the rate of relaxation. This finding is predicted by the voltage–tension curve.

The similarity in tension responses (generated by the action potential and an approximating clamp pulse) and the predictability of the deviations are reassuring in that they indicate that the same portion of the muscle giving rise to the normal contraction is also under clamp control.

Effect of holding potential. In the above experiments, the holding potential prior to the step clamp was adjusted as closely as possible to the normal resting potential. Two possibilities now to be considered are: (1) does the level of membrane potential before the step clamp affect the

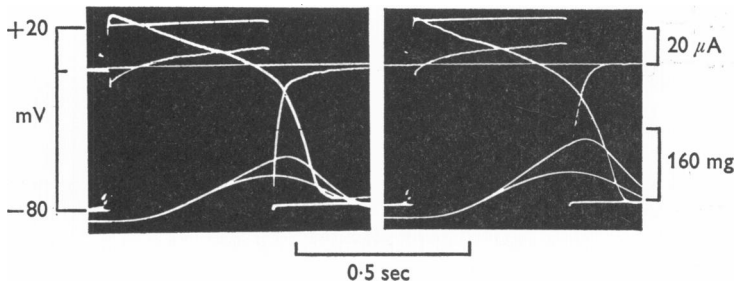

Fig. 13. Superimposed traces of action potential and contraction with an approximating clamp pulse in duration and amplitude (left panel to +20 mV and right panel to +30 mV). Note that the initial rate of rise of tension is the same. Tension deviates only when the normal plateau of the action potential slopes away from the clamped potential. Some transient inward current (downward deflexions) still persists at +30 mV.

magnitude of tension produced by a step depolarization to a given membrane potential? (2) is developed tension related to the amplitude of the depolarization step or only to the absolute value of the membrane potential?

These questions were examined in experiments (total of seven) such as that illustrated in Fig. 14 (inset). The potential was controlled sequentially at the resting potential (−71 mV), 22 mV more negative (−93 mV), and once again at the resting potential for 1 min prior to a step clamp to +40 mV. The tension responses in all three cases are the same, indicating that the final level of the membrane potential determines the tension responses. In some instances repolarization to higher resting potentials after the step depolarization enhanced the rate of relaxation. This effect, however, was very small and often absent. Fig. 14 (graph) shows the relation between membrane potential and tension in a preparation where step depolarizations were carried either from a holding potential of −71 (circles) or −93 mV (triangles). The relation between voltage and tension seems to be independent of the level of previous holding potential (from

-60 to -110 mV examined). That is, the absolute magnitude of the membrane potential rather than the amount of depolarization seems to determine the amplitude of the developed tension.

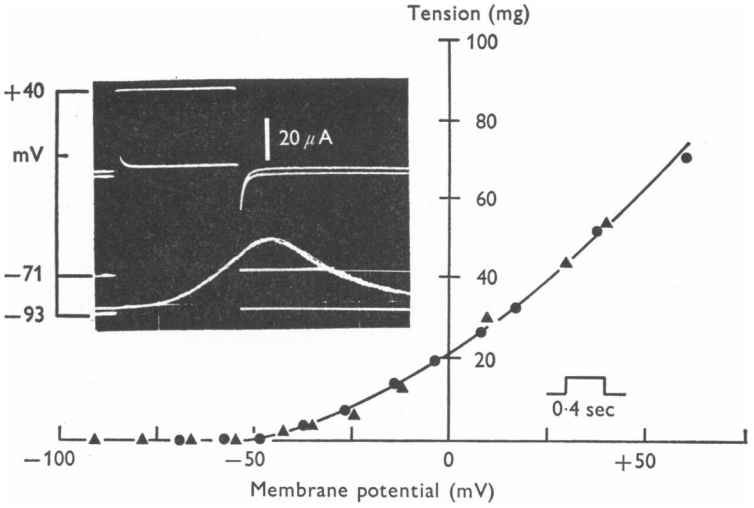

Fig. 14. Voltage–tension relation at two different holding potentials (triangles -93 and circles -71 mV). Note that the development of tension is related only to the absolute membrane potential (graph). The inset panel shows superimposed recordings of step depolarizations from holding potentials of -71, -93, and again -71 mV to $+40$ mV for 0·4 sec. Steady-state inward current (downward deflexion) flows with -93 mV holding potential. This preparation has been treated with tetrodotoxin.

