Limitations of the Double Sucrose Gap Voltage Clamp Technique in Tension-Voltage Determinations on Frog Atrial Muscle

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SUMMARY The purpose of this study was to evaluate the limitations of the double sucrose gap voltage clamp technique in the determination of tension-voltage relationships for frog atrial muscle. Tension-voltage relationships were determined under two conditions. In one case we determined both the tension response and slow inward current associated with an apparent step depolarization (step-clamp) as a function of the magnitude of the step depolarization. In the second case, an action potential was elicited, the voltage clamp was applied early during the plateau phase of the action potential, and the tension response was determined as a function of the clamp potential (action potential-clamp). Under both step-clamp and action potential-clamp conditions, the waveform of the tension response rose to a peak value (T_p) and then decayed with time to a tension that was maintained for the duration of the depolarization. The T_p-clamp potential relationships obtained under step-clamp and action potential-clamp conditions were similar. Microelectrode measurements of transmembrane potential of cells in the "voltage-clamped" region of the preparation demonstrated the lack of temporal and spatial voltage control under both step-clamp and action potential-clamp conditions, and also demonstrated that acquisition of spatial voltage control occurred at about the same time that the tension response reached its peak value. These data indicate that this voltage clamp technique does not allow an accurate determination of the so-called phasic tension-voltage relationship in frog atrial muscle because of a lack of temporal and spatial control of voltage during the rising phase of the tension response.

RECENTLY, the double sucrose gap voltage clamp technique has been applied in studies on frog atrial and ventricular tissue to investigate relationships between contractile activity, transmembrane voltage, and membrane current. The voltage clamp technique presumably would allow a determination of the dependency of contractile activity on transmembrane ionic current independently of its dependency on membrane potential, and in this regard the role of the delayed inward calcium current in controlling contractile activity of frog cardiac muscle has been of particular interest. The assumption that has been made in the interpretation of data obtained by this technique is that voltage control of the cells in the "voltage-clamped" region of the tissue was accomplished rapidly with respect to the onset of both the delayed inward current and the tension response and thus allowed an interpretation of tension-voltage relationships in terms of current-voltage relationships. However, no experimental data validating this assumption have been published.

In 1972 we investigated the tension-voltage-current relationships in frog atrial tissue with the double sucrose gap voltage clamp technique and obtained data (unpublished) similar to those obtained by other investigators who have used this technique. In particular, the change in tension associated with step depolarizations had a waveform suggestive of two components: i.e., the tension response rose to an initial peak and then decayed with time to a maintained value which persisted for the duration of the depolarization.

Also there appeared to be a close correlation between the initial peak tension and the slow inward calcium current (I_Ca) in that (1) short-lasting depolarizations which elicited I_Ca also elicited contraction, (2) the threshold potentials for both contraction and I_Ca were similar, and (3) both the initial peak tension-clamp potential relationship and the I_Ca-clamp potential relationship were maximum at the same potential. Alterations in the magnitude of I_Ca by changes in the extracellular calcium concentration were accompanied by alterations in both the initial peak tension and the sustained tension; reduction in extracellular calcium reduced the magnitude of these parameters. The data suggested that the slow inward calcium current played a predominant role in controlling the contractile activity of frog atrial muscle.

After the investigation of the tension-voltage-current relationships, we began a study of the uniformity of spatial voltage control among cells in the so-called voltage-clamped region (test node) of the preparation. The results clearly demonstrated that, in our preparations, it was not possible to control transmembrane voltage of all cells in the test node region of frog atrial bundles 200–400 μm in diameter during either the fast or slow inward currents elicited by step-clamp depolarizations. In the absence of spatial voltage control the interpretation of current-voltage relationships becomes obscure, and these data raised in our minds some serious reservations about the use of the double sucrose gap voltage clamp technique for the determination of tension-voltage-current relationships in frog atrial muscle. The present study extends our previous investigation of the limitations of double sucrose gap voltage clamp technique to include observations on the spatial uniformity of voltage control and the tension response of the test node region under so-called voltage clamp conditions. The data to be presented demonstrate that the double sucrose gap voltage clamp technique is severely limited in terms of the determination of relation-
ships between contractile activity, membrane voltage, and membrane current in frog atrial muscle.

