

# Active and Passive Electrical Properties of Single Bullfrog Atrial Cells

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**ABSTRACT** Single cells from the bullfrog (*Rana catesbeiana*) atrium have been prepared by using a modification of the enzymatic dispersion procedure described by Bagby et al. (1971. *Nature [Lond.]*. **234**:351-352) and Fay and Delise (1973. *Proc. Natl. Acad. Sci. U.S.A.* **70**:641-645). Visualization of relaxed cells via phase-contrast or Nomarski optics (magnification, 400-600) indicates that cells range between 150 and 350  $\mu\text{m}$  in length and 4 and 7  $\mu\text{m}$  in diameter. The mean sarcomere length in relaxed, quiescent atrial cells is 2.05  $\mu\text{m}$ . Conventional electrophysiological measurements have been made. In normal Ringer's solution (2.5 mM  $\text{K}^+$ , 2.5 mM  $\text{Ca}^{++}$ ) acceptable cells have stable resting potentials of about -88 mV, and large (125 mV) long-duration ( $\sim 720$  ms) action potentials can be elicited. The  $V_m$  vs.  $\log[\text{K}^+]_o$  relation obtained from isolated cells is similar to that of the intact atrium. The depolarizing phase of the action potential of isolated atrial myocytes exhibits two pharmacologically separable components: tetrodotoxin ( $10^{-6}$  g/ml) markedly suppresses the initial regenerative depolarization, whereas verapamil ( $3 \times 10^{-6}$  M) inhibits the secondary depolarization and reduces the plateau height. A bridge circuit was used to estimate the input resistance ( $220 \pm 7 \text{ M}\Omega$ ) and time constant ( $20 \pm 7$  ms) of these cells. Two-microelectrode experiments have revealed small differences in the electrotonic potentials recorded simultaneously at two different sites within a single cell. The equations for a linear, short cable were used to calculate the electrical constants of relaxed, single atrial cells:  $\lambda = 921.3 \pm 29.5 \mu\text{m}$ ;  $R_i = 118.1 \pm 24.5 \Omega\text{cm}$ ;  $R_m = 7.9 \pm 1.2 \times 10^3 \Omega\text{cm}^2$ ;  $C_m = 2.2 \pm 0.3 \mu\text{Fcm}^{-2}$ . These results and the atrial cell morphology suggest that this preparation may be particularly suitable for voltage-clamp studies.

## INTRODUCTION

Quantitative studies of the electrical activity in cardiac tissue have been hindered by the complex syncytial nature of most in vitro cardiac preparations that are presently available. Thus, voltage-clamp techniques applied to multicellular cardiac preparations have been limited by problems such as spatial inhomogeneity, a considerable series resistance because of intercellular clefts, and an inability to inject current uniformly (Johnson and Liebermann, 1971;

Attwell and Cohen, 1977; Noble, 1979). In addition, depletion and accumulation of ions in restricted extracellular spaces often occur, and may distort the time-course and reversal potential of ionic currents in bullfrog atrial trabeculae (Maughan, 1973; Noble, 1976) as well as other cardiac preparations (Baumgarten and Isenberg, 1977; Attwell et al., 1979; DiFrancesco et al., 1979; Kline et al., 1980; Kline and Morad, 1978; Morad, 1980). These inherent difficulties, which arise from the three-dimensional geometry of most available cardiac preparations, often complicate quantitative determinations of the specific electrical constants of cardiac muscle, (Tarr and Trank, 1971). Finally, the existence of gap junctions between cells may contribute to nonuniform voltage control if transjunctional polarization produces electrotonic uncoupling of cardiac cells (Spray et al., 1979). A viable, isolated single-cardiac-cell preparation may reduce or remove many of these problems, and thus would offer considerable advantage for the quantitative study of cellular cardiac electrophysiology.

A variety of enzymatic dispersion techniques have been used to isolate ventricular myocardial cells from rat (Glick et al., 1974; Powell and Twist, 1976; Farmer et al., 1977; Rajs et al., 1978; Clark et al., 1978; Brady et al., 1979), rabbit (Dani et al., 1979), dog (Vahouny et al., 1979), and guinea pig (Bustamante et al.; 1981 [in press]). Recent studies have applied voltage-clamp techniques to isolated rat ventricular cells to study the fast inward sodium current (Lee et al., 1979; Undrovinas et al., 1980; Brown et al. 1981 *b*); and the slow inward current (Isenberg and Klöckner, 1980). Although these voltage-clamp experiments have yielded quantitative data describing the voltage and time dependence of the fast and slow inward currents, relatively little information has been provided about the basic electrophysiological properties of these cells. Therefore, it is important to know the resting membrane potential of the isolated cells and their specific electrical constants, and to compare the basic electrophysiological properties of isolated cells with those of intact tissue. Such information seems essential for monitoring the extent of recovery of the cells from the enzymatic dissociation, so that the physiological significance of the voltage-clamp data from isolated cells can be critically assessed. Previous studies of the electrical properties of isolated rat ventricular cells have been restricted to cells with maintained resting potentials that are relatively depolarized, ranging from  $-20$  to  $-60$  mV (Powell et al., 1980; Brown et al., 1981 *a* [in press]). This raises the question of whether the electrical properties of depolarized cells are comparable to properties of cells that maintain a normal resting membrane potential (see Table II).

Action potential amplitudes and shapes recorded from separate regions of the heart vary considerably, and voltage-clamp studies using intact multicellular preparations suggest that significant differences in ionic currents are responsible for the observed variations in action potential wave forms (see Coraboeuf [1978]). The frog heart has been studied extensively and has provided important information regarding basic electrophysiology of atrial myocardium (Haas et al., 1971; Rougier et al., 1969; De Hemptinne, 1971; Brown and Noble, 1969; Barr et al., 1965; Giles and Noble, 1976). Voltage-

clamp studies of intact atrial tissue, however, are limited by the same factors inherent in any multicellular cardiac preparation. For example, potassium accumulation in the extracellular space of frog atrial trabeculae complicates a quantitative analysis of time-dependent outward current(s) (Maughan, 1973; Noble, 1976; Brown et al., 1980).

We have developed a relatively simple procedure in which fragments of the right atrial wall of the frog heart are enzymatically digested, yielding single atrial cells that have normal resting membrane potentials. In this report, we (*a*) detail the method of enzymatically dispersing isolated atrial cells, (*b*) describe relevant features of the morphology of the cells, (*c*) compare some basic electrophysiological properties of these cells with properties that have been recorded in intact atrium, and (*d*) determine the specific electrical constants of isolated atrial myocytes. Our findings indicate that isolated cells from the frog atrium may be particularly suitable for voltage-clamp analysis of the ionic currents underlying electrical activity in atrial myocardium.

Preliminary results of these experiments have been presented previously (Giles and Hume, 1980; Hume and Giles, 1981 [in press]).

## METHODS

### *Cell Isolation*

The enzymatic dispersion procedure that was used is a modification of the methods previously used to isolate smooth muscle cells (Bagby et al, 1971; Fay and Delise, 1973). Adult frogs (*Rana catesbeiana*) were pithed and their hearts removed and transferred to a dissecting dish containing standard Ringer's solution. The right atrium was separated and several large pieces of tissue were cut off and placed in a second dish containing calcium-free Ringer's solution.

That tissue was then cut into 2-mm pieces and transferred with forceps into a 5-ml solution of calcium-free Ringer's solution containing 0.15% crude bacterial collagenase (type I, *Clostridium histolyticum*; 200 U/mg; Sigma Chemical Co., St. Louis, Mo. and 0.1% bovine pancreatic trypsin (type III, dialyzed, lyophilized, and substantially salt-free; 10,100 U/mg protein; Sigma Chemical Co.). A small (10 × 2 mm) Teflon-coated magnetic stirring bar was included and the flask was sealed. The contents were stirred continuously for 45 min at a low speed (~60 rpm) at room temperature. The incubation medium was then pipetted off, and the tissue fragments were reincubated in 5 ml of calcium-free Ringer's solution containing 0.1% bovine albumin (essentially fatty acid-free, prepared from fraction V; Sigma Chemical Co.), and stirred for 5 min. Next, this solution was pipetted off and the fragments were reincubated in 5 ml of calcium-free Ringer's solution containing 0.05% crude bacterial collagenase and stirred for ≥30 min. After 30–45 min, the incubation medium becomes quite opaque, and visualization under a microscope reveals that the solution contains a high concentration of the two cell types illustrated in Fig. 1, as well as debris. It was helpful during the final incubation stage to periodically withdraw small samples for visualization under a microscope to monitor the progression of the digestion procedure. When a high density of single cells was observed, the suspended cell solution was pipetted off, diluted 1:3 with calcium-free Ringer's solution, and stored at room temperature. Small aliquots of this solution were periodically withdrawn and added to the recording chamber for the electrophysiological experiments.

