

Relationships between Voltage and Tension in Sheep Cardiac Purkinje Fibers

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ABSTRACT The two-microelectrode technique of voltage clamping sheep cardiac Purkinje fibers was used to examine the changes in contraction which occur during trains of voltage clamps. (A "train" is defined as a series of voltage clamps delivered at a particular rate, beginning after a rest long enough that the effects of previous stimulation have died away.) Contractions showed striking staircases, or progressive changes in peak isometric tension, during trains. Short clamps, clamps to voltages more negative than -20 or -30 mV, or holding potentials less negative than the resting potential favored negative staircases, while long clamps, clamps to positive voltages, and holding potentials near the resting potential each favored positive staircases. The staircase behavior appeared to be due to changes in the initial rate of recovery of the ability to contract. The changes in staircase behavior as a function of clamp voltage suggested that the relationship between peak tension and clamp voltage should depend on the experimental design. When the steady-state contraction was plotted as a function of clamp voltage, voltage-tension relations like those recently reported for working ventricle were obtained, with a threshold between -30 and -40 mV and a steep relation between tension and voltage. When the first contraction after a rest was plotted, the threshold voltage was more negative, the curve was flatter, and the peak tensions at inside positive voltages were reduced.

INTRODUCTION

As information about the electrical and contractile behavior of heart muscle has accumulated, it has become clear that membrane electrical activity can not only trigger contraction but also control or modify it. Methods of directly controlling the membrane voltage of heart muscle ("voltage clamp" techniques) have been of great help in determining how contraction depends on voltage, and in providing insight into the mechanism of excitation-contraction coupling in the heart.

After a stepwise depolarization of mammalian heart muscle, two types of

contractile response may be seen: a phasic contraction which resembles a normal twitch and, if the depolarization is maintained, a considerably slower and smaller contraction which lasts for the duration of the depolarization. This dual contractile response has been observed in several species and in both the Purkinje fiber and working ventricular muscle (Kavalier, 1959; Morad and Trautwein, 1968; Fozzard and Hellam, 1968; Beeler and Reuter, 1970; Gibbons and Fozzard, 1971; Ochi and Trautwein, 1971; New and Trautwein, 1972). Most workers have focused on the phasic part of the contractile response, because it appears to be the voltage clamp counterpart of the normal twitch both in time-course and in voltage dependence.

Although the basic features of the contraction of different types of ventricular muscle from different species have been found to be similar, the correspondence between quantitative results from different laboratories has been less satisfying. Intuitively, at least, one of the most useful experiments is to determine how the phasic response depends on clamp voltage. This approach has been used to determine the voltage-tension relation of the Purkinje fiber (Fozzard and Hellam, 1968; Gibbons and Fozzard, 1971) and of working ventricular muscle of several species (Morad and Trautwein, 1968; Beeler and Reuter, 1970; Ochi and Trautwein, 1971; New and Trautwein, 1972; Tritthart et al., 1973). Threshold voltages between -60 and -30 mV have been reported, and above threshold the shapes of the curves relating tension and voltage have often been different. These differences are discussed in detail on pages 355, 56. It is tempting to ascribe the differences to differences in the species or tissue, or to problems in the technique used. While this may be part of the reason for the different results, we have argued (Fozzard and Gibbons, 1973) that some of the variability may be the result of the different experimental approaches used by various laboratories, since in this tissue contraction depends heavily on the rate and rhythm of stimulation.

In this paper, we report the results of experiments designed to examine some of the contractile changes during trains of voltage clamps, and we examine directly the dependence of the voltage-tension relation on the experimental approach used. In the following paper, (Gibbons and Fozzard, 1975), we will consider some of the relations between membrane current and contraction.

METHODS

Some of the experiments to be described were performed at the University of Chicago, while others were done at a laboratory established later at the University of Vermont. Detail improvements were made in the Vermont laboratory, and substitutes had to be found for some equipment items which were no longer available. Because the equipment used in the Chicago laboratory has been described in detail (Gibbons

and Fozzard, 1971), our discussion of methods and references to equipment will be to those used in the Vermont laboratory, and the emphasis will be on the changes made. None of these changes has changed the results of the experiments, so it will not be necessary to indicate which equipment was used for a particular experiment.

Procedure

Sheep hearts were taken from animals killed by electrocution. The free-running portions of the Purkinje network were dissected from the left ventricle and kept in oxygenated Tyrode's solution until needed. Under a dissecting microscope, fine branches that appeared to have only a single column of cells were selected. These were taken from the same part of the Purkinje system used in the ultrastructural analysis by Mobley and Page (1972). Short segments, approximately 1.5 mm long, were isolated by tying with unbraided silk suture. The ties also fastened each end of the preparation to connectors made of two twisted strands of 40-gauge stainless steel suture wire. The preparation was attached to the force transducer as described by Gibbons and Fozzard (1971).

The Tyrode's solution contained (in mM): NaCl, 137; KCl, 5.37; MgCl₂, 1.05; NaHCO₃, 13.5; NaH₂PO₄, 2.4; CaCl₂, 2.7; dextrose, 11.1. The solution was saturated with a 95% O₂, 5% CO₂ gas mixture. Dissection was usually done in Tyrode's solution with CaCl₂ added to bring the calcium concentration to 4.5 mM, to aid in "healing over" in the regions injured by the ties (Deleze, 1970), and the preparation was transferred to the normal Tyrode's solution when it was fastened to the force transducer.

Tension Recording

The photoelectric force transducer used was similar to that described by Hellam and Podolsky (1969), with the modifications detailed by Gibbons and Fozzard (1971). The transducer output was filtered by a second-order low-pass filter (Haykin, 1969, p. 105), which was 3 dB down at 42 Hz. Fourier analysis of the filtered signal showed that most of the energy was in frequencies below 40 Hz and contractions were essentially unchanged from the original wave-forms if they were reassembled from the Fourier components of frequencies less than 20 Hz, so the filter did not distort the tension signal. It did, however, introduce a delay. The delay was almost constant at 5 ms for frequencies of 1–20 Hz, rising to 6 ms at 30 Hz. The filter delay was small enough that we have not altered the records to compensate for it, but any time measurements on the tension record must be corrected for filter delay. Forces were expressed as milligrams weight (1 mg-wt is approximately 10 μ N).

In a few of the experiments reported here, the transducer rested on a shock mounted 700-lb balance table. This gave only partial isolation from building vibrations. With a preparation mounted in this system, the noise level of the tension trace ranged from about 0.2 to 0.4 mg-wt, depending on the level of activity in the building. To improve the isolation, the chamber, transducer, and micromanipulators for the electrodes were mounted on a 500-lb granite slab resting on an air piston type vibration isolation system with a resonant frequency of 1.1 Hz (71.401 Isolation System, Lansing Research Corp., Ithaca, N. Y.). This slightly reduced the noise, to approxi-

mately 0.15–0.2 mg-wt, and there was much less dependence on activity in the building. Records obtained with the former system (e.g. Fig. 3) therefore show somewhat greater variability at low force levels than those obtained with the better isolation system.