Methods

The experiments were performed on thin bundles (200–400 μm in diameter) of frog (Rana catesbeiana) atrial muscle. The double sucrose gap voltage clamp technique as applied to frog atrial muscle, and the general experimental procedure, have been discussed extensively in a previous paper. A discussion of the method as applied in this investigation follows.

In the sucrose gap chamber, a short segment (100–200 μm) in the middle of the preparation (test node) was isolated from the ends of the bundle by two streams of isosmotic sucrose solution which filled the extracellular space. The center pool (test pool) was perfused with normal Ringer’s solution or an appropriate test solution. The right (potential measuring) and left (current injecting) pools were perfused with isosmotic KC1. The membrane potential of the tissue segment (test node) in the center pool was measured as the potential of the right pool with respect to the center pool. The tension response of the test node was measured with a sensitive semiconductor force beam (Königsberg Instruments) attached to the end of the fiber in the right pool by a fine silk thread. Passive tension of approximately 100–200 mg was applied to the bundle. High sensitivity to dynamic tension and high resolution were maintained in the face of relatively high static tensions by applying a force to the transducer to oppose the passive muscle tension. The opposing force was generated by a calibrated spring the transducer to oppose the passive muscle tension.

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The combined transducer-spring-amplifier system provided: (1) a range of static tension from 0–500 mg, (2) ranges of dynamic tension of 0–10 mg to 0–600 mg with a resolution of 5% of full scale, (3) a system compliance of 1.5 x 10^-8 mm/mg, and (4) a system response rise time of less than 6 msec. The contractile response of the fiber, elicited either by an action potential or by a step depolarization under voltage clamp conditions, was observed with a microscope to ensure that the observable contractile activity of the bundle was localized to the test node. Perfusion of both end pools with isosmotic KC1 was mandatory because a tension response frequently was elicited on termination of a step depolarization when the left pool was perfused with normal Ringer’s solution. This tension response, always associated with clearly visible contractile activity of the fiber in the left pool, was eliminated by perfusion of that pool with isosmotic KC1.

Under voltage clamp conditions, two types of experiments were performed. In one case, the potential of the test node was displaced in rectangular steps and the resulting changes in current and tension were measured (step-clamp). In the second case an action potential was elicited in the test node; an action potential or by a step depolarization under voltage clamp conditions, was observed with a microscope to ensure that the observable contractile activity of the bundle was localized to the test node. Perfusion of both end pools with isosmotic KC1 was mandatory because a tension response frequently was elicited on termination of a step depolarization when the left pool was perfused with normal Ringer’s solution. This tension response, always associated with clearly visible contractile activity of the fiber in the left pool, was eliminated by perfusion of that pool with isosmotic KC1. In experiments concerned with the spatial homogeneity of voltage control, the intracellular potentials of cells in the test node were measured with conventional glass microele-
current and tension responses were recorded. Data obtained from both experiments on any particular bundle then were compared. Figure 1 demonstrates the temporal relationships between current, tension, and transgap voltage under conditions of step-clamp and AP-clamp.

The rationale behind the AP-clamp approach lies in the observations that (1) the slow inward current elicited by a step-clamp depolarization appears to be largely inactivated within about 200 msec after the initiation of the step depolarization (Fig. 1), and (2) several investigators have presented data that suggests the slow inward current plays a predominant role in controlling contractile activity in frog cardiac muscle. Assuming that a similar rapid inactivation of the slow inward current occurs during the plateau of the action potential, it would seem that rapid acquisition of spatial voltage control could be accomplished by applying the voltage clamp approximately 200 msec after the initiation of an action potential. Use of this experimental technique would eliminate the need for spatial voltage control during the period of low membrane resistance associated with the slow inward current; from these experiments we hoped to obtain information on the voltage dependency of the tension response under conditions of reasonable spatial uniformity of voltage of the cells in the test node region. Furthermore, if the slow inward current plays a major role in controlling the contractile activity of frog cardiac muscle, then it seemed reasonable to anticipate that the tension responses would be markedly different under step-clamp and AP-clamp conditions. Under AP-clamp conditions a major part of the slow inward current would be that associated with the rising phase of the action potential, and therefore the magnitude of the slow inward current presumably would be relatively constant under AP-clamp conditions. In contrast, in response to step depolarizations, the slow inward current appears to be dependent on the magnitude of the depolarization and therefore the slow inward current presumably would be variable in the step-clamp experiments. At the outset of these experiments we anticipated that by comparing AP-clamp tension-voltage data with step-clamp tension-voltage data we might gain insight into the contribution of the slow inward calcium current in controlling contractile activity in frog cardiac muscle.