### *Experimental Setup and Electrophysiology*

The bottom of the experimental recording chamber consisted of a lid for a 35- × 10-mm tissue culture dish (3001; Falcon Labware Div. Becton, Dickenson & Co., Oxnard, Calif.) that was coated with polylysine (50  $\mu\text{g}/\text{ml}$ ) and mounted on the stage of an inverted binocular phase-contrast microscope (Swift Instruments Inc., San Jose, Calif.). This coating procedure promoted adherence of the cells to the bottom of the chamber and thus facilitated microelectrode impalement. At the start of each experiment, a 1-ml aliquot of cell suspension was added to the chamber and 10 min was allowed for the cells to settle to the bottom. Superfusion with standard Ringer's solution at a rate of  $\sim 0.5$  ml/min was then begun. The total volume of the chamber was  $\sim 2.5$  ml. Solution in the chamber was removed by gravity flow into a second chamber where negative pressure was applied. During electrophysiological experiments, the cells were visualized at a magnification of 400–600.

Membrane potentials were recorded with conventional glass microelectrodes filled with 3 M KCl and had resistances of 20–60 M $\Omega$ . Microelectrodes were positioned using two hydraulic micromanipulators (MO-102; Narishige Scientific Laboratory, Tokyo), the drive units of which were mounted directly on the microscope stage. The potential difference between the intracellular microelectrode and a bath reference electrode was measured differentially with a KS-700 Dual Microprobe System (W-P Instruments, Inc., New Haven, Conn.). Current was injected into the cell through the recording microelectrode. The resulting voltage drop across the microelectrode could be cancelled by balancing an active bridge circuit incorporated into one channel of the KS-700. In the initial experiments, current was measured directly from the monitor of this amplifier. Current pulse duration and frequency were controlled with a digital pulse generator (series 800; W-P Instruments, Inc.). Current and voltage were displayed on a storage oscilloscope (model 1223A; Hewlett-Packard Co., Palo Alto, Calif.), amplified by a factor of 100 (501 amplifiers; Tektronix, Inc., Beaverton, Oreg.), and recorded on an FM instrumentation tape recorder (model 3964A; Hewlett-Packard Co.). In addition, a digital monitor on the KS-700 provided constant readout of membrane potential during the course of an experiment.

For experiments in which a second independent intracellular microelectrode was used, shielding was accomplished by painting this electrode to within 100  $\mu\text{m}$  of the tip with conductive silver paint and then coating with polystyrene Q-dope (Hydro-metals, Inc., Rockford, Ill.). This shield was driven from the unity gain output of the input stage amplifier. Current was measured in the bath with an operational amplifier in the current-to-voltage converter mode. To compare the steady-state voltage response recorded by the two intracellular microelectrodes, 7–20 responses on each electrode in a cell were signal-averaged on a PDP 11-03 laboratory computer (Digital Equipment Corp., Maynard, Mass.), and the difference  $V_1' - V_2'$  was displayed at a high gain on an analog oscilloscope. In the averaging procedure, the data were sampled every 250  $\mu\text{s}$ ; a total of 1,024 data points were taken. Experiments in which the bridge microelectrode signal showed any sign of imbalance were rejected.

Data describing the relation between  $\log[\text{K}^+]_0$  and  $V_m$  in intact atrial tissue were obtained from multiple impalements in the endocardial surface of the atrium. Conventional fiber-filled microelectrodes containing 3 M KCl were used. The normal Ringer's solution contained tetrodotoxin (TTX) ( $10^{-6}$  g/ml) to ensure that the preparation remained quiescent during the course of multiple impalements and thus facilitate the comparison with data obtained in quiescent single cells. Twenty stable impalements were made at each  $[\text{K}^+]_0$ , and the average  $V_m$  was calculated. The data were measured, displayed, and recorded as previously described.

*Solutions and Drugs*

All solutions were made with glass-distilled water and were kept saturated with 95% O<sub>2</sub>/5% CO<sub>2</sub>. Standard Ringer's solution contained: NaCl, 90.6 mM; NaHCO<sub>3</sub>, 20 mM; KCl, 2.5 mM; MgCl<sub>2</sub>, 5.0 mM; CaCl<sub>2</sub>, 2.5 mM; and glucose, 10 mM. The measured osmolarity of this solution was 241 mosM. Unless otherwise stated, all electrophysiological experiments were carried out in standard Ringer's solution. Calcium-free Ringer's solution was identical to standard Ringer's solution, except that CaCl<sub>2</sub> was omitted. In experiments in which the concentration of KCl was varied, the osmolarity was held constant by removal or addition of NaCl. TTX was purchased from Sigma Chemical Co. and verapamil was kindly supplied by Knoll Pharmaceutical Co., Whippany, N. J.

*Electron Microscopy*

A small aliquot of the suspended cell solution was added to the experimental chamber and the cells were allowed to adhere to the bottom. The cells were then superfused with standard Ringer's solution for ~20 min. Fixation was accomplished by superfusing with standard Ringer's solution containing 5% glutaraldehyde for 15 min. Perfusion was then stopped and the cells were kept in this solution for 3 h. Superfusion with glutaraldehyde did not result in contraction of the isolated cells. Fixation was followed by three rinses with a 0.1 M cacodylate buffer solution and postfixation with 1% OsO<sub>4</sub> in 0.1 M cacodylate buffer for 30 min. After three additional rinses with cacodylate buffer, the cells were dehydrated with an ethanol series containing 4% uranyl acetate and embedded in Epon 812. 600-Å sections were cut with a diamond knife on an LKB 4 ultramicrotome (LKB Instruments, Inc., Rockville, Md.). The sections were suspended on Formvar-coated copper grids and viewed with a JEOL CX-100 electron microscope (JEOL USA, Electron Optics Div., Medford, Mass.).

## SYMBOLS FOR PASSIVE ELECTRICAL PROPERTIES

$V_0$	Steady-state deviation of intracellular potential from resting potential, $V_0 = V_m - V_r$ (mV), recorded at site of current injection
$V$	Steady-state deviation of intracellular potential from resting potential, $V = V_m - V_r$ (mV), recorded by second microelectrode
$l$	Distance from the current injection electrode to the opposite end of the cell ( $\mu\text{m}$ )
$x$	Interelectrode separation ( $\mu\text{m}$ )
$\lambda$	Space constant ( $\mu\text{m}$ )
$R_{\text{in}}$	Cell input resistance ( $\text{M}\Omega$ )
$r_i$	Intracellular resistance to axial flow of current ( $\Omega\text{cm}^{-1}$ )
$r_m$	Cell membrane resistance per unit length ( $\Omega\text{cm}$ )
$R_m$	Cell membrane resistance ( $\Omega\text{cm}^2$ )
$R_i$	Intracellular resistivity ( $\Omega\text{cm}$ )
$a$	Cell radius ( $\mu\text{m}$ )
$C_m$	Cell membrane capacitance ( $\tau_m/R_m$ ) ( $\mu\text{Fcm}^{-2}$ )
$\tau_m$	Cell membrane time constant (ms)

## RESULTS

*Cellular Morphology*

Approximately 70% of the cells observed in the final suspension medium are long and thin, ranging from 150 to 350  $\mu\text{m}$  in length and from 4 to 7  $\mu\text{m}$  in diameter (Fig. 1A). These closely resemble frog atrial cells as previously