For tension recording, the preparation was stretched to 140% of slack length and lowered against a 0.75-mm wide by 0.25-mm thick Plexiglas bridge (Rohm and Haas Co., Philadelphia, Pa.) across the chamber to provide a stable base for microelectrode impalement. The physical arrangement was similar to that described by Gibbons and Fozzard (1971, Figure 1). The muscle was located in a 6-mm by 6-mm channel in the Plexiglas chamber, and the spiral silver-silver chloride ground electrode was mounted parallel to the preparation. Tyrode's solution flowed through the channel at approximately 5 ml/min. Temperature was kept between 35 and 37°C, and did not vary more than $\pm 0.3^\circ$ during an experiment.

Voltage clamp

The essentials of the voltage clamp circuitry are shown in Fig. 1. Voltage was recorded differentially between an intracellular microelectrode (VE) and an extra-

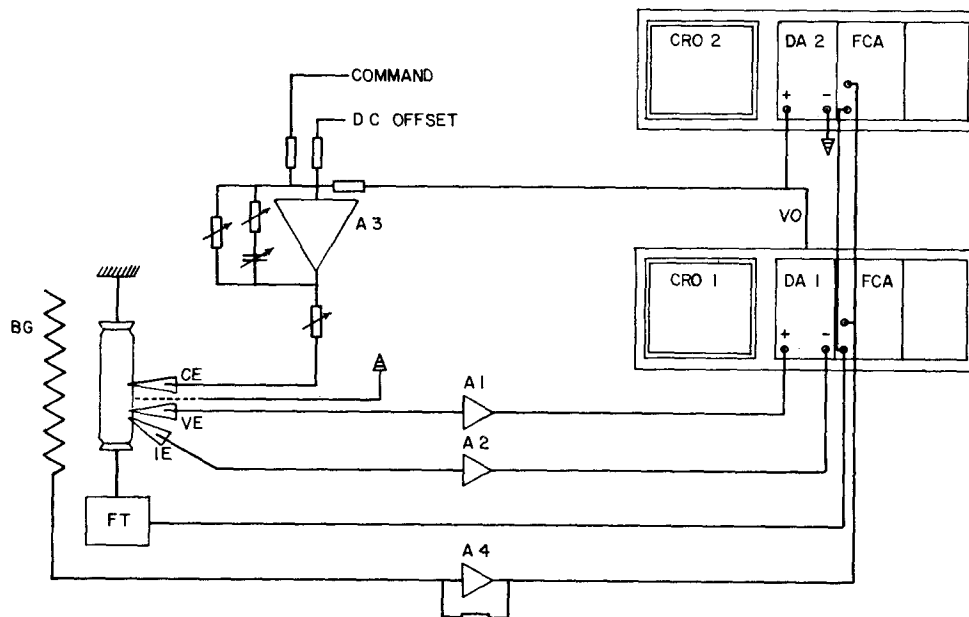


FIGURE 1. Schematic of the voltage clamp circuitry. VE is a 3 M KCl-filled microelectrode, IE is the indifferent electrode which is filled with 3 M KCl-1% agar. The current-passing electrode, CE, is filled with 2 M potassium citrate. A 1 and A 2 are unity gain amplifiers, A 3 is the voltage clamp amplifier, and amplifier A 4 is used to monitor membrane current. Other abbreviations: BG, silver-silver chloride bath ground; FT, force transducer; CRO 1 and CRO 2, cathode ray oscilloscopes; DA 1 and DA 2, differential amplifiers; FCA, four-channel amplifiers. See text for further description and equipment details.

cellular microelectrode (IE) just outside the preparation, using unity gain amplifiers A 1 and A 2 (Picometric model 181, Instrumentation Laboratory, Inc., Lexington, Mass.). The membrane voltage was amplified for display on oscilloscope CRO 1 (Tektronix 5103N D12 Mod 768T, Tektronix, Inc., Beaverton, Ore.) by a differential amplifier DA 1 (Tektronix 5A21N Mod 769Q). In addition to displaying the membrane voltage, CRO 1 was an integral part of the voltage clamp circuit; the vertical signal out from this oscilloscope was fed to the summing point of the voltage clamp amplifier A 3 (Teledyne Philbrick 1022, Teledyne Philbrick, Dedham, Mass.).

Other inputs to the summing point of A 3 were an adjustable DC voltage and a command signal from a specially built digital timer and command voltage source. The clamp amplifier A 3 supplied current through the intracellular current electrode CE to make the membrane voltage follow the command input.

Electrode VE was filled with 3 M KCl by boiling under a vacuum, and resistance was typically 5–7 M Ω . Extracellular electrodes (IE) were made in the same way, but the tip was broken after filling and the electrode was then refilled to a point near the tip with 3 M KCl-1% agar. In most experiments, the electrodes used to pass current were filled with 2 M potassium citrate (pH 6.4–6.8) by the glass fiber method (Tasaki et al., 1968). Electrodes filled with 3 M KCl were used for the earlier of these experiments, but those filled with potassium citrate tended to have more stable rectifying properties.

Electrode CE was placed in the center of the preparation, VE was placed halfway between CE and the end of the preparation, and IE was placed at the surface of the preparation, as close as possible to VE. It was necessary to draw the electrodes in Fig. 1 as if they were in the same plane passing through the axis of the preparation, but in practice CE and VE were in different planes, with an included angle of 20–30°. They were separated by a grounded shield to reduce capacity coupling.

Membrane current was monitored, using operational amplifier A 4 (Bell and Howell 20-007, Bell & Howell Co., Control Products Div., Bridgeport, Conn.) to maintain a virtual ground. Current and tension outputs were amplified for display by a four-channel amplifier (Tektronix 5A14N). Records were made by a kymograph camera (Grass C4, Grass Instrument Co., Quincy, Mass.), operating in a moving film mode, from a second oscilloscope CRO 2 set to display the same signal seen on the main oscilloscope but with the beam sweep stopped.

This technique of voltage control for heart muscle has been criticized on theoretical grounds by Johnson and Liebermann (1971). To counter their criticisms in detail is beyond the scope of this paper, but it should be noted that the question of radial nonuniformity has been discussed by Fozzard and Schoenberg (1972), who estimated that the expected nonuniformity should be on the order of a few millivolts. The possibility of longitudinal nonuniformity has been examined by Fozzard and Hiraoka (1973), and such nonuniformity is small after the first few milliseconds of the clamp, as theory would predict. The problem remains, as we have indicated (Gibbons and Fozzard, 1971), that voltage control is incomplete during the first few milliseconds after a clamp to a voltage above the threshold for the fast inward sodium current. In spite of this, sodium inward current and tension are each smoothly

graded functions of voltage above their respective thresholds, and the two do not appear to be causally related (Gibbons and Fozzard, 1975). While one would wish better control in order to examine in detail the kinetics of the fast sodium current, we know of no evidence which indicates that the technique is not satisfactory for purposes of characterizing tension or later currents.