Figure 2 shows data obtained from one preparation which compares the tension responses elicited by a step-clamp depolarization to the tension response associated with the early phase of an action potential followed by a voltage clamp to the same level of potential as that attained under step-clamp conditions; these data were obtained from the same bundle for which data are presented in Figure 1 to show temporal relationships between the step-clamp and AP-clamp. In response to a step-clamp depolarization, the tension response rose to a peak and then decayed with time; this response was similar to those reported previously by other investigators. A tension response with similar waveform occurred under AP-clamp conditions. In response to a 30-mV depolarization (Fig. 2A) it is apparent that the tension response under AP-clamp conditions was larger than the tension response under step-clamp conditions. However, with larger depolarizing steps (50, 70, and 90 mV) the magnitude of the tension responses were very similar for both techniques (Fig. 2B-D).

The tension-voltage relationships for the peak tension for this preparation obtained under step-clamp and AP-clamp conditions are shown in the upper panel of Figure 3. The lower panel of Figure 3 shows the current-voltage relationship for the slow inward current under step-clamp conditions. It is apparent that the magnitude of the tension responses obtained under step-clamp and AP-clamp conditions were similar over a large range of depolarizations. It also is apparent that this similarity occurred over the range of potentials in which the magnitude of the slow inward current elicited by step depolarizations was highly variable. It should be pointed out the magnitude of the tension response associated with an uninterrupted action potential was equal to that elicited by a step depolarization of +70 mV; i.e., the maximum tension on the tension-voltage curve.

The interpretation of the above tension-voltage and current-voltage data depends critically on knowledge of the quality of spatial voltage control of the cells in the test node and the temporal relationships between the acquisition of spatial voltage control and the tension response. If acquisition of spatial voltage control was rapid under both step and AP-clamp conditions, then the data suggest that the slow inward current, or at least that part associated with the transient peak inward current, plays a negligible role in controlling contractile activity in frog atrial muscle. However, if the acquisition of spatial voltage control occurred slowly with respect to the onset of the tension response, then interpretation of tension-voltage data in terms of current-voltage data would be difficult if not impossible. In the
present experiments, the similarity of the $T_p$-voltage relationships under step- and AP-clamp conditions might be the result of a similar lack of spatial voltage control under both experimental conditions. Under AP-clamp conditions the magnitude of the tension response for potentials on the ascending limb of $T_p$-voltage relationship might be related to variable rates of acquisition of spatial voltage control, thereby leading to variable magnitudes of contractile activity elicited by the action potential activity in the test node. Similarly, under step-clamp conditions the tension response might be influenced by contractile activity elicited by action potentials in the test node.

Intracellular potentials from cells within the test node were measured with microelectrodes in order to assess the quality and speed of voltage control when the voltage clamp was applied during the plateau phase of the action potential. Typical data obtained from one impalement are shown in Figure 4. In Figure 4A the intracellular potentials ($V_{t}$) are compared with the transgap potential ($V$), recorded when the transgap potential was clamped at two different levels during the plateau of the action potential, and are compared along with the tension responses (upper traces; increased tension is a downward deflection) associated with these AP-clamps. Figure 4B shows another comparison, and Figure 4D presents a family of intracellular potentials showing the temporal aspects of the voltage control of the impaled cell when the transgap potential was clamped at different levels. From these data it is clear that voltage control of the impaled cell is gained slowly when the clamp is applied during the plateau phase of the action potential. Furthermore, it appears that the rate at which the action potential is terminated depends on the level of the clamp potential. The action potential is terminated fairly rapidly and a small tension response is recorded (Fig. 4A and B) when the clamp potential is close to the resting potential of the cell. In comparison, a considerable amount of time (several hundred milliseconds) is required for the termination of the action potential and a larger tension response is recorded (Fig. 4A and B) when the clamp potential is at a depolarized level with respect to the resting potential. It should be noted that the time when peak tension occurred corresponds with the time when voltage control of the impaled cell was obtained, and this was the case for all potential steps shown in Figure 4D.