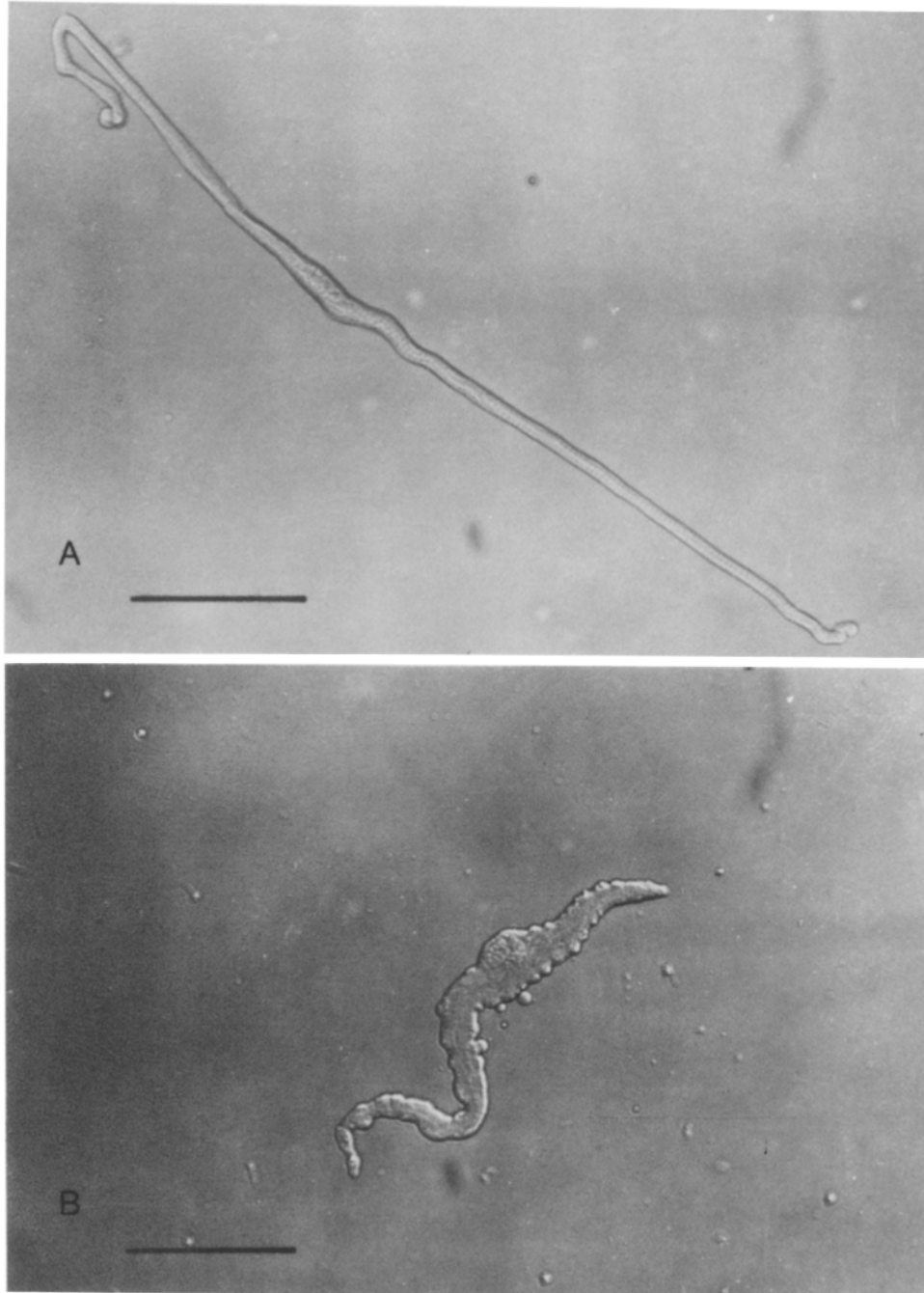


FIGURE 1. Photomicrographs of isolated single bullfrog atrial cells taken using Nomarski optics. A relaxed cell is shown in *A* and a damaged, partially contracted cell is shown in *B*. Bar, 50  $\mu\text{m}$ .

described by Barr et al., (1965) and Tarr and Trank (1976). These cells remain quiescent and relaxed in Ringer's solution containing 2.5 mM calcium. Thus, they do not exhibit the so-called calcium paradox (Zimmerman and Hulsman, 1966), i.e., massive tissue disruption and contracture upon reintroduction of physiological concentrations of calcium. Phasic contractions can be elicited in response to extracellular or intracellular electrical stimulation. The characteristic banded pattern of sarcomeres can be observed using phase-contrast optics; however, the use of interference-contrast optics (Nomarski) allows the sarcomere spacing to be measured more accurately. In photographs of 29 cells such as those shown in Fig. 1 *A*, the sarcomere lengths ranged from 1.7 to 2.3  $\mu\text{m}$ . The mean sarcomere length was  $2.05 \pm 0.03 \mu\text{m}$  (SD), which is similar to measurements of resting sarcomere lengths in intact atrium (Winegrad, 1974).

About 20–30% of the cells observed in the suspension medium resemble the structure in Fig. 1 *B*. These cells are believed to have been damaged by the isolation procedure. This is supported by two observations: (*a*) microelectrode impalements of such cells yield low resting membrane potentials, and action potentials cannot be elicited; and (*b*) unsuccessful attempts to impale long, thin, relaxed cells results in damage to the sarcolemma, which manifests itself as an irreversible contracture, as illustrated by the cell in Fig. 1 *B*.

The fine ultrastructure of isolated frog atrial cells was examined with transmission electron microscopy. Long, thin cells remained relaxed after glutaraldehyde fixation. These cells are mononucleated, with the nucleus centrally located in the wider region of the cell. They rarely contained more than three myofibrils, and usually contained only one myofibril, near the ends of the cell; similar observations have been made in intact frog ventricle (Sommer and Johnson, 1969). *Z* lines are clearly distinguishable, and *A* and *I* bands can be observed (Figs. 2 *A* and 3). *M* lines were not evident. In appropriate sections, an intact sarcolemma can be observed (Fig. 3, arrows). Cross sections (Fig. 2 *B*) verify that these are single cells rather than two- or three-cell aggregates.

Numerous small vesicles are found in close apposition with the sarcolemma and in other regions of the cells. In some sections, these vesicles appear to fuse with the sarcolemma (Fig. 3, *inset*). Vesicles such as these are also observed near the myofibrils and could constitute part of the sarcoplasmic reticulum (Page and Niedergerke, 1972). No transverse tubule system can be observed, which is consistent with previous more detailed studies (Sommer and Johnson, 1969; Page and Niedergerke, 1972). Numerous mitochondria, containing an abundant cisternal network, are prominent. These are distributed quite irregularly, often sandwiched between myofibrils. Overall, the intracellular structure and organization of isolated atrial cells is very similar to that described for intact frog myocardium (Kirsch, 1961; Barr et al., 1965; Baldwin, 1970).

#### *Single-Microelectrode Experiments*

Occasionally, microelectrode penetrations of relaxed isolated cells are abrupt and initial membrane potentials of from  $-80$  to  $-90$  mV are recorded. More

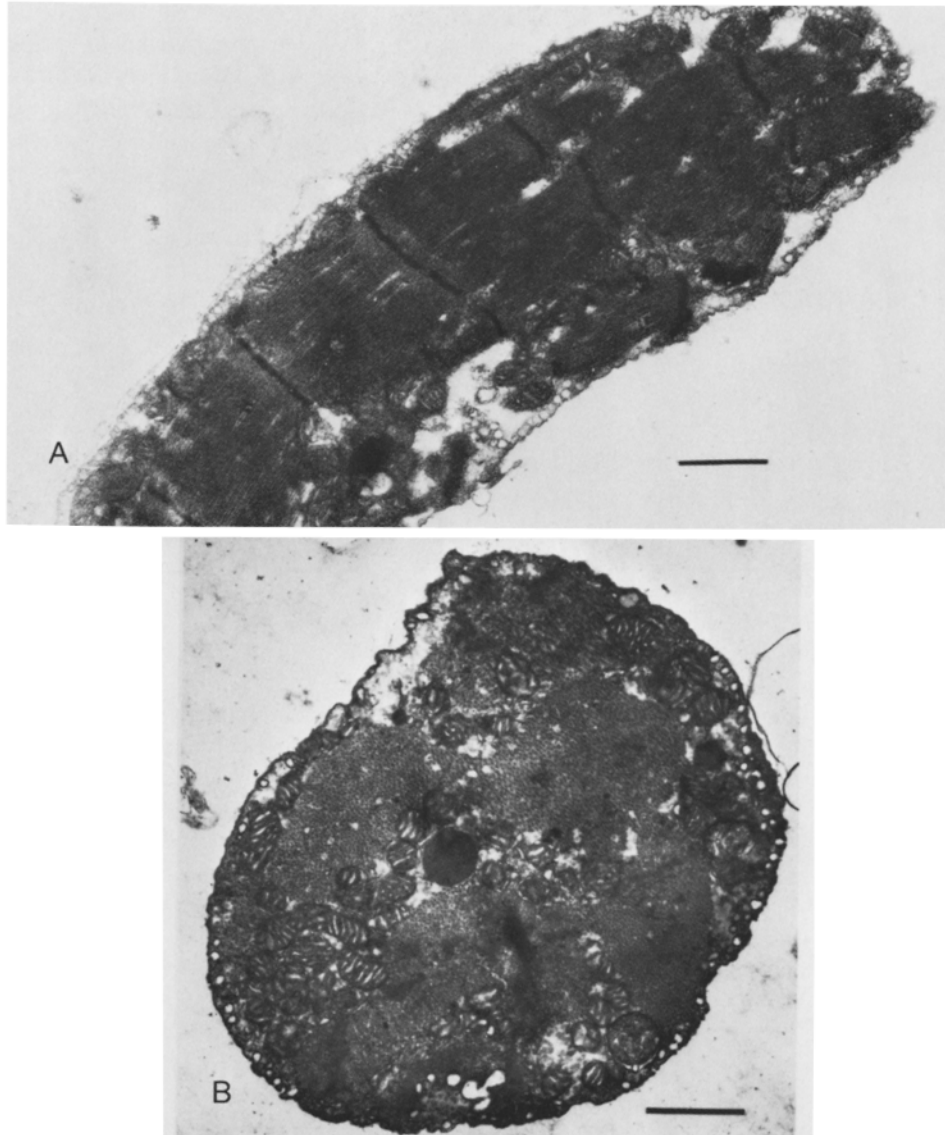


FIGURE 2. Transmission electron micrographs of longitudinal section (*A*) and cross section (*B*) of isolated single bullfrog atrial cell. Bars, 1.0  $\mu\text{m}$ .

