

# AN ASSESSMENT OF THE DOUBLE SUCROSE-GAP VOLTAGE CLAMP TECHNIQUE AS APPLIED TO FROG ATRIAL MUSCLE

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**ABSTRACT** The homogeneity of voltage clamp control in small bundles of frog atrial tissue under double sucrose-gap voltage clamp conditions was assessed by intracellular microelectrode potential measurements from cells in the test node region. The microelectrode potential measurements demonstrated that (1) good voltage control of the impaled cell existed in the absence of the excitatory inward currents (e.g., during small depolarizing clamp pulses of 10–15 mV), (2) voltage control of the impaled cell was lost during either the fast or slow excitatory inward currents, and (3) voltage control of the impaled cell was regained following the inward excitatory currents. Under nonvoltage clamp conditions the transgap recorded action potential had a magnitude and waveform similar to the intracellular microelectrode recorded action potentials from cells in the test node. Transgap impedance measured with a sine-wave voltage of 1,000 Hz was about 63% of that measured either by a sine-wave voltage of 10 Hz or by an action potential method used to determine the longitudinal resistance through the sucrose-gap region. The action potential data in conjunction with the impedance data indicate that the extracellular resistance ( $R_e$ ) through the sucrose gap is very large with respect to the longitudinal intracellular resistance ( $R_i$ ); the frequency dependence of the transgap impedance suggests that at least part of the intracellular resistance is paralleled by a capacitance. The severe loss of spatial voltage control during the excitatory inward current raises serious doubts concerning the use of the double sucrose-gap technique to voltage clamp frog atrial muscle.

## INTRODUCTION

Recent attempts to characterize the membrane ionic currents responsible for the cardiac action potential have used either the single or double sucrose-gap voltage clamp techniques on a variety of mammalian and amphibian cardiac preparations. Because of the multicellular nature, the size, and the complexities of the cardiac preparations, these voltage clamp techniques have been severely criticized on theoretical grounds by Johnson and his colleagues (Johnson and Lieberman, 1971; Kootsey and Johnson, 1972; Harrington and Johnson, 1973) who argue that adequate spatial and temporal uniformity of potential control of the cells within the test compartment is not possible during the excitatory inward currents associated with membrane depolarization.

Despite the apparent limitations of these voltage clamp techniques, the consistent

findings that the fast inward sodium currents recorded from a variety of cardiac preparations are similar to those recorded from uniformly polarized preparations (e.g., squid axon) tempts one to the conclusion that the methodological technique is satisfactory. Indeed, Tarr et al. (1973) demonstrated that the fast inward sodium currents recorded from frog atrial tissue would be those expected from the voltage clamp of an equivalent circuit containing a single patch of membrane obeying a slightly modified set of Hodgkin-Huxley equations in series with a constant resistance. It appears that in much of the literature the primary justification for the acceptance of the cardiac voltage clamp data is this similarity between the experimental data and the anticipated results. However, the danger involved in such a justification has been dramatically demonstrated by the theoretical work of Kootsey and Johnson (1972). They demonstrated that the conclusion that the voltage control is adequate and therefore the method satisfactory for the analysis of the ionic currents is not justified simply because the current records have an essentially normal waveform and do not differ very much from those expected from a perfectly voltage-clamped preparation.

Experimental verification of spatial uniformity of potential control in the test compartment requires the use of a roving microelectrode to sample the intracellular potential of cells in the test compartment for comparison of these potentials with the potential at the control point. Unfortunately, published demonstrations of such experimental verification in a single or double sucrose gap have been somewhat limited and have given a variety of results. The most convincing experimental tests of spatial uniformity have been done with the single-gap technique and the results of these experiments have generally not been encouraging.

In single-gap experiments on dog ventricular trabeculae (dimensions of preparation in test compartment; 0.7 mm diam or less by about 0.8 mm in length) it was found that there was good control of membrane potential over the length of the test compartment during the flow of the slow inward current and poor control during the large fast inward sodium current (Beeler and Reuter, 1970; Reuter, 1973). In similar experiments on cat trabeculae (0.5 mm  $\times$  0.7 mm test compartment), loss of spatial voltage control often occurred during both the fast inward sodium current and the slow inward current (New and Trautwein, 1972). However, in some preparations spatial voltage control occurred within 0.5 ms after the onset of a step depolarization; i.e., spatial voltage control occurred during both the fast and slow inward currents (New and Trautwein, 1972).

In double-gap experiments there is not such a thorough documentation of the quality of spatial voltage control in the preparations most commonly used: frog atrial tissue and cat ventricular trabeculae. Perhaps, this lack of documentation is at least in part due to the technical difficulties of the experiments. In our laboratory initial attempts in 1968 to perform the independent microelectrode recordings from cells in the test compartment in frog atrial preparations were unsuccessful. Other investigators using frog atrial preparations have also stated they were unable to perform these experiments (Rougier et al., 1968; Maughan, 1973). In contrast, Haas et al. (1970) re-

ported preliminary microelectrode experiments showed that when a rectangular step depolarization was indicated by the potential across the sucrose gap the potential change between an internal microelectrode and a nearby reference microelectrode was almost the same except for a small hump (up to 5 mV) at the peak of a large inward current. Tests for spatial control during the slow inward current in frog atrial muscle apparently were not performed and it was presumed that no loss of voltage control would occur during the slow inward current (Einwächter et al., 1972). In the case of double-gap experiments on cat ventricular trabeculae, Kohlhardt et al. (1972) reported that microelectrode testing showed a nearly homogeneous potential within the test compartment. It is unclear under what experimental conditions such a homogeneous potential existed.