RESULTS

Contractions were elicited by action potentials or voltage clamps delivered at a steady rate, beginning after a 90-s or longer rest period. When 30–45 s had elapsed after a stimulus or the end of a series of stimuli, the ability of the preparation to contract appeared to reach a steady level which was independent of the preceding activity; the 90-s rest was used to be as sure as possible that any effects of prior activity had died away. In almost every case, contractions reached a steady state, as judged by unchanging peak tension and tension time-course, after 10 or fewer stimuli. We will use the term “train” to describe a series of stimuli delivered at a particular rate and beginning after a rest period.

The first five of a train of action potentials at a frequency of 0.5 s^{-1} are illustrated in the upper record of Fig. 2. The contraction in response to the first action potential was the largest, and there was a negative “staircase” as the peak tension of succeeding contractions declined to a steady state. In the center record of the same figure, stimuli were delivered at 1 s^{-1} in the same Purkinje fiber. Again the first contraction was the largest (and the same size as the first contraction of the 0.5-s^{-1} record). The negative staircase was more pronounced, and the steady-state contractions were smaller at the

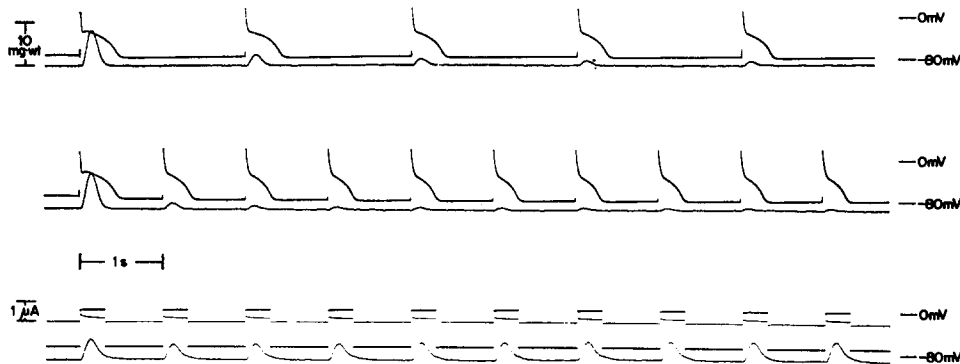


FIGURE 2. Response of the Purkinje fiber to trains of action potentials and voltage clamps. Upper panel: five action potentials and contractions at a frequency of 0.5 s^{-1} . Center panel: train of action potentials and contractions at 1.0 s^{-1} in the same preparation. Action potentials were stimulated by current passed through an intracellular microelectrode. Lower panel: a train of voltage clamps and associated contractions at 1.0 s^{-1} in another preparation. Holding voltage -68 mV , clamp voltage $+2\text{ mV}$, duration of clamps 300 ms .

higher frequency. In other preparations, with rates of stimulation greater than 1 s^{-1} , the negative staircase of the first few beats was followed by a slight positive staircase, but the peak tension of the steady-state contractions did not exceed that of the first contraction. These results from the sheep Purkinje fiber are similar to those obtained by Bautovich et al. (1962) who used a similar approach to examine the contraction of rabbit papillary muscles.

The bottom record in Fig. 2 is from a different preparation, in which the membrane voltage was clamped from a holding voltage of -68 mV to a clamp voltage of $+2 \text{ mV}$. Clamps were 300 ms long and the frequency was 1 s^{-1} . The second contraction was smaller than the first, and there was then a positive staircase to a steady state. As succeeding experiments will show, a purely negative staircase like those in the two upper panels could also be obtained with trains of voltage clamps. The voltage clamp results therefore resemble those obtained when contractions are elicited by action potentials. With action potentials, only the effects of frequency can be examined, but with voltage clamps the effects of a wide range of voltage and time parameters can be studied.

Clamp Duration

It is difficult to examine the effect of clamp duration on a train of voltage clamps and contractions. Between stimuli, cardiac muscle recovers the ability to contract (Kruta and Braveny, 1961; Bautovich, et al., 1962; Gibbons and Fozzard, 1971), and if the duration of the clamps is changed at a constant frequency, the time available for this recovery or "restitution" process is changed. If the interval between clamps is held constant as the duration is changed, of course the frequency changes. To get a qualitative idea of the effect of duration, we have done the experiment both ways.

Figure 3 A shows the effect of changing clamp duration with the frequency held constant at 0.5 s^{-1} . The tension of each contraction in the series, relative to the tension of the first contraction in the series, is plotted for each of the first 10 contractions of the train. Each of the clamp durations used was long enough to cause a full-sized contraction, so the tension of the first contraction was essentially the same for each train. The relative plot therefore gives the same qualitative result as plotting the force of each contraction. In this and in succeeding graphs showing similar data, the lines connecting the points serve only to show which points were obtained during a particular train. In particular, the resulting "curve" does not imply any relationship which may exist between tension and time between the points.

When the clamp duration was 1,000 ms, the second contraction was smaller than the first. There was then a marked positive staircase, and the steady-state tension was greater than that of the first contraction. As the duration was decreased in successive trains, the positive staircase disappeared at a

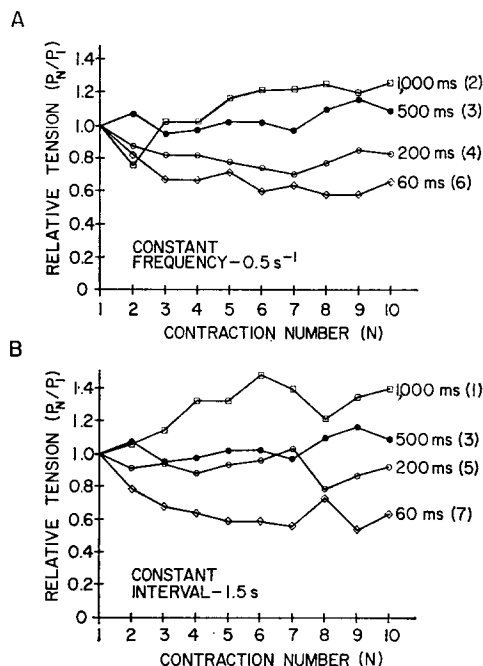


FIGURE 3. Effect of clamp duration on staircase responses to trains of voltage clamps. In this and in subsequent plots of staircase behavior, a train consists of a series of voltage clamps at a regular frequency, beginning after a rest of at least 90 s. For all trains in A and B, the holding voltage was -80 mV and the clamp voltage was -23 mV. The peak tension of the response to the N th clamp, relative to the peak tension of the first response, is plotted as a function of ordinal rank. The duration of the clamps in a particular train is indicated, and the number in parentheses indicates the order in which the trains were given. In A, the frequency was kept constant at 0.5 s⁻¹. In B, the interval between the end of one clamp and the beginning of the next was kept constant at 1.5 s. Train 3 is the same for both graphs. Absolute values of the peak tensions of the first contractions of the trains were: (1) 1.9 mg-wt; (2) 2.0 mg-wt; (3) 1.9 mg-wt; (4) 2.0 mg-wt; (5) 2.1 mg-wt; (6) 2.1 mg-wt; (7) 2.2 mg-wt.

duration of 500 ms, and there was a negative staircase at clamp durations of 200 and 60 ms, in spite of the fact that the time available for the muscle to recover the ability to contract was increased each time the duration was decreased.