often, microelectrode penetrations yield initial membrane potential measurements of from  $-40$  to  $-50$  mV. In  $\sim 50\%$  of these cases, the membrane potential gradually becomes more negative and eventually stabilizes at levels between  $-80$  and  $-90$  mV. The time-course of this stabilization process (1–3 min) closely matches that of the healing-over phenomenon, which has been studied extensively by De Mello et al. (1969) in amphibian cardiac muscle. In some trials, spontaneous, oscillatory electrical and mechanical activity is initiated by the impalement. This subsides as the membrane potential hyper-



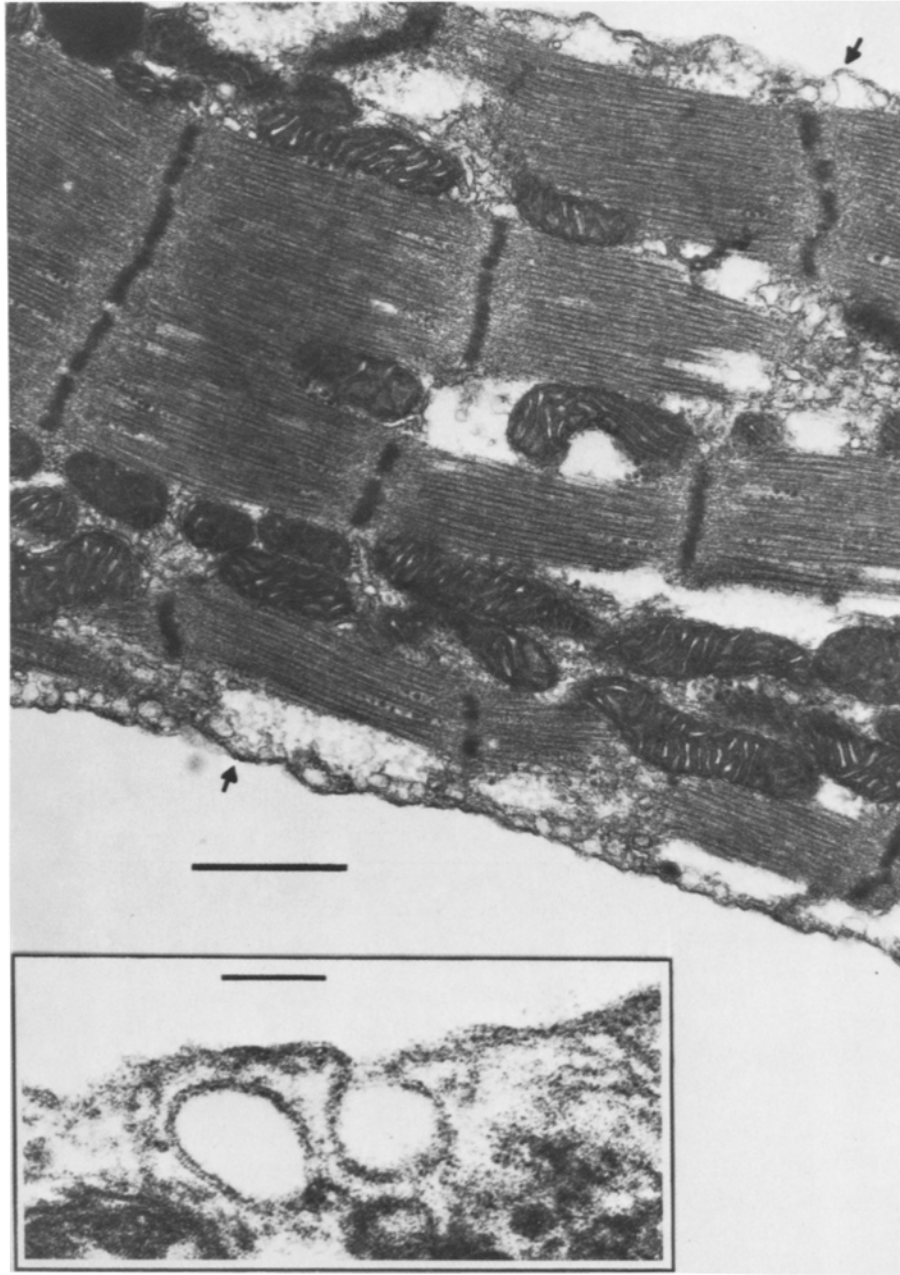


FIGURE 3. Transmission electron micrograph of a longitudinal section of an isolated single bullfrog atrial cell. Bars, 1.0  $\mu\text{m}$ ; 0.1  $\mu\text{m}$  (*inset*). Arrows point to sarcolemma.

polarizes to  $-65$  or  $-70$  mV. Unsuccessful impalements are characterized by a steadily depolarizing membrane potential while the cell undergoes irreversible contracture. Cells that heal over and recover from the impalement exhibit

relatively stable resting membrane potentials that may be continuously recorded for periods of ~30 min.

It is worth noting that successful impalements of small cells having such high input resistances (Table I) require careful adjustment of leakage current from the preamplifier. In our experiments, these bias currents were nulled to  $<5 \times 10^{-12}$  A.

Action potentials can be elicited by either extracellular or intracellular application of current (Fig. 4 A) and are accompanied by visible contraction and relaxation. The application of long-duration depolarizing current pulses typically elicits repetitive electrical (Fig. 4 B) and mechanical activity. This phenomenon, termed "induced pacing," is a characteristic of intact frog atrium (Brown and Noble, 1969) as well as intact mammalian myocardium (Katzung, 1975).

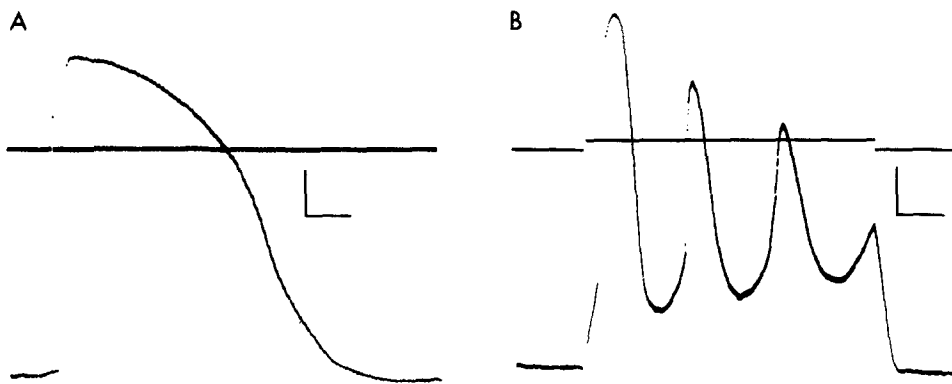


FIGURE 4. Electrical activity recorded from an isolated atrial cell. *Top trace* is current (and the zero reference for current and voltage) and the *bottom trace* is transmembrane potential. (A) An action potential is elicited in response to a brief intracellular stimulus. (B) Prolonged intracellular current injection elicits induced pacing (note improper bridge balance). Vertical calibration, 20 mV for A and B,  $2.5 \times 10^{-9}$  A for A, and  $1 \times 10^{-9}$  A for B. Horizontal calibration, 100 ms for A and 500 ms for B.