Certain aspects concerning the voltage clamp of a multicellular preparation such as cardiac muscle have become apparent from the experimental work of New and Trautwein (1972) and Beeler and Reuter (1970) and the theoretical work of Kootsey and Johnson (1972). (1) The loss of voltage control is not necessarily reflected in abnormal looking current waveforms; thus, the waveform of the current cannot be used to decide whether or not spatial voltage control is adequate to allow interpretation of the data. (2) The quality of spatial voltage control can depend on the individual preparation; thus, the results of preliminary experiments on a few cardiac preparations does not justify the conclusion that all similar cardiac preparations are spatially voltage-controlled simply because the currents look similar. (3) The pattern and degree of the spatial inhomogeneity and the current waveform depend strongly on the position of the control electrode in the test compartment; thus, extrapolation of the results of spatial voltage control experiments obtained from single-gap experiments to the double-gap situation or vice versa must be done cautiously.

As a result of these recent findings, it seemed a prerequisite for any future voltage clamp experiments in our laboratory that we reinvestigate the spatial voltage control problem. The limitations of a technique dictate to a large extent the interpretation of data obtained by the use of a technique, and it is our opinion that a proper documentation of the possible limitations of the double sucrose-gap voltage clamp technique as applied to cardiac muscle is essential for the proper evaluation of past, present and future cardiac voltage clamp data by the scientific community. Since frog atrial tissue is one of the most common double sucrose-gap voltage-clamped preparations, and considering the technical difficulties involved in performing the spatial control experiments in conjunction with the technical difficulties involved in the voltage clamp of any cardiac preparation, we have for the present limited our investigation to frog atrial tissue.

## METHODS

The upper part of Fig. 1 shows schematically the sucrose-gap chamber and the lower part of Fig. 1 shows an equivalent circuit of the preparation and the electronic apparatus associated

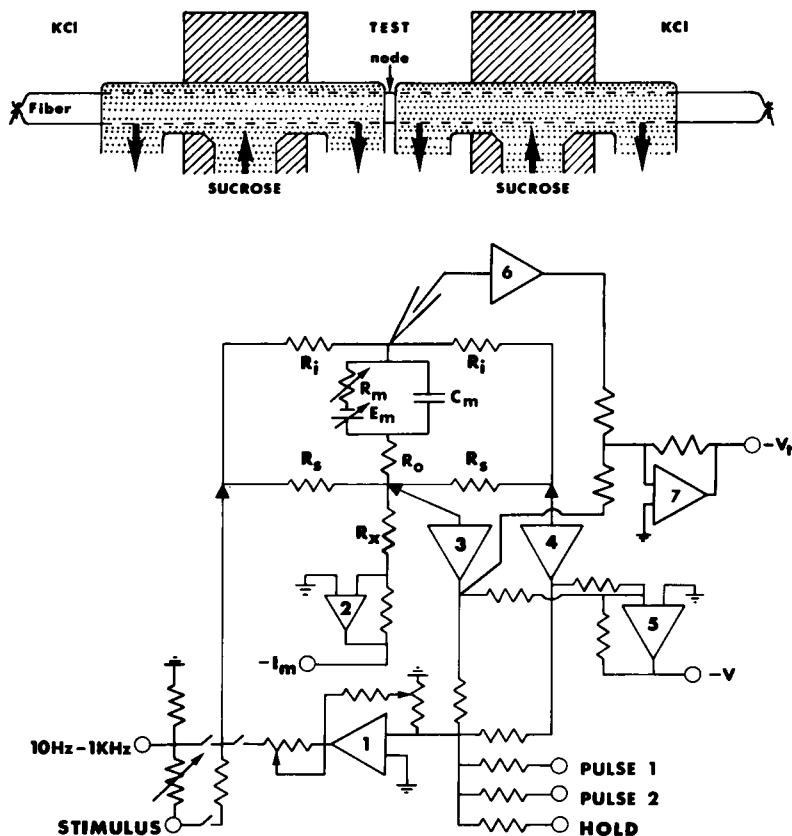


FIGURE 1 Schematic representation of the sucrose-gap chamber (upper part) and an equivalent circuit of the preparation with the associated electronic apparatus (lower part). Amplifiers 1, 2, 5, and 7 are inverting operational amplifiers. Amplifier 4 is a high input impedance operational amplifier follower. Amplifier 3 is a high input impedance inverting amplifier and amplifier 6 is a high impedance, negative capacitance electrometer amplifier. Under voltage clamp conditions the switch in series with the output of the control amplifier (no. 1) was closed thereby placing the test node in the feedback loop. The control amplifier supplied, through an Ag-AgCl electrode in the left pool, the current required to make the potential of the test node (measured as the potential across the right sucrose gap) equal the magnitude of the summed command signals (holding potential and pulses 1 and 2). The test node compartment was actively grounded through an Ag-AgCl electrode connected directly to the summing point (a virtual ground) of operational amplifier no. 2. This amplifier serves as a current to voltage converter to measure the total current flowing into or out of the test compartment. Under nonvoltage clamp conditions, a stimulus could be applied to the test node either by connecting the Ag-AgCl electrode in the left pool to a constant current (very high impedance) source or by connecting the left electrode to a low impedance voltage source: the latter method was used in the determination of longitudinal impedance. To prevent prolonged current flow between the left pool and the test node, the switch connecting the left pool to the voltage source was closed only during the time required to make the resistance measurements. A sine-wave voltage (10 Hz and 1,000 Hz) could also be applied to the Ag-AgCl electrode in the left pool from a low impedance source. (See text for further discussion.)

with the experiments discussed in this paper. A detailed discussion of the double sucrose-gap voltage clamp technique as applied to bundles of frog atrial muscle has been presented previously (Tarr and Trank, 1971). The experiments to be described below were performed on small bundles (300–500  $\mu\text{m}$  diam) of frog (*Rana catesbeiana*) atrial muscle; the test node width was 100  $\mu\text{m}$ .