Data obtained from another bundle are shown in Figure 5. For this impalement we determined the voltage control of the impaled cell under both AP-clamp and step-clamp conditions as well as the tension response under the same conditions. In Figure 5A the intracellular potential ($V_{t}$) is compared with the transgap potential ($V$). It is again apparent that a period of several hundred milliseconds was required for acquisition of voltage control of the impaled cell when the voltage clamp was applied during the action potential. In Figure 5B, $V_{t}$ is compared with $V$ under step-clamp conditions. It is apparent that the impaled cell is producing an abortive action potential in response to the step change in transgap potential and that voltage control of the impaled cell is obtained only after a considerable period of time. In Figure 5C the $V_{t}$ responses under AP-clamp and step-clamp conditions are compared and these responses, in addition to the tension responses, are shown in Figure 5D. It is apparent that the time required to gain voltage clamp...
FIGURE 5 Comparison of transgap potential (V) and intracellular microelectrode recorded potentials (V1) under action potential (AP-clamp and step-clamp conditions. The 1-second time calibration in C applies also to A and B. The test node was superfused with Ringer's solution containing tetrodotoxin (TTX). See text for further discussion.

The data presented in Figure 5 clearly demonstrate that action potential activity can be elicited by long duration step-clamp depolarizations and it seemed possible that variable action potential activity might also be elicited by short duration step-clamp depolarizations. Figure 6 demonstrates the results of a typical experiment demonstrating that this is the case. In this figure, the potentials recorded by the intracellular microelectrode and tension responses associated with 600-msec step-clamp depolarizations of 40 mV, 70 mV, and 130 mV have been superimposed. In response to a depolarization of 40 mV (trace 1) it is apparent that an abortive action potential is produced. In response to a depolarization of 70 mV (trace 2) an action potential again is elicited but the delay between the onset of the step depolarization and the onset of the action potential is shorter than in response to the 40-mV depolarization; the tension response also begins with a shorter delay. In response to a depolarization of 130 mV (trace 3) the intracellular recorded potential falls below the transgap potential (horizontal bar at left of figure), nevertheless the intracellular potential is markedly depolarized with respect to the other two examples. In this case, the tension response (trace 3) begins after considerable delay and has a small amplitude. These data demonstrate that action potential activity of variable magnitude can be elicited by short duration, step-clamp depolarizations. Nevertheless, the greater the magnitude of the step-clamp depolarization the more depolarized is the intracellularly recorded potential.

FIGURE 6 Tension responses (upper panel) and intracellular microelectrode-recorded potentials (lower panel) from one cell in the test node when the transgap potential was clamped at three different levels by step-clamp depolarizations of 600-msec duration. The magnitude of the step-clamp depolarizations (40 mV, 70 mV and 130 mV) are indicated by the horizontal bars to the left of the microelectrode recordings. The potential and tension responses associated with these step-clamp depolarizations are numbered accordingly. The test node was superfused with Ringer's solution containing tetrodotoxin (TTX).

control under step-clamp conditions is approximately the same as under AP-clamp conditions and the tension responses are similar in both cases.

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Discussion

Much of our recent knowledge of excitation-contraction coupling in frog myocardium has been derived from voltage-clamp experiments on either frog atrial or ventricular fibers. The emphasis of much of this research has been the investigation of the relationship between the tension response and the membrane voltage as well as the relationship between the tension response and the slow inward calcium current (I_{Ca}).