In these and other similar electrophysiological experiments, extreme care must be taken to ensure that single cells, as opposed to two- or three-cell aggregates, are impaled. Careful visual inspection with well-adjusted optics is essential. We chose relatively short cells (average length, 204  $\mu\text{m}$ ; range, 150–258  $\mu\text{m}$ ) in a further attempt to avoid this important source of error. Table I contains a summary of a number of electrical parameters of 12 cells successfully impaled with a single microelectrode. In these initial studies, current was injected intracellularly through the recording microelectrode.

The mean resting potential of  $-88.6$  mV is similar to resting membrane potentials that have been recorded with microelectrodes in intact frog atrium (Walker and Ladle, 1973). Action potentials with prominent plateaus and

relatively long durations are common in the frog heart, and the average maximum upstroke velocity ( $\dot{V}_{\text{MAX}}$ ) of 42.2 V/s is similar to previously measured values of 17–38 V/s (Antoni and Delius, 1965; Aceves and Erlj, 1967) in intact frog atrium.

At the resting potential,  $R_{\text{in}}$  was calculated from the steady-state hyperpolarization produced by small, inward current pulses of 50–150-ms duration.  $R_{\text{in}}$  estimated this way ranged from 107 to 380 M $\Omega$ , with an average of 219.8 M $\Omega$ . These measurements of  $R_{\text{in}}$ , obtained with a single microelectrode for simultaneously passing current and measuring potential, are subject to error arising either from improper bridge balance or from complex, three-dimensional patterns or current flow near the pipette tip (cf. Eisenberg and Engel, [1970]; Engel et al. [1972]). However, subsequent experiments utilizing a second independent microelectrode (see below: *Two-Microelectrode Experiments*) have confirmed these  $R_{\text{in}}$  values.

The time-course of decay of the steady-state voltage responses after cessation

TABLE I  
SUMMARY OF ELECTRICAL PARAMETERS OF FROG ATRIAL  
CELLS ( $n = 12$ )

	Mean $\pm$ SD
Resting membrane potential	-88.6 mV $\pm$ 1.7
Action potential amplitude	124.4 mV $\pm$ 8.1
Action potential overshoot	35.8 mV $\pm$ 7.1
Action potential duration	722.5 ms $\pm$ 106.3
Upstroke velocity ( $\dot{V}_{\text{MAX}}$ )	42.2 V/s $\pm$ 11.8
Input resistance ( $R_{\text{in}}$ )	219.8 M $\Omega$ $\pm$ 77.4
Membrane time constant ( $\tau_m$ )	19.7 ms $\pm$ 7.3
Cell length	204.0 $\mu\text{m}$ $\pm$ 34.8
Cell diameter (measured at the widest region of the cell near the nucleus)	7.1 $\mu\text{m}$ $\pm$ 0.8

of the current pulses is a single-exponential function. This result indicates that the membrane capacitance discharges through a simple parallel resistance-capacitance equivalent circuit. Fig. 5 shows an approximately linear relationship between the measured membrane time constant ( $\tau_m$ ) and the calculated values of  $R_{\text{in}}$  for a number of cells, which suggests that the variability in  $\tau_m$  can be attributed to changes in  $R_m$ .

**DEPENDENCE OF  $V_m$  ON  $[\text{K}^+]_0$**  Previous data from intact frog atrium indicate that measured resting potentials deviate significantly from the estimated potassium equilibrium potential ( $E_K$ ) (Lüttgau and Niedergerke, 1958; Glitsch et al., 1965; Walker and Ladle, 1973), suggesting that some steady inward current contributes to the resting membrane potential. For example, a 10-fold change in  $[\text{K}^+]_0$  has been reported to produce a 43-mV change in membrane potential in intact frog atrium (Haas et al., 1966).

To assess the functional integrity to the sarcolemma of isolated frog atrial myocytes, the effects of changes in  $[\text{K}^+]_0$  on resting membrane potential were

compared in both intact bullfrog right atrium and in isolated cells derived from the same tissue. Fig. 6A shows the effect of changes in  $[K^+]_0$  on resting membrane potentials recorded from intact right atrial tissue in TTX containing ( $10^{-6}$  g/liter) Ringer's solution. Elevation of  $[K^+]_0$  from 2.5 to 112 mM produced progressive depolarization. The data were fitted with a straight line with a slope of 52 mV/decade. A best-fit line, which includes the values at

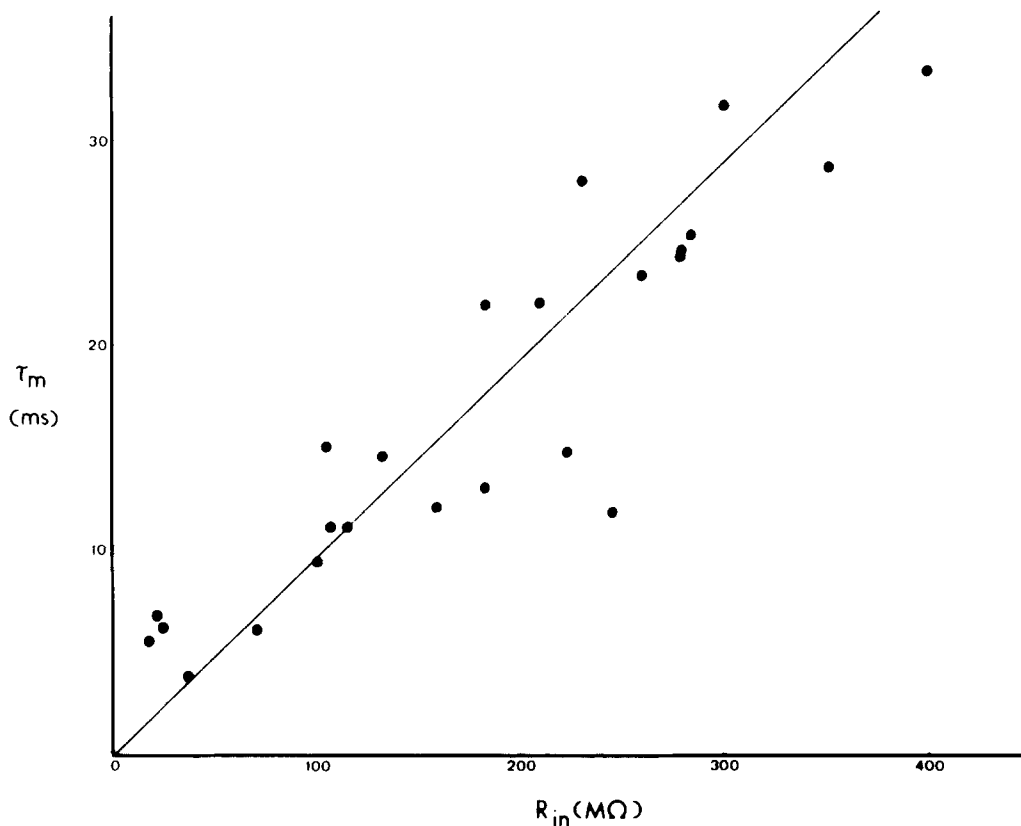


FIGURE 5. Relationship between membrane time constant ( $\tau_m$ ) and input resistance ( $R_{in}$ ) in 24 single atrial cells.  $\tau_m$  and  $R_{in}$  were measured from the time-course and magnitude of steady-state hyperpolarizations elicited by intracellular current injection via a bridge circuit. For cells in which  $R_{in} > 100 \text{ M}\Omega$ , the resting membrane potential was  $-80 \text{ mV}$ , or more negative.

$[K^+]_0 < 2.5 \text{ mM}$ , gives a slope of 48 mV/decade. At  $[K^+]_0$  levels  $< 2.5 \text{ mM}$ , a slight deviation was observed. This deviation indicates that when  $[K^+]_0$  is low, some ion other than  $K^+$  carries a significant amount of net transmembrane current. Because normal  $[K^+]_0$  is 2.2–2.5 mM, this phenomenon was not studied in detail.