The intracellular potentials of cells in the test node were measured with conventional 3 M KCl-filled glass microelectrodes (resistances of 10–25 M $\Omega$ ) connected to an electrometer-input, negative capacitance amplifier (no. 6). In order to compare directly the intracellular potential recorded by the microelectrode with that recorded across the right sucrose gap, the potential drop across  $R_x$  (a combination of solution and electrode resistance which exists between the outside of the node membrane and the summing point of amplifier no. 2) was measured by amplifier no. 3 connected to an Ag-AgCl electrode placed in close proximity to the tissue in the test compartment. This potential was electronically subtracted from the outputs of amplifiers 6 and 4. The resultant is a representation of the transmembrane and gap potentials without the contribution of  $IR_x$ . A microelectrode impalement was accepted only if (1) the impalement occurred abruptly as indicated by a sharp drop in the recorded potential to that of the resting potential of the cell, (2) the resting potential recorded by the microelectrode was stable, (3) the resting potential recorded by the microelectrode was equal to or more negative than that recorded across the sucrose gap, and (4) the action potential or the potential change associated with a 10–15 mV depolarizing voltage clamp pulse recorded by the microelectrode had an amplitude equal to or greater than that recorded across the sucrose gap. Less than 50% of the impalements met all of these criteria. Although it was not possible to determine accurately the position of the tip of the microelectrode within the test node, its position with respect to the length of the test node was assigned according to it being in the left third (LTN), center third (CTN), or right third (RTN) of the test node. It was not possible to assess whether superficial or deep cells were impaled.

The longitudinal impedance through the preparation bathed by the left sucrose gap was determined by two methods. In one case, following a stimulus pulse both the left pool and the test pool were held at ground potential (i.e. no potential difference across  $R_x$ ) and the impedance was obtained by measuring the current flow (output of amplifier 2 gave a direct measure of the total current crossing the gap) between the test node and the left pool resulting from the action potential elicited in the test node. The magnitude of the action potential was taken as the potential recorded across the right sucrose gap, the difference between the input potentials of amplifiers 3 and 4. The impedance between the left pool and the test pool was also determined by applying a sinewave voltage (40 mV peak to peak, 10 Hz and 1,000 Hz) to the left pool and measuring the resulting current flow through the gap into the test pool. These measurements give the absolute magnitude of the impedance and do not distinguish between the resistive and reactive components of the intracellular and extracellular current paths.

The Ringer's solution had the following composition (in millimoles per liter): NaCl, 111, KCl, 5.4;  $\text{CaCl}_2$ , 1.8; tris(hydroxymethyl)aminomethane, 10. The pH of this solution was adjusted to 7.0 at 25°C by adding 12.4 N HCl. Isosmotic KCl was 121 mM; isosmotic sucrose was 193 mM. All solutions were maintained at 14–18°C during the experiments. For solutions containing tetrodotoxin (TTX), TTX was added to the Ringer's solution to obtain a TTX concentration of 200 nM. For solutions containing  $\text{CoCl}_2$ ,  $\text{CoCl}_2$  was added to the Ringer's solution to obtain a concentration of 3 mM.

Statistical analyses were done by the methods described by Goldstein (1964). Data are expressed as mean  $\pm$ SE or as the ratio of the mean of one value to the mean of another value with the 95% confidence limits of the ratio.

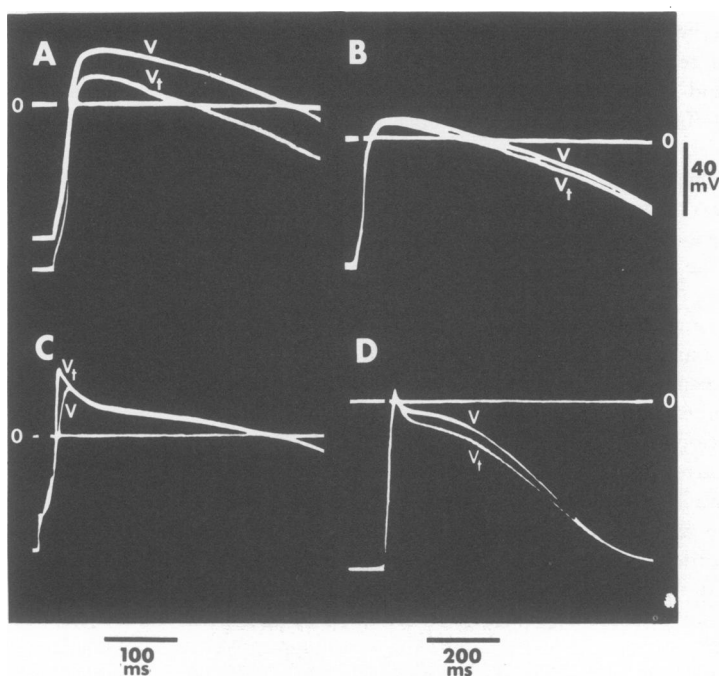


FIGURE 2 Action potentials as recorded with an intracellular microelectrode ( $V_i$ ) and by extracellular Ag-AgCl electrodes across the right sucrose gap ( $V$ ). In A, B, and D the zero voltage trace indicates zero potential for both  $V_i$  and  $V$ . In C,  $V_i$  and  $V$  were artificially superimposed; the resting potential as measured with the microelectrode was 30 mV negative with respect to  $V$ . In A (exp. A013173) and B (exp. D020273) the test node was exposed to normal Ringer's solution; in C (exp. C022173) and D (exp. C031573) the test node was exposed to Ringer's solution containing 3 mM  $\text{CoCl}_2$ . Voltage calibration (40 mV) applies to A, B, C, and D. Time calibration of 200 ms applies to A, B, and D; 100 ms applies to C.