In the same experiment, alternate trains were delivered in which the repolarized interval between clamps, rather than the frequency, was held constant as the duration was changed. The results of these trains, in which the repolarized interval was always 1.5 s, are shown in Fig. 3 B. The result is qualitatively the same as in Fig. 3 A except that the positive staircase in response to 1,000-ms clamps is much more marked when the muscle is given more time to recover between clamps.

Holding Potential

Beeler and Reuter (1970) and Gibbons and Fozzard (1971) showed that changes in the holding or resting potential altered the contractile response to a voltage clamp, with depolarization reducing the contraction caused by a clamp to a given voltage. We examined the effects of a change in the holding potential in the experiment illustrated in Fig. 4. In this and succeeding experiments, the duration and frequency were kept constant at 300 ms and 1 s^{-1} , respectively. In the experiment of Fig. 4, the preparation was always clamped to +2 mV. Several trains were given from each of the following holding voltages: -68, -58, -48, and -38 mV, and the peak tensions of

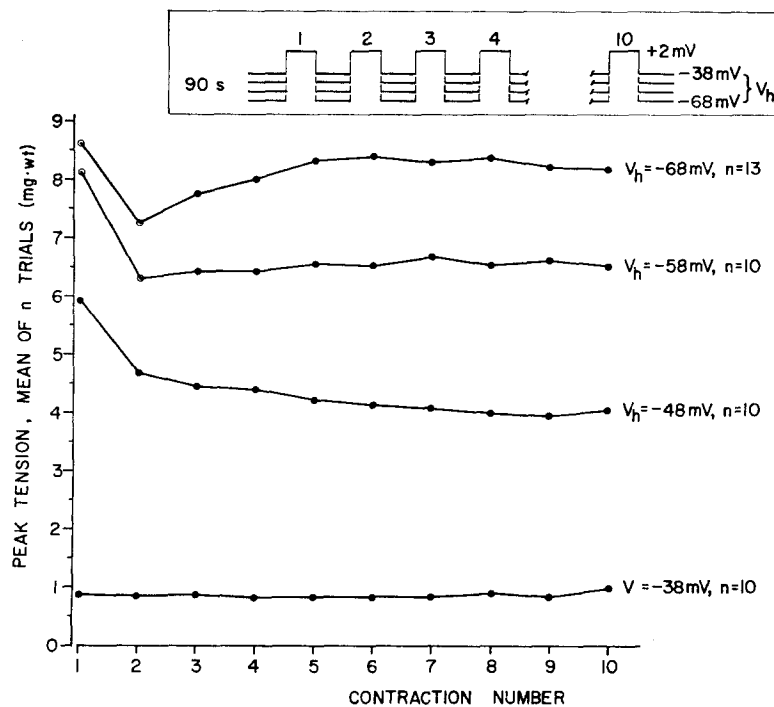


FIGURE 4. Effect of the holding voltage on staircase responses. For all trains, the clamp duration was 300 ms, the clamp voltage was +2 mV, and the frequency was 1 s^{-1} . The holding voltage was varied between -68 and -38 mV, as illustrated in the inset. For the trains at each holding voltage, the average peak tension is plotted as a function of rank in the train. The open circles for the first two points of the two upper curves indicate that there was some overlap of the range of observations for these points, i.e. some of the largest first contractions for the holding voltage of -58 mV were as large as some of the smaller contractions for the -68 mV holding voltage, and the same thing was true for the second contractions at these holding voltages. There was no overlap of any of the rest of the data (see text).

the corresponding contractions were averaged for each holding voltage. Thus the first point for a holding voltage of -68 mV represents the mean of all of the first contractions, for 13 trains with voltage steps from -68 mV to $+2$ mV.

Repeating the trains several times at each voltage turned out to have been unnecessary. The results were very reproducible from trial to trial, so that the ranges of the observations for each holding voltage overlapped only for the first and second contractions obtained at holding voltages of -68 and -58 mV. The first and second points for these two voltages are shown as open circles; solid circles are used for the rest of the data in which the ranges of the observations did not overlap.

As expected, making the holding potential less negative reduced the tension of the first contraction. In addition, there was a dramatic change in the staircases seen during the trains. In each of the three upper curves, the second contractions were smaller than the first ones. Depending on the holding voltage, there was then a positive staircase to a steady state when the holding voltage was -68 mV, a very slight positive staircase at -58 mV, and a negative staircase when the holding potential was -48 mV. The result at -38 mV is curious. The cumulative effect of repetitive stimulation, or perhaps more accurately the time dependence of contraction, seemed to be absent and there was no staircase effect.

Clamp Voltage

Having examined the effects of duration and holding voltage, we investigated the effect of clamp voltage by altering the clamp voltage in successive trains, keeping the duration at 300 ms and using a frequency of 1 s^{-1} . The result of such an experiment is illustrated in Fig. 5. Peak tension in milligrams weight is again plotted for each contraction in a train and for the train at each voltage. The clamp protocol is illustrated in the inset, and the small numbers next to each curve indicate the order in which the observations were made. The lines connecting the points again have no significance other than to show which points were obtained during a single train; two are shown dashed to help separate them from others which they cross.

For a train of clamps from the holding voltage of -78 to -65 mV, no contractions were observed in response to any clamp. In the next train from -78 to -52 mV, the voltage was above the threshold for tension development for the first and second contractions of the series, but there were no measurable contractions in response to the 3rd through the 11th clamps. Not until the clamp voltage was raised to -34 mV was the voltage unequivocally above tension threshold for all clamps in the train; it may also have been above threshold at -38 mV but at this tension level it was difficult to separate twitches from noise.

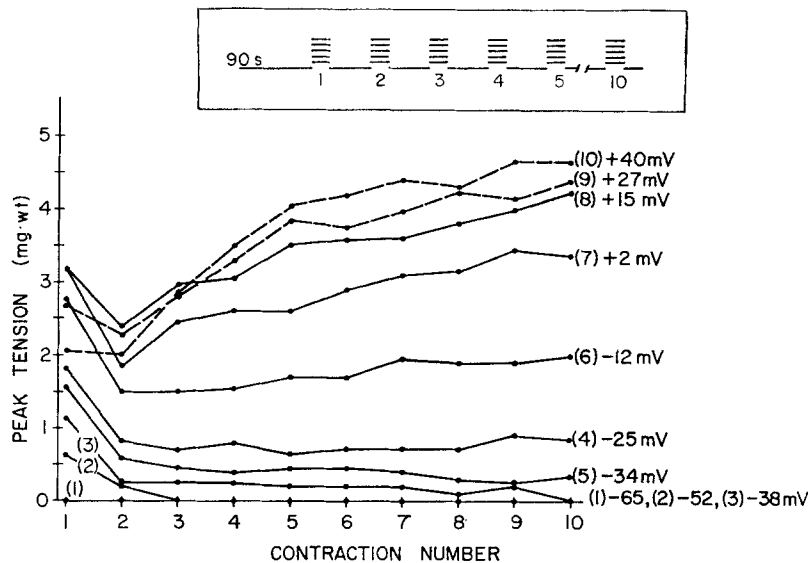


FIGURE 5. Effect of clamp voltage on staircase responses to trains. Clamp voltage was varied in successive trains as shown in the inset. The peak tension of each of the first 10 contractions is plotted as a function of rank in the train. The clamp voltage for each train is shown at the right of each curve; the number in parentheses indicates the order in which the observations were made. The clamp duration was 300 ms, the frequency was 1 s^{-1} , and the holding voltage was -78 mV for all trains. (See text.)