DISCUSSION

The main conclusion drawn from these results is that in frog ventricle the membrane potential exerts continuous control over the contractile response. This is true for all membrane potentials tested from the mechanical threshold, about -50 mV, to $+150$ mV and for durations between 100 msec to 30 sec. Relaxation occurs only with repolarization. The capacity for prolonged tensions in response to prolonged depolarization is not surprising in view of known ability of frog and turtle heart to produce long action potentials and large and maintained tensions at low temperatures (Brady, 1964; Weidmann, 1959).

The voltage–clamp results described above are compatible with a scheme where the membrane potential serves as a direct regulator for the availability of activator-calcium to the myofibrils. The duration and amplitude of membrane depolarization, as well as the concentration of extracellular calcium, influence the amount of activator available to the contractile proteins and the tension generated. When the membrane is repolarized, closing the opened 'gate', the activator entry stops and that

which has entered is taken up from the myoplasm by some internal structure, sarcoplasmic reticulum or mitochondria and/or by the surface membrane. These factors are incorporated in a scheme diagrammed in Fig. 15.

The major features of the model are as follows: (1) the rate of rise of tension, i.e. the rate of entry of activator calcium, is a continuous function of membrane potential. Tension threshold would be determined by the

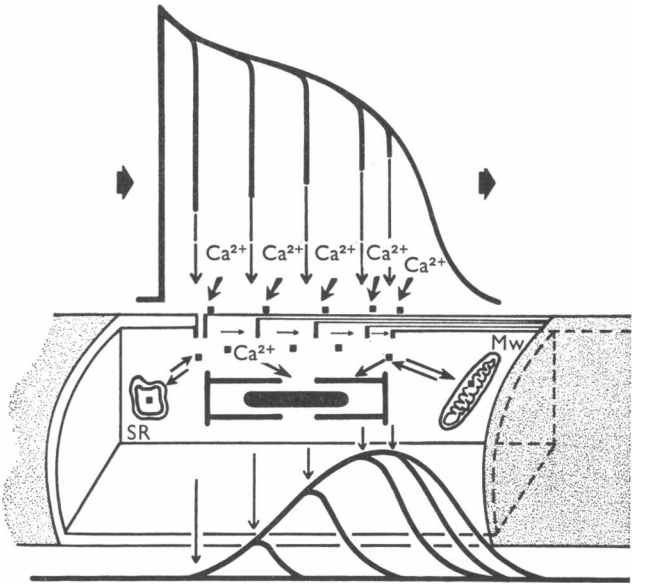

Fig. 15. Schematic representation of control of contraction by the action potential superimposed on a diagram of a myocardial cell. At the vertical arrows, the action potential is repolarized and contraction is terminated. The 'latches' in the membrane withdraw with depolarization (opening the 'gate') for various times (horizontal arrows). Calcium (squares) is either released from the membrane or is bound and then released (indicated by short and heavy arrows). Mw, mitochondria; SR, sarcoplasmic reticulum. For a discussion of model, see text.

level at which the rate of entry exceeds the capacity of the relaxing system to sequester the activator, (2) the duration of depolarization determines the duration of entry and permits the activator to accumulate in the myoplasm, leading to a progressive increase in tension. Contraction plateaus when the rate of entry and uptake of activator become equal, (3) with repolarization, the rate of entry falls to rest level and the activator is taken up before expulsion from the cell. This scheme, though based on electrophysiological data, has much in common with one proposed by Niedergerke (1963*b*) based primarily on a study of the movement of radioactive calcium (see also Chapman & Niedergerke (1970).

The above model does not distinguish between the two possible sources of activator calcium – that in the extracellular fluid $[Ca]_o$ or that bound to any cellular structure controlled by the membrane $[Ca]_m$. The model is *incompatible* with a trigger release of preset amount of intracellular calcium by the action potential. However, if the 'calcium-source' were electrically continuous with the plasma membrane through low resistance pathways, it is plausible that electrical activity of surface membrane could also control in a continuous manner (see Figs. 3, 4, 5 and 10) the release of calcium from the internal 'source'. In the absence of T-tubular system, lateral sacs or prominent subsarcolemmal cisternae (Niedergerke, 1963*a*; Staley & Benson, 1968), it is highly improbable that there is significant electrical continuity with the small amount of sarcotubular membranes that exist in the frog heart.

If an external or membrane-bound source is primarily responsible for activator calcium, two likely mechanisms for the transport of calcium across the cell membrane should be considered: (1) calcium moving down its electrochemical gradient; (2) calcium bound to a carrier in the membrane, which is activated by change in membrane potential or by another cation crossing the membrane.