## RESULTS

### *Intracellular vs. Transgap Potentials*

The magnitudes of resting and action potentials recorded from cells in the test node with intracellular microelectrodes compared with those recorded across the right sucrose gap are presented in Fig. 2; data from impalements in four different preparations are shown. In some impalements (Fig. 2 A and C) the resting potentials as measured by the microelectrode were negative with respect to those recorded across the sucrose gap, whereas the action potentials were of similar magnitudes. In other impalements (Fig. 2 B and D) both the resting potentials and action potentials recorded with the intracellular microelectrode had magnitudes similar to those recorded across the sucrose gap. We feel that the more negative resting potentials recorded by the microelectrodes were due to variable tip potentials, since a given microelectrode would in some impalements give a resting potential equal to that recorded across the sucrose gap and in another impalement give a resting potential negative to the sucrose-gap

recorded resting potential. However in both impalements the potential change due to the action potential as recorded by the microelectrode equaled that recorded across the sucrose gap. Exposure of the preparation to 3 mM  $\text{CoCl}_2$  blocked the slow depolarizing phase and suppressed the plateau potential of the transgap recorded action potential; the action potential recorded simultaneously with an intracellular microelectrode showed similar changes of waveform (Fig. 2 C and D).

#### *Voltage Clamp Control during the Fast Inward Current*

In order to test the homogeneity of the voltage clamp control the intracellular potentials ( $V_i$ ) of cells in the test node as recorded by a microelectrode were compared with the apparent clamp potential ( $V$ ) as recorded across the right sucrose gap. In order to increase the reliability of the microelectrode data, double pulse experiments were per-

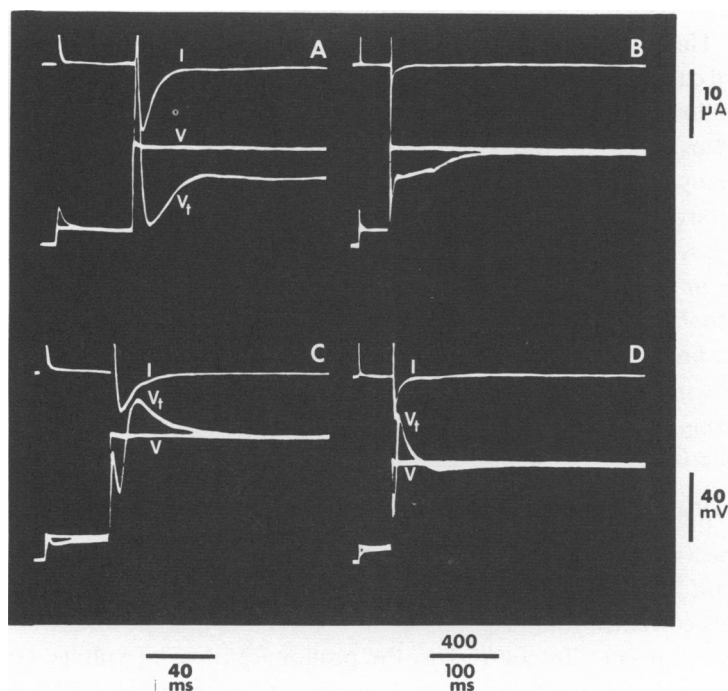


FIGURE 3 Voltage clamp control during the fast inward current. In each case  $V$  is the clamp potential as recorded across the right sucrose gap and  $V_i$  is the intracellular potential of a cell in the test node as recorded by an intracellular microelectrode. Two successive clamp pulses were applied to the preparations. The clamp pulses were as follows: A, B, and D (+10 mV and +50 mV), C (+15 mV and +60 mV). The 40 ms time calibration applies to A and C, the 400 ms calibration applies to B, and the 100 ms calibration applies to D. Voltage and current calibrations apply to A, B, C, and D. In each impalement the resting potential recorded by the microelectrode was the same as that recorded across the sucrose gap; i.e.,  $V_i$  equaled  $V$  in the resting state. Microelectrode position was in the center third (CTN) of the test node for A and B (exp. B033073: fiber diam. of 500  $\mu\text{m}$ , node length of 100  $\mu\text{m}$ ) and RTN for C and D (exp. B032973, different cells: fiber diam. of 300  $\mu\text{m}$ , node length of 100  $\mu\text{m}$ ).

formed on each impalement. A 10–15 mV clamp pulse was first applied in order to assess the quality of voltage control in the absence of any excitatory inward current. A second pulse was then superimposed on the first pulse and this second pulse depolarized the preparation sufficiently to elicit the excitatory inward current. Since the time interval between the first and second pulse was brief, a comparison of the quality of voltage control at the two potential levels seems justified. In some cases several successive double pulse experiments were performed during one impalement and the quality of voltage control during a small depolarizing step was the same before or after a large depolarizing step (Fig. 3 A and B). In experiments concerned with the fast inward currents, the slow inward current was suppressed by exposure of the test node to 3 mM  $\text{CoCl}_2$ .

The results obtained from two different preparations are presented in Fig. 3. In Fig. 3 A the data were recorded at a relatively fast sweep speed on the oscilloscope. It is apparent that following the initial capacitive current  $V_i$  equaled  $V$  during the +10 mV pulse. However,  $V_i$  deviated markedly (about 45 mV) from  $V$  during the excitatory inward current associated with the +50 mV pulse. Data taken at a slower sweep on the oscilloscope (Fig. 3 B) revealed that  $V_i$  deviated from  $V$  for a long period (about 500 ms) following the inward current, but voltage control of the impaled cell was eventually regained. Another type of loss of voltage control frequently seen during the fast inward current is demonstrated in Fig. 3 C and D. In these impalements the loss of voltage control during the inward current is followed by what appears to be an attenuated action potential. The presence of such an action potential in the noncontrolled cell did not generally produce notches on the current waveform.

The data presented in Fig. 3 are representative of the type of data obtained on all preparations investigated (13). In no case were we able to demonstrate spatial voltage control during the fast inward sodium current, whereas spatial voltage control during small depolarizing or hyperpolarizing pulses was easily demonstrable.