The interpretation of tension-voltage data obtained from voltage clamp experiments is straightforward if voltage control of all cells in the "voltage-clamped" region of the tissue is accomplished rapidly with respect to the onset of the tension response. To relate the tension response to membrane current, it also is required that spatial voltage control exists during both the current and tension responses under investigation. Morad and Orkand found that a homogeneous distribution of potential was very critical in determining the nature of the tension response elicited by membrane depolarization with the hybrid sucrose gap voltage clamp technique (single sucrose gap with microelectrode as voltage control point). In preparations in which the lack of spatial uniformity of potential was apparent, the tension response associated with long duration (about 1 second) step-clamp depolarizations had an initial peak. Also, short duration depolarizations (less than 100 msec) elicited tension responses. These investigators demonstrated that such tension responses could be eliminated or minimized when the homogeneity of the distribution of membrane potentials was improved by shortening the voltage-clamped region of the preparation. There have been no data presented by investigators who used the double sucrose gap voltage clamp technique that acquisition of spatial voltage control is rapid enough to allow a straightforward interpretation of their published tension-voltage-current data. We found that acquisition of spatial voltage control occurred well after the onset of the tension response elicited by step-clamp depolarizations. The slow acquisition of spatial voltage control, in conjunction with the demonstrated presence of action poten-
tial activity associated with apparent step-clamp depolarizations, indicate that the double sucrose gap voltage clamp technique has severe limitations, at least in our laboratory, for the determination of the relationship between tension, membrane voltage, and I\(_t\). In addition to the limitations imposed by the lack of spatial voltage control, the method of tension recording also may have limitations due to the series elasticity of the several millimeters of muscle imposed between the test node region, where tension is developed, and the end of the bundle where tension is recorded. Possible limitations of the tension-recording system have not been investigated.

Voltage clamp data from both frog atrial and frog ventricular fibers obtained by the double sucrose gap voltage clamp technique have led some investigators\(^{1,4}\) to conclude that I\(_t\) is responsible for controlling a so-called phase tension component (i.e., the tension response associated with relatively short duration step-clamp depolarization). This conclusion has been based primarily on the apparent close correlation between the magnitude of the phase tension and the magnitude of I\(_t\). In contrast, Morad and co-workers\(^{10}\) have concluded from experiments on frog ventricular muscle using the hybrid sucrose gap voltage clamp system that I\(_t\) plays little if any role in controlling the contractile activity of frog cardiac muscle. It should be recognized that our data do not eliminate the role for I\(_t\) in controlling contractile activity, since in both step-clamp and AP-clamp experiments an I\(_t\) would have been elicited by the membrane depolarization. This I\(_t\) might have played a role in controlling the early rising phase of the tension response, but a cause-and-effect relationship between I\(_t\) and tension would be difficult to establish, because of the lack of spatial voltage control. We feel, however, that our data strongly support the conclusion of Morad and co-workers\(^{10}\) that the membrane potential and/or membrane current during the latter phase of the action potential (after the first 100–200 msec) plays the predominant role in controlling the tension response associated with a normal action potential. This conclusion is supported by the following observations indicating a lack of correlation between the apparent membrane potential during the first 200 msec of depolarization and the tension response: (1) The tension responses under AP-clamp and step-clamp conditions were quite similar under conditions for which there appeared to be marked differences in the membrane potential during the first 200 msec of membrane depolarization (Fig. 5). (2) The rising phase of the action potential responses associated with step-clamp depolarizations were highly variable and depended on the level of the clamp depolarization, yet the tension responses were quite similar to those obtained under AP-clamp conditions for which the membrane potential during the first 200 msec of depolarization was relatively constant.