As the clamp voltage was increased in successive trains, the contraction in response to the first clamp in the train increased, as expected since conditions for the first clamp after a 90-s rest are similar to those used to obtain the voltage-tension relationship which we reported earlier (Gibbons and Fozzard, 1971). In the voltage range we could examine, the second contraction was always smaller than the first. After the second contraction, there was a negative or a positive staircase depending on the clamp voltage.

During the train of clamps to $+27 \text{ mV}$, the first contraction decreased as a function of voltage (train 9), and it became still smaller when the voltage was increased to $+40 \text{ mV}$ for the last train (train 10). There was still a steep positive staircase, however, for succeeding contractions at these voltages.

Voltage-Tension Relation

The relation between membrane voltage and tension has been examined under voltage clamp conditions by several laboratories, using several different mammalian cardiac muscle preparations and techniques. Beeler and Reuter (1970) used a hybrid sucrose gap technique on dog trabecular muscles to obtain the relation between peak steady-state tension and the voltage of 500-ms voltage clamps at a frequency of 0.33 s^{-1} . When they examined

voltages between -80 and $+30$ mV, there was a small tension developed at all voltages positive to the threshold for I_{Na} (-61 mV), and a sigmoid relationship between tension and voltage which began at -35 mV and plateaued at 0 mV. They concluded that the small "foot" on the curve between -61 and -35 mV was due to poor voltage control during the sodium inward current. The same basic result was obtained by New and Trautwein (1972) in cat ventricular muscle, apparently using a similar experimental design, but the foot on the curve occurred only when voltage control appeared to be less than optimal. In the latter experiments, the plateau of the curve extended to $+40$ mV. Tritthart et al. (1973), using the double sucrose gap technique in cat papillary muscles, obtained a voltage-tension curve in which there was a sudden appearance of tension coincident with the threshold of I_{Na} .

Ochi and Trautwein (1971) used a different experimental design to examine the voltage-tension behavior of guinea pig papillary muscles, switching on the voltage clamp after establishing a steady state with action potentials stimulated at 1 s^{-1} . The relationship between voltage and tension obtained in this way was a double sigmoid relationship, with tension threshold at approximately -50 mV, an inflection and secondary rise at about -20 mV, and no clear plateau up to $+45$ mV. We (Gibbons and Fozzard, 1971) used single 500-ms clamps after a rest to examine the voltage-tension relation of sheep Purkinje fibers. Tension threshold appeared unrelated to sodium inward current. Above the tension threshold of -55 to -60 mV, tension was a steep function of voltage; there was an inflection at about -20 mV, and a secondary rise at more positive voltages.

Inadequate voltage control when the sodium inward current is large, acknowledged to be a problem with all voltage clamp techniques for heart muscle, apparently is responsible for some of the wide variation between results from different laboratories. Even so, there are striking differences not likely to be due to this problem, and it is important to find out why these differences exist. We think the experiment illustrated in Fig. 5 offers a clue to the source of some of the differences. Taking the train of clamps to $+2$ mV as an example, the relationship between voltage and tension clearly is different for every clamp in the train since the tension is changing in a complex way while the voltage of each clamp is the same. Plotting the tension of the steady-state contractions as a function of clamp voltage will give one voltage tension curve, while plotting the tension of the first contraction as a function of voltage will give another. In the latter case, the conditions of the determination would be those we used earlier (Gibbons and Fozzard, 1971), while in the former case the experiment would be analogous to that used by Beeler and Reuter (1970), New and Trautwein (1972), and Tritthart et al. (1973). The voltage-tension relations obtained in this way from the data of Fig. 5

are plotted as curves *a* and *b* in Fig. 6. The curves were drawn by eye to include almost all the experimental points, even though that caused some bumps and dips in the curves. The threshold voltage for curve *b* was between -35 and -40 mV, within the range reported by New and Trautwein (1972).

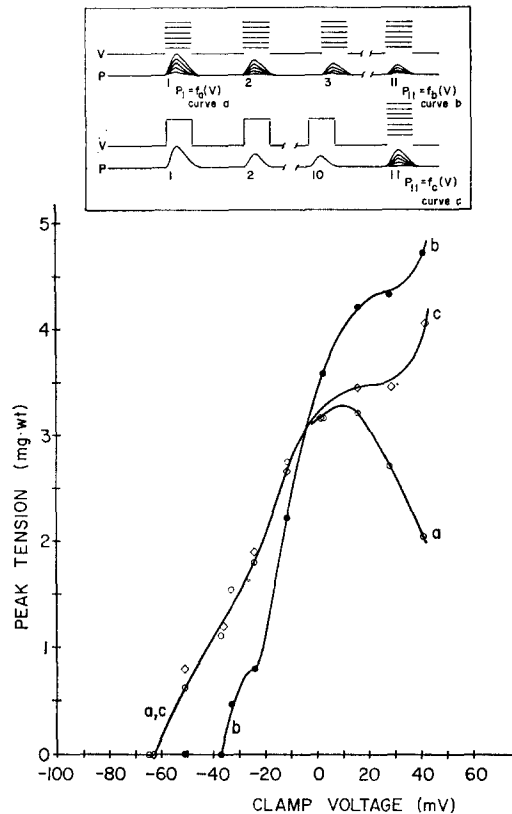


FIGURE 6. Dependence of the voltage-tension relation on the experimental design. The inset illustrates three general approaches which have been used to determine the voltage-tension relation of heart muscle. Curves *a*, *b*, and *c* are the voltage-tension curves obtained when these three methods are used in the same Purkinje fiber preparation. Clamp duration 300 ms, frequency 1 s^{-1} , holding voltage -78 mV. For curve *c*, the steady state was established by 10 clamps to $+1$ mV. (Same preparation as in Fig. 5, and the data for curve *a* are the first contractions of the trains in that figure.)