#### *Voltage Clamp Control during the Slow Inward Current*

In experiments concerned with the slow inward current, the fast inward current was blocked with 200 nM TTX. It was anticipated that the voltage control during the slow inward current would be good due to the small amplitude and slow rate of activation of the current. In contrast to the pronounced loss of voltage control which was always seen during the fast inward current, it was possible to demonstrate that cells within the test node were voltage controlled during the slow inward current. An example of data taken from an impalement in which voltage control was good during the slow inward current is shown in Fig. 4 A: pulses of +20 mV and +40 mV. However, such data were not obtained routinely. Furthermore, there was a great variability in the quality of voltage control between different cells within a preparation. For example, another impalement made on the same preparation clearly demonstrated that the voltage control of this cell was very poor (Fig. 4 C and D) and that the quality of voltage control depended on the clamp potential. When the clamp pulses were +10 mV and +40 mV (Fig. 4 C)  $V_i$  was greater than  $V$  but the control was fairly stable



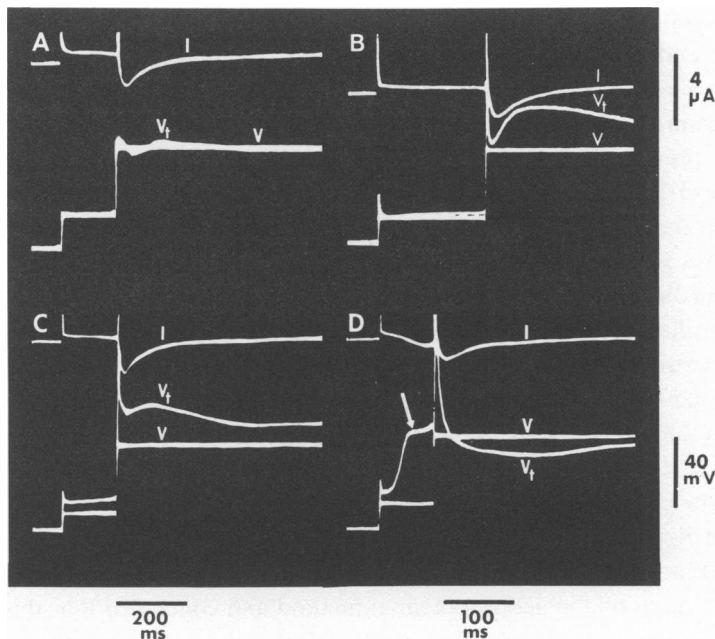


FIGURE 4 Voltage clamp control during the slow inward current. Voltage, and current calibrations apply to A, B, C, and D. The 200 ms time calibration applies to A, C, and D; 100 ms calibration to B. In the resting state,  $V_t$  equaled  $V$  in each impalement. Microelectrode position was RTN for A, CTN for C and D; microelectrode position was not recorded in B. A, C, and D (exp. A032273: fiber diam. of 500  $\mu\text{m}$ , node length of 100  $\mu\text{m}$ ), B (exp. E030273: fiber diam. of 300  $\mu\text{m}$ , node length of 100  $\mu\text{m}$ ).

with time during the slow inward current. However, when the first clamp pulse was increased to +15 mV where an inward current was activated the impaled cell began to show the upstroke phase of an action potential (arrow) just prior to the second clamp pulse. Another example of an action potential type response is shown in Fig. 4 B; these data were obtained from a preparation other than that presented in Fig. 4 A, C, and D. Other preparations demonstrated different types of loss of voltage control during the slow inward current. In some cases  $V_t$  remained negative with respect to  $V$  during the slow inward current: a response similar to that seen during the fast inward current (see Fig. 3). The magnitude of this type of loss of voltage control depended, at least in one case, on the absolute value of the clamp potential. When the clamp pulses were +20 mV and +60 mV the loss of voltage control during the slow inward current was about 10 mV. However, when the clamp pulses were +10 mV and +60 mV the loss of voltage control was about 30 mV even though the magnitude of the inward current did not change appreciably.

#### Gap Resistance

The microelectrode experiments clearly demonstrated that the intracellular change in potential recorded by a microelectrode was of a magnitude similar to the transgap re-

corded potential ( $I$ ) during an action potential, (2) in response to a small depolarizing clamp pulse and (3) following recovery from the loss of voltage control during the excitatory inward current. These findings suggest that in this preparation the extracellular resistance ( $R_e$ ) through the sucrose gap is very large with respect to the intracellular resistance ( $R_i$ ) through the preparation bathed by the sucrose. In contrast, New and Trautwein (1972) presented data from single-gap experiments on cat ventricular trabeculae which they interpreted as indicating a relatively low ratio of  $R_e$  to  $R_i$ : ratio seldom larger than 5 (see Trautwein, 1973). Their conclusions were based on data obtained from the following experiments. The parallel sum of the longitudinal intracellular resistance ( $R_i$ ) between the test compartment and the current injecting compartment and the longitudinal extracellular resistance through the gap ( $R_e$ ) was determined by measuring the current flow through the gap in response to a sine-wave signal (1,000 Hz) applied to the test compartment. The individual resistance  $R_i$  was determined by holding the extracellular potential on both sides of the sucrose gap at ground potential (i.e. no potential difference across  $R_e$ ) and measuring the current flow through  $R_i$  in response to an action potential elicited in the test compartment. They found that the transgap impedance measured at 1,000 Hz was significantly less than that measured by the action potential method and concluded that this difference was due to the existence of a significant extracellular shunt through the sucrose gap. However, these investigators did not compare the magnitude of an intracellular micro-electrode recorded action potential with that of a transgap recorded action potential in order to confirm their interpretation of the impedance data.

We performed experiments similar to those of New and Trautwein on frog atrial tissue in order to compare the transgap impedance determined by an action potential method ( $R_{AP}$ ) with that determined by the sine-wave signal method. In these experiments the magnitude of the action potential was taken as the potential recorded across the right sucrose gap and therefore  $R_{AP}$  in our method gives the parallel combination of  $R_i$  and  $R_e$ . Experiments on 22 preparations gave the following results: action potential magnitude =  $95 \pm 2$  mV;  $R_{AP} = 288 \pm 38$  K $\Omega$ ;  $R_{1,000} = 182 \pm 25$  K $\Omega$  and  $R_{1,000}/R_{AP} = 0.63$  (0.56–0.70). These data suggest that the parallel combination of  $R_i$  and  $R_e$  may be frequency dependent.