Fig. 6B illustrates the results of a similar series of experiments performed in

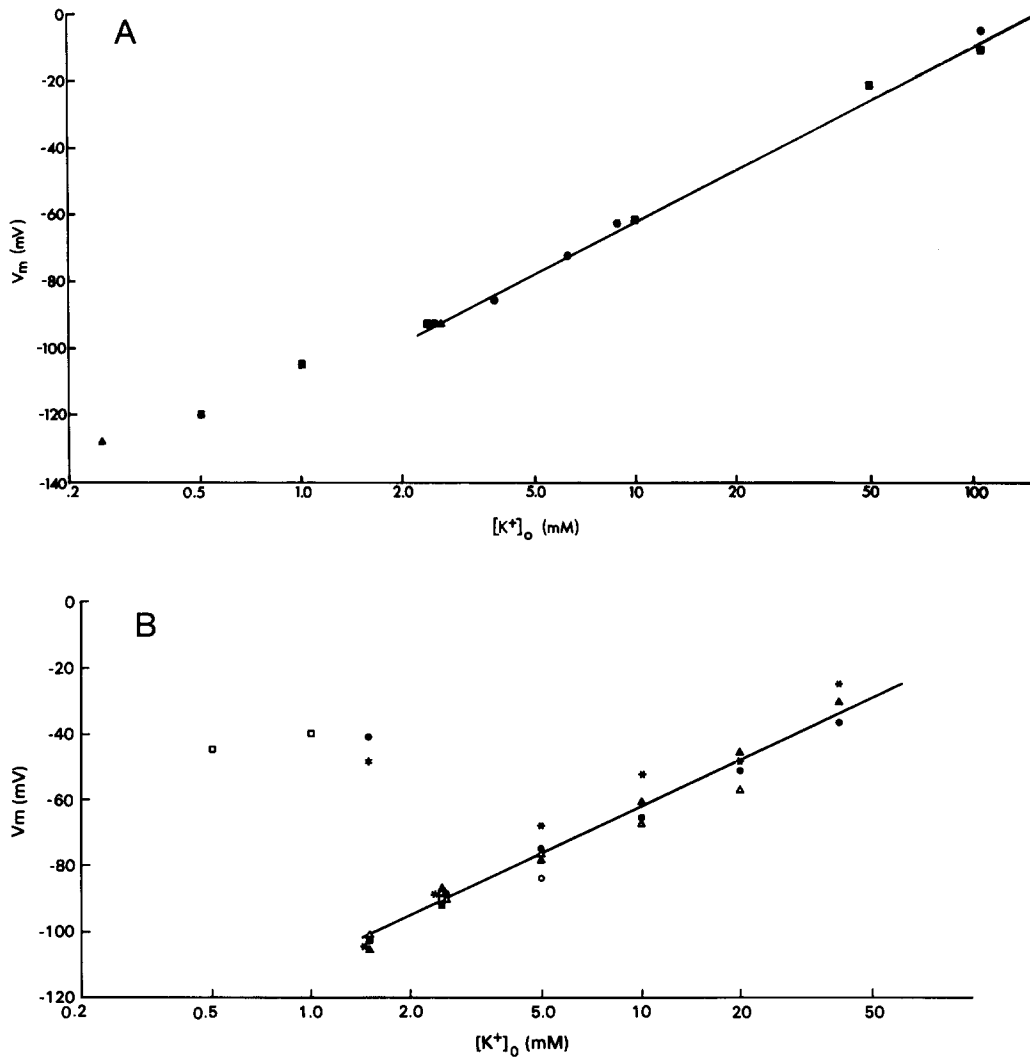


FIGURE 6. Relationship between  $[K^+]_o$  and resting membrane potential in intact bullfrog atrium (*A*) and in isolated, single bullfrog atrial cells (*B*). The different symbols in *A* refer to an average  $V_m$  measured in different preparations; the different symbols in *B* refer to  $V_m$  measured in different isolated cells. Slope of the line is 52 mV/decade in *A* and 46 mV/decade in *B* (see text for further explanation).

isolated atrial cells. Raising  $[K^+]_o$  from 2.5 to 40 mM produced graded depolarizations. These data could be fit with a straight line with a slope of 46 mV/decade. This indicates that the ratio  $P_K:P_{Na}$  of the sarcolemma in quiescent, isolated atrial cells is very similar to that of the intact atrium. Hence, it provides strong evidence that the passive ionic conductances of the sarcolemma have not been modified significantly by the enzymatic isolation

procedure. The small differences between the intact tissue and the single-cell data are within the range of measurement errors.

At  $[K^+]_o$  concentrations  $<2.5$  mM, a dramatic depolarization was observed in the isolated cell experiments. A similar phenomenon has been studied in canine Purkinje fibers (Gadsby and Cranefield, 1977). Additional experiments are needed to resolve the mechanism of this phenomenon in isolated atrial cells.

**BIPHASIC DEPOLARIZATION OF THE ACTION POTENTIAL** Two distinct inward currents are believed to generate the depolarizing phase of the action potential in both atrial and ventricular cardiac tissues (Coraboeuf, 1978; Reuter, 1979). Niedergerke and Orkand (1966) first observed that the initial depolarization of the action potential in frog ventricle was biphasic and that the amount of overshoot was strongly dependent on  $[Ca^{++}]_o$ . Subsequent voltage-clamp experiments on intact frog atrium have suggested that both a fast inward Na current and a slow inward ( $Ca^{++}/Na^+$ ) current contribute to the depolarizing phase of the action potential (Rougier et al., 1969; Connor, et al., 1975). These findings, however, have been criticized on the grounds that radial voltage nonuniformity could, in theory, give rise to the false appearance of a second or slow inward current (Johnson and Lieberman, 1971). It was therefore of interest to examine whether two distinct components contribute to the upstroke of action potentials in isolated frog atrial cells.

Fig. 7A illustrates a typical action potential recorded from an isolated cell. In Fig. 7B, the upstroke is shown on a faster time scale. Two phases of depolarization are clearly seen. Fig. 7C shows an action potential elicited in the same cell as in Fig. 7A after addition of TTX ( $10^{-6}$  g/ml). The overshoot of the action potential was virtually unchanged, but the duration was slightly decreased and the upstroke was slowed significantly (Fig. 7D). In five such experiments, this concentration of TTX reduced the maximum upstroke velocity ( $V_{MAX}$ ) from 42 to 2.2 V/s. Responses such as these have been called "slow responses" (Cranefield et al., 1972). This finding strongly suggests that an appreciable TTX-resistant inward current exists in these isolated atrial cells.

Fig. 8 illustrates the effects of verapamil ( $3 \times 10^{-6}$  M), which at this concentration primarily blocks slow inward current (Fleckenstein, 1977). Verapamil caused (a) a reduction in action potential overshoot, (b) a prolongation of action potential duration, and (c) a nearly complete removal of the plateau. Other slow-channel blockers, such as manganese and nickel, have also been used and produce very similar results. It therefore appears that cells isolated from the frog atrium have retained functional TTX-sensitive fast sodium channels, and that they also exhibit a separate inward current mechanism.

#### *Two-Microelectrode Experiments*

A second intracellular microelectrode was used to evaluate the accuracy of potential measurements made with the active bridge circuit and to measure the electrical constants of single atrial cells. In these experiments, current was

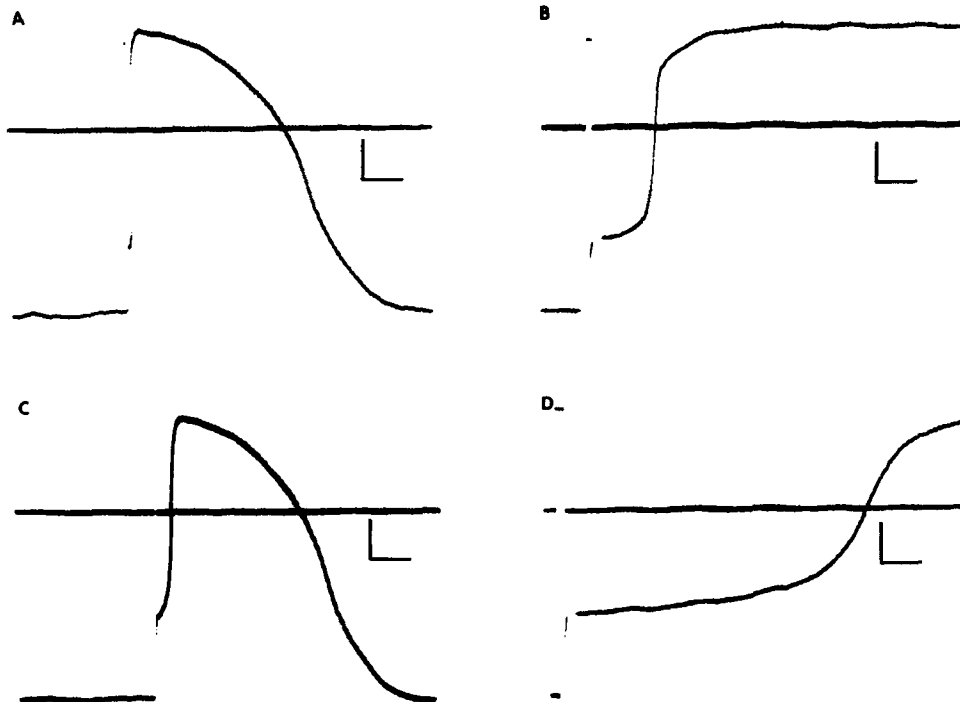


FIGURE 7. Action potentials elicited from an isolated bullfrog atrial cell are shown at two sweep speeds under control conditions (*A* and *B*) and in the presence of  $10^{-6}$  g/ml TTX (*C* and *D*). Vertical calibration, 20 mV;  $2.5 \times 10^{-9}$  A for *A*-*D*; horizontal calibration, 100 ms for *A* and *C*; 5 ms for *B* and *D*.