As it is drawn, there is a foot on the curve which may or may not be real in this particular case, since to draw the foot may place undue reliance on our ability to measure accurately tensions smaller than 0.5 mg-wt. In any case, the appearance of tension above -40 mV was not coincident with the threshold for I_{Na} (Gibbons and Fozzard, 1975). If the foot on the curve is ignored, the curve is sigmoid with a tension threshold near -40 mV, and

we could easily have drawn the same curve in the voltage range -40 to $+30$ mV that Beeler and Reuter (1970) and New and Trautwein (1972) drew.

Curve *a*, for voltages up to $+10$ mV, is the same as that which we previously obtained under similar conditions (Gibbons and Fozzard, 1971), including the slight inflection at -30 to -20 mV. At voltages positive to $+10$ mV, outside the voltage range we were able to examine in our earlier work, the tension of the first contraction after a rest declined sharply with increasing voltage.

Our equipment was not arranged to allow us to reproduce exactly the experimental approach used by Ochi and Trautwein (1971), but we did determine the voltage-tension relation by establishing a steady-state contraction with 10 300-ms voltage clamps to $+2$ mV in this same preparation, and then interrupting the steady state with an 11th clamp to various voltages. The steady state was reestablished before each test clamp. The resulting relation is curve *c* of Fig. 6, which is indistinguishable from curve *a* for voltages between -70 and 0 mV. It does not decline at more positive voltages, although as the curve is drawn there is a slight dip. Again, it would have been reasonable to draw the same curve through these points as that drawn by Ochi and Trautwein (1971, Figure 6).

These data illustrate that the various experimental designs used to examine the voltage-tension behavior of cardiac muscle can give strikingly different results. Even the correspondence between curves *a* and *c* may have been fortuitously good. We showed (Gibbons and Fozzard, 1971) that a change in the holding potential changed the shape of the voltage-tension relation (determined in the manner of curve *a*) and shifted it along the voltage axis. We changed the holding voltage from -78 mV, used to obtain the data of Fig. 6, to -58 mV and redetermined the voltage-tension relations of this preparation. The curves *a'*, *b'*, and *c'* of Fig. 7 are the result.

The tension thresholds of curves *a'* and *c'* were shifted by about the same amount and the shapes of the curves changed slightly so that the correspondence was not as good as in Fig. 6. The tension threshold of curve *b'* was unchanged by the shift in the holding potential, and one might still conclude that there was a foot on the curve. The tension at all voltages in curve *b'* was reduced with very little change in the shape of the curve. In Figs. 6 and 7, the behavior of tension at voltages in the $+30$ to $+50$ mV range must be regarded as a tentative description, both because we seldom obtained a preparation which lasted through such a long and rigorous experiment and because voltage uniformity may not be as good as one would like at such extreme voltages. The dependence of the voltage-tension relation on the experimental design, however, and especially the difference between the methods which gave curves *a* and *b*, was observed regularly.

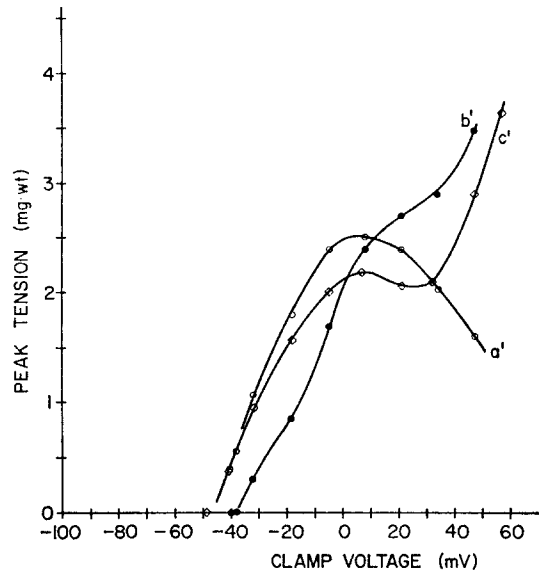


FIGURE 7. Changes in the voltage-tension relations in response to a shift in the holding voltage. The preparation is the same as that used for Fig. 6, and the experiments were done in the same way except that the holding voltage was -58 mV. The steady state for curve c' was established by a train of clamps to 0 mV.

DISCUSSION

One general conclusion of this work is that, under a variety of voltage clamp conditions, the contractile behavior of the Purkinje fiber is very similar to that of working ventricular muscle. Although the contraction in Purkinje fibers is much smaller, this is likely to be due to the small cross-sectional area of the fiber and the reduced content of myofibrils. Page and co-workers (Mobley and Page, 1972; Page et al., 1971) report that in rat ventricular muscle the volume fraction of myofibrils is 0.48 and in the sheep Purkinje fiber it is 0.234. Comparable studies on relative quantities of sarcoplasmic reticulum membrane are not available.

In the case of one rather rigorous test of the voltage dependence of contraction, the determination of the voltage-tension relation, we obtain results almost identical to those obtained by others in ventricular muscle (Beeler and Reuter, 1970; Ochi and Trautwein, 1971) if we use experimental procedures similar to theirs. The main qualitative difference between the contraction of the cardiac Purkinje fiber and the contraction of working ventricle appears to be that the first contraction after a rest is large in the Purkinje, whereas it has been reported to be very small in ventricular muscle (Beeler and Reuter, 1970). This presumably explains why others have tended to examine the voltage-tension relation during steady stimulation. If the

calcium which activates the first contraction after a rest comes primarily from intracellular stores, it would appear that a long rest depletes the intracellular stores of ventricular muscle. We cannot presently explain the different behavior of the rest contraction, but it is interesting to note that Bautovich et al. (1962) reported contractile behavior of the rabbit papillary muscle similar to that reported here for Purkinje fibers.

Given that a number of different voltage-tension relations can be obtained in the same tissue when different procedures are used, it is appropriate to ask which of them is most useful, and whether any of them gives the true dependence of tension on voltage. The method which we initially used (Gibbons and Fozzard, 1971, and curve *a* of Fig. 6) is relatively easy. The conditions before the test appear to be a true steady state, for the state of the excitation-contraction coupling mechanism seems to be constant and, perhaps more important, the same conditions should exist before each test. This method has the disadvantage that the steady state is artificial, since the heart never rests for 90 s to 2 min, and the same experiment apparently cannot be done in working ventricular muscle. However, the fact that the conditions are not physiological does not mean that the method may not be useful in inferring normal physiological mechanisms.

The method used by Beeler and Reuter (1970) and New and Trautwein (1972) may also be of great use in determining the mechanism of cardiac excitation-contraction coupling, but we find it more than usually difficult to interpret the results of this type of experiment. Beeler and Reuter (1970) quite carefully and correctly refer to the data as "the relation between steady-state tension and membrane potential." This is very different from considering this to be a steady-state voltage-tension relation, at least within the narrow definition of steady state used above, since the steady state is different for each determination.

The technique used by Morad and Trautwein (1968) and Ochi and Trautwein (1971), in which a steady state is established by action potentials before the clamp is turned on for the voltage-tension determination, is clearly the most physiological of the three general methods. Furthermore, the steady state should be the same before each test. This method is probably closest to the "true" voltage-tension relation, but even if action potentials are used to establish the steady state, different relations between voltage and tension might be obtained at different stimulation frequencies.