To test this hypothesis, the gap impedance on nine preparations was measured by the action potential method ( $R_{AP}$ ) and with sine-wave signals of 10 Hz ( $R_{10}$ ) and 1,000 Hz ( $R_{1,000}$ ). The following results were obtained:  $R_{10}/R_{AP} = 1.02$  (0.95–1.07);  $R_{1,000}/R_{AP} = 0.70$  (0.55–0.81); and  $R_{1,000}/R_{10} = 0.69$  (0.53–0.81). These data demonstrate that the gap impedance measured at 1,000 Hz was significantly less than that at 10 Hz; i.e. the gap impedance is frequency dependent. Membrane resistance measurements on six of the nine preparations demonstrated that the membrane resistance of the preparation in the test node was about 5% of  $R_{10}$  and therefore the frequency-dependent reduction of gap impedance could not be accounted for by the impedance of the cell membranes in the test node. The frequency dependence of the gap impedance indicates that a sine-wave signal of 1,000 Hz cannot be used on frog

atrial muscle to determine the parallel sum of the intracellular and extracellular resistive elements (i.e.,  $R_i$  and  $R_e$ ).

## DISCUSSION

### *Gap Resistance*

The data presented in this paper demonstrate that the action potential from frog atrial muscle as recorded across the sucrose gap had an amplitude and waveform similar to the transmembrane action potentials of the cells in the test node as recorded by an intracellular microelectrode. The conclusion to be reached from these data is that the extracellular resistance ( $R_e$ ) through the sucrose gap is very large with respect to the intracellular resistance ( $R_i$ ) through the preparation bathed by the sucrose. This conclusion is further supported by the observation that under voltage clamp conditions the intracellular potential of cells in the test node as measured by a microelectrode equaled the clamp potential as recorded across the sucrose gap during the holding potential, during a small depolarizing clamp pulse, and following recovery from the loss of voltage control during the excitatory inward currents.

Our gap resistance measurements indicate that the transgap impedance is frequency dependent. Similar data have been obtained by other investigators. Sperelakis and Hoshiko (1961) found that the impedance of strips of cat cardiac muscle soaked in isotonic sucrose was frequency dependent and their data gave a ratio for the impedance at 1,000 Hz to that at 10 Hz of about 0.75 which agrees with our value of 0.69. They calculated that the specific resistance of the intracellular pathway was about 5,560  $\Omega$ -cm, and they divided this resistance into a component due to the myoplasmic resistance (454  $\Omega$ -cm) and a component due to transverse membranes (perhaps the intercalated discs) of high resistance and high capacitance. From our gap resistance data we would calculate a specific resistance for the intracellular pathway of frog atrial muscle to be on the order of 1,000  $\Omega$ -cm. Freygang and Trautwein (1970) concluded from longitudinal impedance measurements on cardiac Purkinje strands that about 17% of the intracellular resistance is in parallel with a capacity and they presumed that this resistance represents the resistance of the junction between cells for the longitudinal flow of current. Although the exact equivalent circuit for the longitudinal intracellular pathway in frog atrial tissue cannot be deduced from our impedance data, the frequency dependence of the transgap impedance suggests an equivalent circuit in which at least part of the intracellular resistance is paralleled by a capacitance.

### *Voltage Clamp in Multicellular Tissue*

The voltage clamp of a multicellular preparation has the possible severe limitation that the potentials of all the cells in the preparation will not be well controlled; that is, a nonhomogeneous clamp will occur. The data presented in this paper demonstrate that the homogeneity of the voltage control is very good during the holding potential or

during a small depolarizing pulse (+10 mV), but is extremely poor when the depolarization elicits an inward current. Our microelectrode experiments, therefore, do not confirm those reported by Haas et al. (1970). Haas et al. stated that the spatial variation of the membrane potential of frog atrial cells in the test node region was less than 2 mV during the inward current and almost zero after the inward current, and that when a rectangular step depolarization was indicated by the potential across the sucrose gap, the potential change between an internal microelectrode and a nearby reference microelectrode was almost the same, except for a small hump (up to 5 mV) at the peak of a large inward current. Part of the difference between our data and those of Haas et al. might be explained by the microelectrode recording techniques employed. It appears that Haas et al. measured the potential difference between an intracellular microelectrode and an extracellular microelectrode placed in close proximity to the membrane of the impaled cell. We chose not to use this technique since the potential changes recorded by the microelectrodes are not directly comparable to those recorded by the external Ag-AgCl electrodes during large current flow (either outward or inward) due to the voltage drop across the resistance in series with the membrane (Tarr and Trank, 1971). Our microelectrode recording technique allows a direct comparison of the intracellular potential and transgap potentials.

The potential difference between two ends of a short cable should be proportional to the current flowing into the short cable (Adrian et al., 1970). Therefore, the waveform of the potential difference between the microelectrode and the potential at the control point (i.e.,  $V_i - V$ ) should have had a waveform similar to the current injected into the cable if the system behaved as a one-dimensional cable. New and Trautwein (1972) found in experiments on cat myocardial fibers that this condition existed in that the pattern of spatial nonhomogeneity depended upon the position of the control electrode along the cable and that the waveform of the spatial nonhomogeneity was similar to that of the current waveform (see Fig. 5 of their paper). In our voltage clamp system with the microelectrode used to test for spatial homogeneity placed between the control point and the site of current injection,  $V_i - V$  should have been positive during the flow of outward (positive) current and negative during the flow of inward (negative) current. The preparation appeared to behave as expected for a one-dimensional cable during the transient outward current associated with the step depolarization (see Fig. 3). However, during the excitatory inward currents there was not good agreement between the waveform of the current and the waveform of  $V_i - V$ . In some cases  $V_i$  had a waveform of an attenuated action potential following the fast inward current (Fig. 3) or slow inward current (Fig. 4). In other cases,  $V_i - V$  was negative for a considerable period of time after the excitatory inward current was completely inactivated (Fig. 3 B). In other cases  $V_i$  indicated that the impaled cell was producing a fairly uncontrolled action potential prior to any pronounced inward current (Fig. 4 D).