If the inward movement of extracellular calcium down its electrochemical gradient were primarily responsible for initiating contraction, it would be expected that the influx of calcium would decrease as the membrane potential approached the calcium equilibrium potential, E_{Ca}. That is, calcium movements, or I_{Ca}, would depend on $I_{Ca} = g_{Ca}(E_{Ca} - V_m)$, where I_{Ca} is transmembrane calcium current, g_{Ca} calcium conductance, V_m the membrane potential, and $(E_{Ca} - V_m)$ the electromotive force for calcium. If I_{Ca} is proportional to developed tension, then where $E_{Ca} = V_m$, $I_{Ca} = 0$ and therefore development of tension would be suppressed. It is implicit in the concept of E_{Ca} that tension–voltage relation should have a maximum far below E_{Ca} and that at E_{Ca} no net force for movement of calcium across the membrane exists, and thus no tension via this mechanism could develop.

Electrochemical gradient of calcium. An estimate of E_{Ca} could be made from the Nernst equation, $E_{Ca} = RT/ZF \ln [Ca]_o/[Ca]_{in}$, assuming the internal free calcium concentration at the moment preceding development of tension is just below that necessary to activate the myofilaments, i.e. 5×10^{-8} to 10^{-7} M (Hellam & Podolsky, 1969 in skinned frog skeletal muscle; Winegrad, 1971 in chemically skinned frog ventricle). With external calcium concentrations of 2×10^{-4} M (used in this study),

$$E_{Ca} = 29 \log \frac{2 \times 10^{-4}}{5 \times 10^{-8}} = +104 \quad \text{or} \quad +95 \text{ mV}$$

when considering $[Ca]_{in}$ of 10^{-7} M. The finding that the voltage–tension curve rises almost linearly from +5 to +80 mV (Figs. 10, 12 and 13) and beyond (in other experiments) and saturating at +200 mV in one occasion, rules out a downhill movement of external ionic calcium as the sole mechanism for the entry of the activator. At +104 mV the calculated E_{Ca} is probably an overestimate, since the internal calcium concentration increases above 5×10^{-8} M during the time course of contraction with a 1 to 2 second clamp pulse. The argument that the internal calcium activity is far below 5×10^{-8} M and that there may be still an inward calcium gradient at +80 mV (Fig. 10) is further weakened by the staircase clamping experiment (Fig. 11). The first depolarization step (for 2–3 sec at +20 mV) generates 3–4 times twitch tension, thus undoubtedly increasing the internal calcium activity. Step clamping with a second or third depolarization above the lowered E_{Ca} would be expected to produce small or no further increase in tension. However, more tension develops and a voltage–tension relation similar to that observed with single step clamps is obtained. It appears, therefore, that E_{Ca} can not by itself explain the influx of calcium into the cell to initiate contraction.

Calcium current and activation of contraction. It has been suggested that activation of a secondary and much slower inward current, highly sensitive to variation in $[Ca]_o$ and not suppressed by the application of tetrodotoxin, initiates contraction in mammalian myocardium (Beeler & Reuter, 1970; Mascher & Peper, 1969). Similar observations of calcium-dependent inward current were made in frog atrium (Rougier, Vassort, Garnier, Garouil & Coraboeuf, 1969). It is agreed by the above investigators, and confirmed by our unpublished data, that almost all of the transient inward currents (in the presence and absence of tetrodotoxin) are inactivated within the first 100 msec of depolarization (see also Fig. 7). The hypothesis that a component of this current is responsible for initiating contraction is inconsistent with our findings that step clamps of about 100 msec in duration are ineffective in producing tension (see Figs. 3, 4, 5, and 7). Although in the normal Ringer solution we could never separate a 'calcium current' from the total transient inward currents, it was possible to show that the transient inward current in the presence of tetrodotoxin was sensitive to variations in $[Ca]_o$ (M. Morad, unpublished observation).

In the mammalian heart where most of the tension is triggered in the first 100 msec of depolarization (Morad & Trautwein, 1968), it is conceivable that the 'secondary' inward current triggers the release of the activator calcium from internal stores associated with the T-tubular system or lateral and subsarcolemmal cisternae. Thus, the initial segment (50–80 msec) of the mammalian action potential may serve primarily as a trigger for activator release, and the rest of the plateau as a replenisher

of Ca^{2+} to the partially depleted stores. Such a scheme is consistent with observations of Wood, Heppner & Weidmann (1969) and Morad & Trautwein (1968) that prolongation of the action potential potentiates the tension of a subsequent beat, and that shortening of the action potential for series of beats progressively diminishes the developed tension (M. Morad, unpublished, in cat, dog, and rabbit hearts; Wood *et al.* 1969 in sheep hearts).