We think that the result obtained with any of these methods constitutes an exhaustive test of the muscle behavior under the conditions of the test, in the sense that any hypotheses about the excitation-contraction coupling mechanism must be able to explain the complex voltage-tension behavior. However, it is difficult to infer much about the excitation-contraction coupling mechanism directly from the voltage-tension curves, and it is necessary to

consider whether there is some orderly way of analyzing our experiments which might ultimately be useful in determining what the cellular mechanisms are.

There have been several attempts to propose a coherent framework for the various rate- and rhythm-dependent contractile phenomena of cardiac muscle. Kruta and Braveny (1961) proposed that the common base linking the phenomena was a variable rate of "restitution" or "recovery" of the ability to contract. Blinks and Koch-Weser (1961) proposed a system which utilized three factors: the strength of the rested state contraction, the negative inotropic effect of activation (NIEA), and the positive inotropic effect of activation (PIEA). A more mathematical analysis has been attempted by Johnson and his co-workers, beginning with a study of contractile recovery by Bautovich et al. (1962). The usefulness of these approaches is that they attempt an orderly dissection of a wide variety of phenomena into basic "processes." This provides at least a conceptual framework, and it is logical to hope that identifying and studying behavior common to the various contractile phenomena may illuminate the cellular mechanisms behind them. Although each of these approaches has advantages, we find it easiest to analyze our experiments in terms of what they imply about the restitution or recovery process described by Kruta and Braveny (1961).

There is an extensive literature on the behavior of contractile recovery, and we can only outline here the basic aspects of recovery behavior which may relate to our experiments. Between contractions in response to stimulation at a steady rate, heart muscle gradually recovers the ability to respond to another stimulus. This recovery is typically slow relative to the interval between steady-state contractions, so that the ability of the muscle to contract is still changing as each regular stimulus is delivered. In fact, the ability of the muscle to contract continues to change as a function of time for several seconds or more after a train of stimuli (Kruta and Braveny, 1961; Bautovich et al., 1962), although only the initial rate of the restitution or recovery process should be crucial in determining the strength of contractions at physiological stimulation rates. This early rate is quite variable. For example, if the stimulation frequency is increased the time available for recovery between stimuli is of course less, but the early rate of recovery increases. For frequencies in the physiological range, the increased rate of recovery usually more than compensates for the shorter interval between contractions, and the strength of the steady-state contraction increases (Kruta and Braveny, 1961).

There have been relatively few attempts to use voltage clamp techniques to further define the behavior of contractile recovery. We examined the time-course of recovery after a single voltage clamp using two identical clamps separated by various intervals (Gibbons and Fozzard, 1971). Recovery began only after the muscle was repolarized, and the final level of

recovery was a function of the membrane voltage during the repolarized interval between the initial and the test clamps (Beeler and Reuter, 1970; Gibbons and Fozzard, 1971). We, along with others (Wood et al., 1969; Gibbons and Fozzard, 1971; Morad and Goldman, 1973), have suggested that as heart muscle relaxes, calcium is taken up by sites from which it cannot be released by depolarization and that recovery, or at least the initial rate of recovery, reflects the return of calcium to an available site or pool. Clearly other processes, such as the gain or loss of calcium by the cells, might also have a profound effect.

The basic aspects of the behavior of contractile recovery outlined above allow us to draw limited conclusions about how the initial rate of recovery must have behaved during the trains of clamps used in these experiments. After each voltage clamp in the train, recovery begins (from zero) when the preparation is repolarized, and each clamp after the first constitutes a test of how much recovery occurred during the preceding interval. Because the interval between clamps is constant during a particular train, a positive staircase in response to the second and succeeding clamps indicates a progressive increase in the initial rate of recovery, while a negative staircase indicates a progressive decrease in the initial rate of recovery. Contractions presumably reach a steady level when the initial rate of recovery is no longer changing.

The first type of experiment reported here (Fig. 3) showed that it is possible to obtain a negative or a positive staircase, depending on the duration of the clamps in a train. We would infer from this that the initial rate of recovery after a clamp depends, in part, on the duration of that clamp. It would also seem that this effect of duration can be cumulative in the course of a train. Because determination of the full time-course of recovery is a laborious process, we have only begun to examine the time-course of recovery. We have, however, checked directly the first of the above conclusions.

Fig. 8 shows the result of an experiment in which contractions were elicited by 90-, 190-, and 500-ms clamps, each after a rest of 90 s or longer. Each of the three initial clamps was long enough to cause a full size contraction characteristic of the clamp voltage. The rate of recovery after each clamp was determined with a 500-ms test clamp to the same voltage as the first, and the tension response to the test clamp, relative to that caused by the initial clamp, is plotted as a function of the repolarized interval between the two clamps. As the data on the behavior of trains suggested, the initial rate of recovery increased considerably as the duration of the first clamp was increased. There was a slight oscillation in the recovery process after the 500-ms clamp. After the 90-ms clamps, there appears to be a slight lag before the recovery process begins. This is consistent with the interpretation that recovery represents cycling of intracellular calcium, since contraction

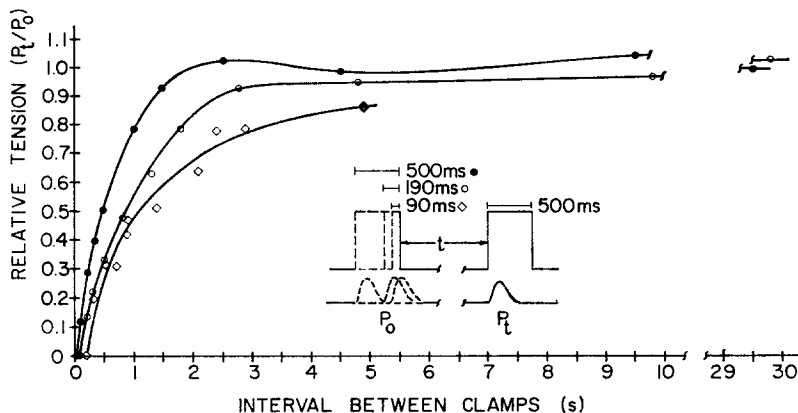


FIGURE 8. Rate of recovery after clamps of different durations. The inset illustrates the method used to examine the rate of recovery after 90-, 190-, and 500-ms clamps. Each of the first clamps was long enough that the initial contraction, P_0 , was not a function of clamp duration. The initial contractions did, however, decline slowly with time during the course of the experiment. The repolarized interval between the initial and the test clamps was varied, and the tension response to the test clamp, relative to the initial response, is plotted as a function of the interval between clamps. The holding voltage was -70 mV, the clamp voltage was -4 mV for both clamps, and the test clamp duration was 500 ms.

is about at a peak at 90 ms and a short time is required for relaxation. The final extent of recovery, after 30 s, is the same for the two curves which were examined for such long times, but of course it would be the initial rate which determines the size of contractions in any train at frequencies such as 0.5 or 1 s^{-1} . For two 500-ms clamps at a frequency of 0.5 s^{-1} , the second contraction should be about equal to the first according to the curves in Fig. 8. This agrees well with the behavior of 500-ms clamps in Fig. 3.