A reasonable explanation for the complex waveform of the loss of spatial voltage control seen in our preparations can be derived from the work of Kootsey and Johnson

(1972). In their theoretical analysis of the early currents obtained from a multipatch computer simulation of a single-gap voltage clamp system they demonstrated that the transmembrane potential deviated markedly from the apparent clamp potential during the flow of the inward current associated with a step depolarizing clamp pulse when a significant resistance was in series with the cell membrane: the transmembrane potential was that of an uncontrolled action potential. In the presence of a series resistance the relation between the true change in transmembrane potential ( $V_m$ ) and the measured change in membrane potential ( $V$ ) at any patch of membrane is given by the equation  $V = V_m + IR_o$  where  $I$  is the current flowing across the resistance ( $R_o$ ) in series with the membrane patch. Analysis of their plots of transmembrane voltage ( $V_m$ ) and membrane current as functions of time and position along the multipatch model (see Figs. 6 and 7 of Kootsey and Johnson, 1972) indicate that the waveform of the measured intracellular potential ( $V$ ) would have had a waveform similar to those we observed experimentally (see Fig. 3 C and D, Fig. 4 B). For example at the 0.2 mm position (Fig. 6 of Kootsey and Johnson, 1972) the  $IR_o$  drop at the time of the peak inward current was on the order of  $-175$  mV (approximately  $-350 \mu\text{A}/\text{cm}^2 \times 500 \Omega\text{-cm}^2$ ). At the time of peak inward current flow across this membrane patch the transmembrane potential change ( $V_m$ ) was that of a 120 mV action potential. Thus, during the flow of inward current the measured membrane potential ( $V$ ) for a transient period would have been negative to the holding potential by as much as 55 mV. The depolarizing clamp pulse in this case was +32 mV (potential change with respect to holding potential) and therefore the difference between the apparent clamp potential and the measured membrane potential at this patch would have been about 90 mV: measured potential negative with respect to apparent clamp potential. For a period of time after the inactivation of the inward current the measured potential would have been positive with respect to the apparent clamp potential due to the existence of the transmembrane action potential. Thus, it would appear that a large series resistance could account for the large negative loss of voltage control during the fast inward current followed by the positive loss of voltage control upon inactivation of the inward current (Fig. 3 C and D).

Our experimental data support other conclusions that can be made from the theoretical work of Kootsey and Johnson (1972). From their analysis it appears that the waveform of the measured potential ( $V_i$ ) would be a function of the waveform of the current flowing across the resistance in series with the cell from which the microelectrode recording was made, and the waveform of this current can be quite different from the waveform of the current recorded from the entire preparation. In this case a good correlation between the waveform of the current measured from the entire preparation and  $V_i - V$  need not occur. Kootsey and Johnson (1972) also demonstrated that the peak inward current at any membrane patch occurred when the action potential propagates into this membrane patch. In this case conduction delays would produce time differences between the peak of the measured inward current and the time of maximum  $V_i - V$ . If the conduction distance between the peripheral and deep cells in our prepa-

rations was on the order of 200  $\mu\text{m}$  and the conduction velocity was about 5–10 cm/s then delays on the order of several milliseconds would occur; such delays were routinely seen in the case of the fast inward current.

Previously we presented data (Tarr and Trank, 1971) which indicated that the apparent series resistance  $R_o$  (see Fig. 1) was small with respect to the resting membrane resistance in frog atrial muscle. In light of the data presented in the present paper it would appear that considerable resistance might exist in series with some cells within the preparation. This is certainly conceivable since contamination of the extracellular space in the test node with sucrose is likely to occur when the test node length is less than the bundle diameter, and cells deep within the preparation might have considerable series resistance. Considerable variability in the quality of spatial voltage control could result from a variability in series resistance due to (1) the size of the preparation and the relationship of test node width to bundle diameter, (2) the position of the test electrode within the preparation and the relationship of series resistance to superficial and deep cells, and (3) the current density close to the recording electrode. It would appear that a reduction in the value of the extracellular resistance in series with the deep cells should improve the quality of spatial voltage control. Whether or not a reduction in series resistance and thereby an improvement of spatial voltage control can be accomplished simply by reducing in the diameter of the preparation remains to be determined. Our previous experience with thin bundles (100–150  $\mu\text{m}$  diameter) was that action potentials recorded from thin bundles as well as voltage clamp data obtained from these bundles were generally not as good as those obtained from the thicker bundles. New and Trautwein (1972) also reported greater success with larger diameter bundles and suggested that lateral connections within the bundle had a salutary effect in achieving spatial homogeneity.

Our data support the conclusions of Taylor et al. (1960) and New and Trautwein (1972) that the waveform of the current cannot be used to decide whether or not any given preparation is space clamped adequately enough to allow interpretation of the voltage clamp data. Although a systematic study of the peak inward current vs. voltage relationships was not performed in the present experiments, an extensive number of current-voltage curves for both the fast and slow inward current obtained from a large number of similar preparations during the last four years do not give indications of spatial loss of voltage control; i.e., the current-voltage curves do not show very steep negative slope regions which are generally interpreted as being indicative of poor spatial voltage control (Johnson and Lieberman, 1971; Harrington and Johnson, 1973). Therefore, at present we feel that the only valid check on the adequacy of a space clamp is independent measurement along the preparation with a microelectrode. Our data indicate that a great variability in the degree of spatial voltage control exists between different cells within the preparation (see Fig. 4). Thus, recordings from a representative number of cells within the preparation must be obtained before any conclusion can be reached concerning the adequacy of space clamp within any given preparation. This condition limits the practicality of using a multicellular prepara-

tion, at least frog atrial tissue, for voltage clamp analysis due to the difficulty and time involved in obtaining these recordings.