In the absence of 'mammalian-like' calcium stores in the frog ventricle (Sommer & Johnson, 1969; Staley & Benson, 1968), the action potential's initial 'trigger-release' of activator is not seen. Thus, the duration of the plateau of the action potential serves as quantitative regulator for movement of calcium from or across the surface membrane.

Net increase in intracellular calcium per beat. In frog ventricle, it has been shown that calcium influx per beat under optimum conditions is 1–2 μmole/l. of tissue water.beat (Niedergerke, 1963*b*; Niedergerke, Page & Talbot, 1969). This figure falls far short of the 50–60 μ mole calcium/kg wet weight of tissue necessary to completely saturate troponin-tropomyosin proteins (Katz, 1970). However, it is obvious that the twitch tension of the frog ventricle is much smaller than the maximum tension produced under voltage–clamp conditions (Figs. 10, 12 and 14). In fact, maximum tension may be as much as 5–6 times larger than the twitch tension (extrapolation from tension–voltage relation to +200 mV). A comparison of maximum tension, obtained in chemically skinned frog ventricular strips, with normal twitch tension also gives a similar ratio of 5:1 (Winegrad, 1971). Such observations may suggest that less than 'saturating' calcium (e.g. 10 μ mole/l.beat) is sufficient for the myofibrillar proteins to produce a normal contraction. Measurement of radioactive calcium in ventricular strips (referred to above) still fall short of 10 μ mole/l.beat, by a factor of 5–10 times, even if all the calcium entering were to bind to troponin. It is conceivable therefore, that most of the activator calcium is released continuously, during depolarization, from a 'membrane' and sequestered with repolarization by the surface or an internal membrane system which must be closely coupled to the electrical activity of the outer membrane. A 100 msec delay in onset of activation of contraction observed in these experiments could be explained by a conceivably slow time constant of activation of such an internal membrane system. Although a large portion of the activator calcium in this scheme would be *recirculated* between the myofilaments and the 'membrane' under direct control of action potential, about 20% of the calcium per beat could be crossing the membrane from an extracellular space during depolarization.

Carrier mediated transport of the activator. The precise mechanism of transport of calcium across the membrane or its release from the mem-

brane remains unknown. It is, however, clear that release or entry of calcium is under continuous control of the membrane potential. A carrier mediated transport system could offer a unitary mechanism for the activator transport from both sources of calcium. A fairly linear relation between depolarization and the development and maintenance of tension may suggest that the binding to or release of calcium from such a carrier could be potential dependent. Another possibility for activation of the carrier could be mediated through an outward flux of another cation such as potassium. This hypothesis is attractive because with depolarization the electrochemical gradient for potassium increases favouring its efflux from the cell. If only a small fraction of the potassium efflux were coupled to $[Ca]_o$ or $[Ca]_m$, the net membrane current would be outward with an effective net influx of calcium. According to such a hypothesis, the delay in onset of activation may be explained by the well known delay in the turning on of potassium conductance (Noble, 1966 and Fozzard & Sleator, 1967). The potassium conductance increases with progressive depolarization in the positive region of membrane potential, +10 to +100 mV, and thus the release or influx of calcium would progressively increase, giving rise to the observed tension–voltage relation. The increase in tension response with increase in extracellular calcium for a given depolarization can also readily be explained, i.e. there would be more calcium to exchange for outward potassium flux. The precise mechanism of activation of such a carrier or the ratio of K/Ca exchanges mediated by such a carrier must be left for future experimental development.

Other comments. In the step clamp and shortened action potential experiments, the onset of tension development was delayed 80–100 msec after the membrane was depolarized. Such depolarizations produced no tension. A simple explanation, that the delay represents the time necessary for activator calcium to reach a threshold concentration (Niedergerke, 1963*b*, Fig. 9), would predict that increasing the amount of activator either by increasing the external calcium or by increasing the amplitude of the depolarization should shorten this minimum time for activation. The minimum activation time did not change with larger depolarization (Fig. 7), nor did the threshold for activation with higher calcium concentrations (Fig. 12). However, the calcium studies were conducted over a limited range (0·2–1 mM) and do not provide a definitive test of this hypothesis. In support of the above hypothesis is our observation that these brief ineffective depolarizations did facilitate tension responses to subsequent clamp pulses. Such results might be explained if clamp pulses release activator to the interior which either raises the concentration of activator toward threshold or partially saturates the sequestering system.

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