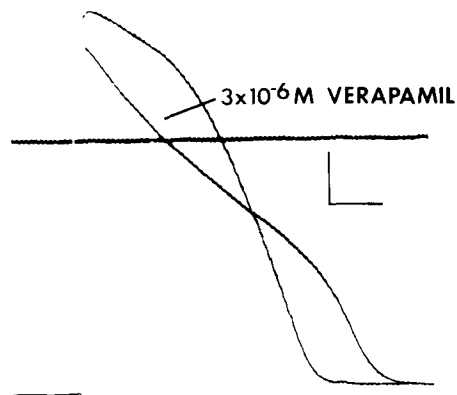


FIGURE 8. Effect of verapamil ( $3 \times 10^{-6}$  M) on an action potential recorded from a single bullfrog atrial cell. Vertical calibration, 20 mV and  $2.5 \times 10^{-9}$  A; horizontal calibration, 100 ms.

measured directly from the bath by using an operational amplifier circuit. When a cell was successfully impaled, as evidenced by return of the membrane potential to near  $-85$  mV, a second impalement was made. This usually

resulted in a depolarization of  $-30$  to  $-50$  mV; the membrane potential either hyperpolarized back to near  $-85$  mV within 1–2 min or it remained at this depolarized level, in which case the cell usually underwent partial contracture. In cells that recovered (hyperpolarized), it was possible to stimulate intracellularly via the bridge electrode and to monitor simultaneously an action potential at the site of stimulation and at some distance away in the same cell. Fig. 9 shows an experiment in which the two microelectrodes were separated by  $113 \mu\text{m}$ . It is evident that the size and time-course of the action potential recorded by the bridge electrode (Fig. 9, *top trace*) are nearly identical to those recorded by the second microelectrode.

Some estimates of the resting space constant ( $\lambda$ ) in intact multicellular cardiac preparations have indicated that it is about 10 times the length of an individual cardiac cell (Tsien and Seigelbaum, 1979). However, estimates of  $\lambda$  in intact frog myocardium yield  $863 \mu\text{m}$  (Scubon-Muleri and Sichel, 1975),



FIGURE 9. An elicited action potential recorded at two sites within a single atrial cell. The *top trace* was recorded by the microelectrode connected to a bridge circuit, the *bottom trace* (offset by  $-5$  mV) was recorded by an independent microelectrode located  $113 \mu\text{m}$  away. Vertical calibration, 20 mV; horizontal calibration, 100 ms.

$690 \mu\text{m}$  (Brown et al., 1976), and  $328 \mu\text{m}$  (Chapman and Fry, 1978). These values of  $\lambda$  are, however, subject to the previously mentioned technical difficulties arising from complex patterns of current flow in syncytial structures. To facilitate accurate measurement of small differences in the electrotonic potentials, we chose relatively long cells to maximize the interelectrode separation. In addition, multiple electrotonic responses in each cell were signal-averaged.

Fig. 10 *A* illustrates records of the applied current and the resulting electrotonic potentials recorded at two sites. The resting membrane potential of this cell was  $-88$  mV, and the electrode separation was  $160 \mu\text{m}$ . The magnitude and time-course of the electrotonic responses to a 150-ms hyperpolarizing current are nearly identical. To resolve any small differences in their magnitudes, responses from each electrode were signal-averaged and then subtracted.



These averaged responses and their difference are shown in Fig. 10 *B* at a higher gain. In this case, a difference ( $V'_1 - V'_2$ ) of 0.16 mV was measured. Assuming a single atrial cell to be a linear finite cable terminated by open circuits at both ends, the steady-state response will be given by

$$V = V_0 \frac{\cosh (1-x)/\lambda}{\cosh (1/\lambda)} \quad (1)$$

$$R_{in} = r_i \coth (1/\lambda) \quad (2)$$

(Hodgkin and Rushton, 1946; Weidmann, 1952). Using these equations and

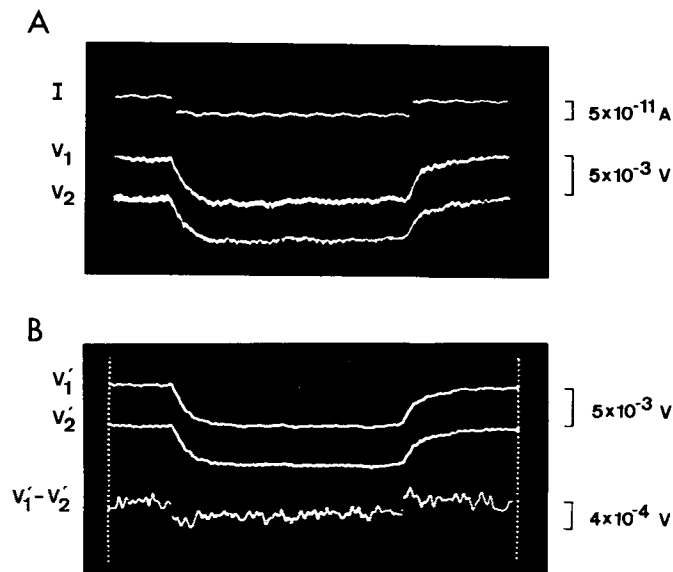


FIGURE 10. Electrotonic potentials recorded at two sites in a single atrial cell in response to intracellular current injection. In *A* the original traces are shown.  $I$  is the current measured from the bath,  $V_1$ , is the electrotonic potential measured by the microelectrode connected to the bridge circuit, and  $V_2$  is the electrotonic potential measured by an independent microelectrode. Electrode separation was  $160 \mu\text{m}$ . *B* Signal-averaged  $V_1$  and  $V_2$  ( $n = 13$ ), denoted ( $V'_1$ ) and ( $V'_2$ ), respectively. The difference ( $V'_1 - V'_2$ ) is displayed at a higher gain.

the measured cell dimensions, the passive electrical constants can be calculated.

Table II summarizes the results from nine single cells.  $R_i$ , the specific internal resistivity of the cell,  $= \pi a^2 r_i$ ; and  $R_m$ , the specific membrane resistance,  $= 2\pi a r_m$ . Cells that remain relaxed and have a membrane potential of  $-80$  mV or more have a radius of  $\sim 2.5 \times 10^{-4}$  cm, which was used for these calculations.  $C_m$ , the membrane capacity per unit length, was calculated from  $C_m = \tau_m / R_m$ . In four of six cells, small differences in electrotonic

potentials recorded by the two electrodes could be reliably detected. We chose the values for the largest apparent differences in  $V_1$  and  $V_2$ . Thus, the calculated mean values for  $\lambda$  (921.3  $\mu\text{m}$ ),  $\tau_m$  ( $5 \times 10^6 \Omega\text{cm}$ ) and  $R_m$  (7.9  $\text{k}\Omega\text{cm}^2$ ) represent minimal values, whereas  $R_i$  ( $6 \times 10^8 \Omega\text{cm}^{-1}$ ), and  $R_i$  (118  $\Omega\text{cm}$ ) and  $C_m$  ( $2.2 \mu\text{Fcm}^2$ ) represent upper limits.

The membrane potential of three cells did not recover after the second electrode penetration, and these cells exhibited partial contracture. Cell length was reduced nearly 50%, and the cell radius was increased about fourfold. In these cells,  $R_{in}$  was reduced considerably. As expected, smaller values of  $\tau_m$  and larger differences between the electronic potentials at the two recording sites were measured. As a result,  $\lambda$  was considerably smaller in these damaged cells. This could be a result of a decrease in  $R_m$ , an increase in  $R_i$ , and/or a result of leakage caused by incomplete sealing around the microelectrodes.