Since determination of the full time-course of recovery bears out the qualitative judgment based on the behavior of trains of clamps, we think it reasonable to make preliminary judgments about the effect of holding voltage and clamp voltage on recovery, pending a more thorough examination. When the duration of the clamps and the interval between them were kept constant, decreases in the holding voltage also changed the staircase behavior (Fig. 4). Unlike the case of the experiments in Fig. 3, the final level of recovery (e.g. at the end of 90 s) should be a function of the holding voltage and should be different for each curve (Beeler and Reuter, 1970; Gibbons and Fozzard, 1971). Note, for example, the decreases in the first contraction after a rest. Even so, the results indicate that the initial rate of recovery changed in different ways during the course of trains at the different holding voltages, since there was a negative staircase for a holding voltage of -48 mV, and a positive staircase at a holding voltage of -68 mV.

Clamp voltage clearly has a very strong effect on the rate of recovery (Fig. 5). For a 1-s^{-1} train of 300-ms clamps from a holding voltage of -78 mV, the initial rate of recovery seemed to stay approximately constant throughout when the clamp voltage was -25 mV. There was a progressive increase in the initial recovery rate for trains to less negative voltages, and a progressive decrease for clamps to more negative voltages.

Given the possibility that recovery reflects the return of activator calcium to a releasable pool, the initial rate of recovery could depend on the amount of calcium in the muscle. Certainly those maneuvers which led to positive staircases are generally thought to do so by augmenting intracellular calcium stores. If this interpretation is correct, however, then it would also follow that negative staircases occur as the intracellular stores are progressively depleted, in spite of the fact that there appears to be inward calcium movement, or at least slow inward current, at clamp voltages which cause a negative staircase (Gibbons and Fozzard, 1975). At this stage in our understanding of heart muscle, this is only speculation, and in order to draw really meaningful conclusions about the mechanisms which govern cardiac contractility, more data are needed. The recovery process seems at least to be a reasonable, and somewhat different, way of approaching the voltage and time dependence of cardiac contraction. Voltage clamp techniques offer a way of taking a more rigorous look at this process in the hope that its behavior may offer clues as to how calcium is gained, lost, and recycled by cardiac cells.

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REFERENCES

- BAUTOVICH, G., D. B. GIBB, and E. A. JOHNSON. 1962. The force of contraction of the rabbit papillary muscle preparation as a function of the frequency and pattern of stimulation. *Aust. J. Exp. Biol. Med. Sci.* **40**:455.
- BEELER, G. W., JR., and H. REUTER. 1970. The relation between membrane potential, membrane currents and activation of contraction in ventricular myocardial fibres. *J. Physiol. (Lond.)* **207**:211.
- BLINKS, J. R., and J. KOCH-WESER. 1961. Analysis of the effects of changes in rate and rhythm upon myocardial contractility. *J. Pharmacol.* **134**:373.
- DELEZE, J. 1970. The recovery of resting potential and input resistance in sheep heart injured by knife or laser. *J. Physiol. (Lond.)* **208**:547.
- FOZZARD, H. A., and W. R. GIBBONS. 1973. Action potential and contraction of heart muscle. *Am. J. Cardiol.* **31**:182.
- FOZZARD, H. A., and D. C. HELLAM. 1968. Relationship between membrane voltage and tension in voltage-clamped cardiac Purkinje fibres. *Nature (Lond.)* **218**:588.

- FOZZARD, H. A., and M. HIRAOKA. 1973. The positive dynamic current and its inactivation in cardiac Purkinje fibres. *J. Physiol. (Lond.)*. 234:569.
- FOZZARD, H. A., and M. SCHOENBERG. 1972. Strength-duration curves in cardiac Purkinje fibres: effects of liminal length and charge distribution. *J. Physiol. (Lond.)*. 226:593.
- GIBBONS, W. R., and H. A. FOZZARD. 1971. Voltage dependence and time dependence of contraction in sheep cardiac Purkinje fibers. *Circ. Res.* 28:446.
- GIBBONS, W. R., and H. A. FOZZARD. 1975. Slow inward current and contraction of sheep cardiac Purkinje fibers. *J. Gen. Physiol.* 65:367.
- HAYKIN, S. S. 1969. Synthesis of RC Active Filter Networks. P. 105. McGraw-Hill, London.
- HELLAM, D. C., and R. J. PODOLSKY. 1969. Force measurements in skinned muscle fibres. *J. Physiol. (Lond.)*. 200:807.
- JOHNSON, E. A., and M. LIEBERMANN. 1971. Heart: excitation and contraction. *Annu. Rev. Physiol.* 33:479.
- KAVALER, F. 1959. Membrane depolarization as a cause of tension development in mammalian ventricular muscle. *Am. J. Physiol.* 197:968.
- KRUTA, V., and P. BRAVENY. 1961. Restitution de la contractilité du myocarde entre les contractions et les phénomènes de potentiation. *Arch. Int. Physiol. Biochim.* 69:645.
- MOBLEY, B. A., and E. PAGE. 1972. The surface area of sheep cardiac Purkinje fibres. *J. Physiol. (Lond.)*. 220:547.
- MORAD, M., and Y. GOLDMAN. 1973. Excitation-contraction coupling in heart muscle: membrane control of development of tension. *Prog. Biophys. Mol. Biol.* 27:257.
- MORAD, M., and W. TRAUTWEIN. 1968. The effect of the duration of the action potential on contraction in the mammalian heart muscle. *Pfluegers Arch. Eur. J. Physiol.* 299:66.
- NEW, W., and W. TRAUTWEIN. 1972. The ionic nature of slow inward current and its relation to contraction. *Pfluegers Arch. Eur. J. Physiol.* 334:24.
- OCHI, R., and W. TRAUTWEIN. 1971. The dependence of cardiac contraction on depolarization and slow inward current. *Pfluegers Arch. Eur. J. Physiol.* 323:187.
- PAGE, E., L. P. MCCALLISTER, and B. POWER. 1971. Stereological measurements of cardiac ultrastructures implicated in excitation-contraction coupling. *Proc. Natl. Acad. Sci.* 68:1465.
- TASAKI, K., Y. TSUKAHARA, S. ITO, M. J. WAYNER, and W. Y. YU. 1968. A simple, direct and rapid method for filling microelectrodes. *Physiol. Behav.* 3:1009.
- TRITTHART, H., R. KAUFMANN, H.-P. VOLKMER, R. BAYER, and H. KRAUSE. 1973. Ca-movement controlling myocardial contractility I. *Pfluegers Arch. Eur. J. Physiol.* 338:207.
- WOOD, E. H., R. L. HEPPNER, and S. WEIDMANN. 1969. Inotropic effects of electric currents. *Circ. Res.* 24:409.