It might be argued that the extremely poor spatial voltage control seen in our preparations during both the fast and slow inward currents suggests that our preparations are highly disorganized and that the normally good electrical communication between cells has been destroyed experimentally. If this were the case, it would seem unlikely that (1) the transgap recorded action potentials would have magnitudes of 100 mV or greater and equal the magnitudes of the intracellularly recorded action potentials, (2) good voltage control of the impaled cell would exist in the absence of the excitatory inward currents (e.g., during small depolarizing or hyperpolarizing clamp pulses), and (3) good voltage control of the impaled cell would exist at large clamp pulses following the loss of voltage control associated with the excitatory inward current. Since these results depend on cells in the test node being electrically coupled to both the current injecting (left) and voltage sensing (right) pools (see Fig. 1) through low resistance intracellular pathways, the experimental findings that these events did occur suggest to us that the loss of the normal electrical coupling between cells in the test node of our preparations is not a reasonable explanation for the poor spatial voltage control seen during the excitatory current.

#### *Conclusions from Cardiac Voltage Clamp Data*

In light of the data presented in this paper which demonstrate loss of spatial voltage control during both the fast and slow inward currents in frog atrial tissue, it would seem that at least a brief discussion of the type of voltage clamp data routinely obtained by us on similar preparations is called for in order to aid the interpretation of our voltage clamp data. Typical voltage clamp data obtained from our preparations have been published previously (see Tarr, 1971; and Tarr et al., 1973). We routinely see a temporal separation of the fast and slow inward currents in frog atrial tissue and the voltage clamp data obtained from our preparations clearly demonstrate the existence of a distinct slow component of inward current in the presence of the fast inward current (see Tarr, 1971). It had been our opinion that temporal separation of the two inward currents would improve with the quality of the voltage clamp. Recently, New and Trautwein (1972) from voltage clamp experiments on cat ventricular muscle demonstrated that a distinct separation of the fast and slow inward currents occurred when spatial voltage control was good. However, in light of the present data it would seem logical to conclude that a temporal separation of the fast and slow inward currents can probably exist in preparations where spatial voltage control does not exist.

The contribution of nonuniform spatial voltage control to the waveform of the currents recorded from our preparations must seriously be considered, since in a multicellular preparation such as cardiac muscle nonuniform voltage control could result in two distinct peaks of inward current (see Johnson and Lieberman, 1971). For example, in response to a depolarizing clamp pulse the voltage of the peripheral cells of the bundle could change more rapidly than the voltage of the deep cells

due to differences in the magnitude of the extracellular resistance in series with the cell membrane. If this occurred, as suggested by Johnson and Lieberman (1971), then the peripheral cells could give rise to the first inward current (the so-called fast inward current) and the cells less accessible to the controlling current could give rise to the slow secondary inward current (the so-called slow inward current). However, if only one ionic current mechanism were responsible for both inward currents and the two currents were due to asynchronous potential control of peripheral and deep cells, then it would seem unlikely that selective inhibition of one current component in the presence of the other current component would be possible; especially selective inhibition of the slow current which by this hypothesis would require selective inhibition of the deep cells. It was demonstrated that fairly selective inhibition of either inward current was possible (Tarr, 1971). Application of tetrodotoxin to the test node abolished the fast inward current and the slow inward current remained. In contrast, application of low concentrations of  $\text{MnCl}_2$  (1–3 mM) abolished the slow inward current and the fast inward current remained. Each current inhibitor was not always without an effect on the other inward current (e.g., in some preparations TTX partially suppressed the slow inward current and  $\text{MnCl}_2$  slightly suppressed the fast inward current) and this lack of complete selectivity might have resulted from some degree of asynchronous potential control of the cells in the test node. However, a qualitative selectivity of TTX for the fast inward current and of  $\text{MnCl}_2$  (in low concentrations) for the slow inward current was clearly apparent. The simplest explanation that can be given to these voltage clamp data is that two distinct inward current components contribute to the cardiac action potential of frog atrial tissue (see Tarr, 1971).

In a previous publication Tarr et al. (1973) presented data on the effect of propranolol on the fast inward current in frog atrial muscle and interpreted these data in terms of the effect of the drug on the sodium conductance and kinetic parameters of the sodium current. Interestingly, the data obtained by Tarr et al. on frog atrial muscle are quite similar to those obtained by Wu and Narahashi (1973) on the effect of propranolol on the inward sodium current in squid axon. However, the interpretation of "membrane currents" recorded under "voltage clamp" conditions is reasonably straightforward in terms of membrane conductance changes only when isopotentiality exists in the region of the preparation from which the current is recorded. The demonstration in the present paper that such isopotentiality does not exist suggests that a quantitative interpretation of the fast inward current voltage clamp data in terms of sodium conductance and kinetic parameters is not justified. The fact that the current-voltage data are reasonable and are similar to those obtained from preparations where isopotentiality exists does not justify the use of such current-voltage data for quantitative purposes until the meaning of such current-voltage data can be rigorously determined both experimentally and theoretically.



### Summary

The data presented in this paper indicate that the spatial loss of voltage control during the inward excitatory currents in frog atrial muscle can be severe and that caution should be exercised in the interpretation of voltage clamp data obtained from similar multicellular preparations. This demonstration that bad voltage control exists in preparations giving ionic currents similar to those obtained under allegedly good voltage clamp conditions certainly raises serious doubts concerning the reliability of the method and forces the conclusion that the adequacy of voltage control must be assessed by independent intracellular microelectrode recording from a number of cells in the test compartment in each and every preparation.

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