TABLE II  
ELECTRICAL CONSTANTS OF SINGLE FROG ATRIAL CELLS

Cell	$V_r$	Cell length	Elec-trode separation	$R_{in}$	$\tau_m$	$V_1 - V_2$	$\lambda$	$R_i$	$R_m$	$r_i$	$\tau_m$	$C_m$
	<i>mV</i>	$\mu\text{m}$	$\mu\text{m}$	<i>M</i> $\Omega$	<i>ms</i>	<i>mV</i>	$\mu\text{m}$	$\Omega\text{cm}$	$\text{k}\Omega\text{cm}^2$	$\times 10^8 \Omega\text{cm}^{-1}$	$\times 10^6 \Omega\text{cm}$	$\mu\text{F cm}^{-2}$
1	-88	225	100	300	31.8	0.1	838	187.4	10.4	9.4	6.6	3.1
2	-87	270	88	224	14.9	0.08	977	121.3	9.3	6.2	5.9	1.6
3	-85	125	48	106	15.1	0.0						
4	-88	333	160	133	14.5	0.16	936	95.2	6.7	4.9	4.3	2.2
5	-84	350	153	400	33.4	0.0						
6	-87	325	173	101	9.5	0.07	934	71.1	5.0	3.6	3.2	1.9
Mean	-86.5	271		210.7	19.9		921.3	118.1	7.9	6.0	5.0	2.2
$\pm$ SEM	0.7	34.8		49.3	4.1		29.5	24.5	1.2	1.2	0.8	0.3
7	-35	138	113	19.2	5.6	1.05	214	1602.2	1.5	5.1	0.23	3.7
8	-55	130	113	47.5	6.5	0.5	367	1382.3	3.7	4.4	0.59	4.5
9	-50	132	105	23.3	6.3	0.35	251	1413.7	1.8	4.5	0.28	3.5
Mean		133.3		30.0	6.1		277.3	1466.1	2.3	4.7	0.37	3.9
$\pm$ SEM		2.4		8.8	0.3		46.1	68.7	0.7	0.2	0.1	0.3

The large value for  $R_i$  in these depolarized cells reflects the increase in cell radius due to contracture. However, the surface area calculated from the measured cell radius, assuming a right-cylindrical geometry, is likely to be in error because the sarcolemma of contracted atrial cells may exhibit prominent infoldings similar to those that have been described in contracted smooth muscle cells (Fay and Delise, 1973). An error in surface area could account for the relatively large calculated values for  $R_i$  and  $C_m$  in these cells because values of  $r_i$  are similar to those calculated for relaxed cells.

#### DISCUSSION

Single atrial cells can be dispersed from fragments of bullfrog right atrium by using a combined enzymatic and mechanical isolation procedure. These cells,

which are relaxed and quiescent, are morphologically very similar to those previously studied in the intact frog atrium. Despite earlier unsuccessful attempts to impale isolated frog atrial cells with microelectrodes (Tarr and Trank, 1978), our findings clearly demonstrate that intracellular impalement with conventional microelectrodes is possible and that these isolated single cells have electrophysiological properties comparable to those recorded from intact atrial tissue. Thus, normal, resting membrane potentials and long-duration action potentials with prominent plateau phases can routinely be recorded with a single microelectrode. Moreover, in both isolated atrial cells and the intact atrium, a similar relationship between  $[K^+]_0$  and resting membrane potential is observed. Two distinct components, which may correspond to a fast inward  $Na^+$  current and a slow inward ( $Ca^{++}/Na^+$ ) current, contribute to the depolarizing phase of action potentials recorded in both preparations. These morphological and electrophysiological similarities strongly suggest that isolated frog atrial cells are viable, despite the rigors of the isolation procedure.

The minimal space constant of a single atrial myocyte ( $921.3 \mu m$ ) is long compared with the length of the cell (mean cell length,  $204 \mu m$ ), provided the cell is relaxed and an appreciable leak is not created by the microelectrode impalement. The effect on the resting membrane potential of a linear leakage current around the microelectrode tip (assuming a reversal potential for the leak current of  $0 mV$ ) is given by

$$E_R^1 = E_R \frac{G_{in}}{G_{in} + G_1} \quad (3)$$

(Jack, 1979).  $E_R^1$  is the measured value of the resting potential and  $E_R$  is the true value.  $G_{in}$  is the input conductance of the cell at the site of the microelectrode insertion, and  $G_1$  is the magnitude of the leak conductance around the microelectrode. It can be assumed that the ratio  $G_1/G_{in}$  is low for cells that regain a resting membrane potential of at least  $-80 mV$ , whereas for cells that remain depolarized, the ratio will be higher. In the latter cells, a significantly shorter space constant ( $277.3 \mu m$ ) was determined. As Hagiwara and Jaffe (1979) have pointed out, even a very small leakage current caused by a microelectrode impalement may have very dramatic effects if the steady-state current-voltage relation exhibits inward-going rectification. Although none of our findings establish that inward-going rectification is observed in quiescent, single atrial cells, this phenomenon has been observed in intact frog atrial trabeculae (cf. Noble [1976]).

Are the calculated electrical constants of single atrial cells consistent with expectations based upon the known dimensions of the cells? The specific membrane resistance can be calculated from  $R_m = R_{in} \times \text{cell surface area}$ , assuming that the cell is uniformly polarized. A mean  $R_{in}$  of  $219.8 M\Omega$  was measured at a resting potential of  $-88.6 mV$  (Table I). For the surface-area calculation, we assume that a typical cell is a cylinder  $200 \mu m$  in length and  $5 \mu m$  in diameter, giving a membrane surface area of  $3.0 \times 10^{-5} cm^2$  ( $3,000 \mu m^2$ ). If the cavaolae present in the sarcolemma (Fig. 3) are assumed to increase the total membrane surface area by 50% (K. Baldwin, personal

communication), then the total surface area of a typical cell becomes  $4.5 \times 10^{-5} \text{ cm}^2$  ( $4,500 \mu\text{m}^2$ ), and  $R_m$  is  $9.9 \text{ k}\Omega\text{cm}^2$ . Using a mean membrane time constant of 19.7 ms (Table I),  $C_m$  is  $2.0 \mu\text{Fcm}^{-2}$ . These values are very similar to values of  $R_m$  ( $7.9 \text{ k}\Omega\text{cm}^2$ ) and  $C_m$  ( $2.2 \mu\text{Fcm}^{-2}$ ) obtained from the two-microelectrode experiments. The low value of  $C_m$  of single atrial cells implies little infolding of surface membrane in relaxed cells, consistent with anatomical studies (Sommer and Johnson, 1969; Page and Niedergerke, 1972), which have verified that frog myocardium lacks an extensive T system. The contribution of caveolae to the total membrane surface area is uncertain, however, and could lead to an overestimate of  $C_m$ .

Similar ranges of values for electrical constants have been obtained in intact multicellular myocardial preparations. An axial space constant of  $880 \mu\text{m}$  was found for sheep and calf ventricular trabeculae (Weidmann, 1970), and  $1,200 \mu\text{m}$  has been reported for dog papillary muscles (Sakamoto, 1969). In these studies, estimates of  $R_m$  and  $R_i$  were also similar to those reported here. Our estimate of the space constant of single frog atrial cells is similar to that obtained in frog atrial trabeculae ( $863 \mu\text{m}$ ) by Scubon-Mulieri and Sichel (1975). This similarity suggests that cell-to-cell resistance normally represents a small fraction of the total longitudinal resistance in intact frog atrium. Conditions that cause cell-to-cell uncoupling, however, would be expected to reduce the value of  $\lambda$  in a multicellular preparation. The recent data of Chapman and Fry (1978) in frog ventricular trabeculae and strips yield considerably lower values of  $\lambda$  ( $328 \mu\text{m}$ ). This discrepancy might reflect real differences in the electrical properties of atrial and ventricular myocardium. However, a considerable difference between the resistance of the total intracellular pathway ( $R_i = 588 \Omega\text{cm}$ ) and the cytoplasmic resistivity ( $282 \Omega\text{cm}$ ) was measured. The low value of  $\lambda$  calculated in frog ventricle may be the result of relatively large intercellular junctional resistances.

These experiments demonstrate that isolated heart cells from adult frog atrium retain many of the electrical properties of cells normally coupled together in a syncytial network. This preparation thus represents the first viable, single-heart-cell preparation derived from adult atrial myocardium. Morphological examination has verified that our enzymatic dispersion procedure yields single atrial cells. The electrical constants of atrial cells measured with two microelectrodes and the absence of an extensive T system indicate that it is feasible to study the transmembrane ion currents in this preparation using a two-microelectrode voltage-clamp technique. Recent voltage-clamp measurements have revealed the presence of a TTX-insensitive inward current in single atrial cells (Giles and Hume, 1981 [in press]).

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