It is well established that modification of the membrane potential of frog cardiac muscle during the plateau of the action potential can markedly affect the magnitude of the tension response.\(^{11,12}\) In general it has been found that hyperpolarizing current shortens the duration of the action potential and reduces the magnitude of the tension response, whereas depolarizing current augments the tension response.\(^{11,12}\) It should be noted that in our preparations the maximum of the tension-voltage relationship obtained under voltage clamp conditions generally corresponded to the magnitude of the tension response associated with a normal action potential. It seems reasonable to conclude that the ascending limb of the tension-voltage relationship obtained under AP-clamp conditions (Fig. 3) resulted from hyperpolarization of the membrane potential during the plateau of the action potential. However, the response to depolarizing current applied during the plateau appears to be different from that reported previously by some investigators.\(^{11,12}\) In our preparations the peak of the tension-voltage relationship under AP-clamp conditions occurred at a voltage below the peak of the action potential. For example, in Figure 3 the peak tension occurred at a depolarization of 70 mV, whereas the action potential in this case had an amplitude of 85 mV. This finding means that depolarizing current applied during the plateau of the action potential also can suppress the tension response. The apparent suppression of tension at large depolarizations is also demonstrated by the short duration step-clamp depolarizations (Fig. 6). In this case, less tension was associated with a step-clamp depolarization of 130 mV than with a depolarization of 70 mV, and intracellular microelectrode recordings from numerous cells in the preparation demonstrated that the membrane potential was more depolarized by the 130-mV depolarization than by the 70-mV depolarization.

At present we cannot give a definitive explanation for the depressed tension response at large depolarizations. Other investigators,\(^{3,4,4}\) using short duration step-clamp depolarizations, have attributed the depressed tension at large depolarizations to the decreased magnitude of the slow inward calcium current, presumably as a result of decreased driving force on the calcium ions flowing into the cell down an electrochemical gradient. Weidmann\(^{13}\) demonstrated that elevation of the extracellular calcium level during the plateau of an action potential in turtle ventricle increases the tension associated with that action potential, and this result suggests that a transmembrane influx of calcium occurs continuously throughout the period of membrane depolarization associated with the plateau of the action potential. Recently, Kavaler\(^{14}\) presented voltage clamp data from frog ventricular fibers which support this conclusion. He demonstrated that alteration of the extracellular calcium concentration 1 second after the initiation of a 10-second step-clamp depolarization affected the magnitude of the tension response during the latter part of the 10-second depolarization. Kavaler's data\(^{14}\) suggest that the tension-voltage relationship in frog ventricular muscle is mediated by a continuous voltage-dependent influx of calcium into the cell. The mechanism by which this continuous influx of calcium is mediated is not clear. The present demonstration that depolarizing voltage clamps applied during the plateau of the action potential suppress tension would be compatible with the influx of calcium being controlled by its electrochemical gradient.

Regardless of what meaning one wishes to attach to the tension-voltage data presented in this paper with regards to the role membrane potential or membrane current, or both, play in controlling contractile activity in frog cardiac muscle, the data clearly demonstrate the need for further
critical evaluation of the double sucrose gap voltage clamp technique by other investigators using this technique in order that the limitations of the technique can be better defined and the tension-voltage-current relationships in frog myocardial tissue better understood.

References

Measurements of Disordered Flows Distal to Subtotal Vascular Stenoses in the Thoracic Aortas of Dogs
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SUMMARY Instantaneous blood velocity measurements employing a constant temperature hot film anemometer were obtained in the region distal to externally enforced, subtotal vascular stenoses in the descending thoracic aortas of anesthetized dogs. Our objectives were to determine alterations in velocity waveforms and energy spectra as the degree of stenosis was increased. We paid particular attention to descending thoracic aortas of anesthetized dogs. Oar objectives were the National Science Foundation under Grant ENG 74-21986.

THE PREDILECTION for cholesterol-laden plaques to cause intimal injury by increasing the local shear stress acting on the endothelial surface1 or by a vibrational effect akin to the phenomenon of fatigue in engineering materials.2

There is also experimental evidence that unstable flow patterns lead to a randomness in endothelial cell orientation,3 Any of these mechanisms might diminish the effectiveness of the normal vascular barrier to lipid accumulation within the intima.

Although the existence of turbulence has been reported in canine4 and equine5 aortas, little is known of the extent of its occurrence in the normal vasculature of man. However, the production of an unstable, fluctuating flow distal to obstructions in a relatively large artery is a natural supposition of the fluid dynamist. Physicians often have attributed various sounds and bruits to turbulence; and recently, direct verification in vivo of the existence of intensely turbulent fields in the presence of vascular stenoses was reported.4

An interesting corollary of these fluctuating velocity fields created by a pathological state is the potential for utilizing the characteristics of the disordered flow to interpret the struc-
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