Voltage Clamp Analysis of Embryonic Heart Cell Aggregates

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ABSTRACT The double-microelectrode voltage clamp technique was applied to small spheroidal aggregates of heart cells from 7-d chick embryos. A third intracellular electrode was sometimes used to monitor spatial homogeneity. On average, aggregates were found to deviate from isopotentiality by 12% during the first 3-5 ms of large depolarizing voltage steps, when inward current was maximal, and by <3% thereafter. Two components of inward current were recorded: (a) a fast, transient current associated with the rapid upstroke of the action potential, which was abolished by tetrodotoxin (TTX); and (b) a slower inward current related to the plateau, which was not affected by TTX but was blocked by D600. The magnitudes, kinetics, and voltage dependence of these two inward currents and a delayed outward current were similar to those reported for adult cardiac preparations. From a holding potential of -60 mV, the peak fast component at the point of maximal activation (-20 mV) was -185 μA/cm². This value was about seven times greater than the maximal slow component which peaked at 0 mV. The ratio of rate constants for the decay of the two currents was between 10:1 and 30:1.

INTRODUCTION

The voltage clamp technique has been used by numerous investigators to describe the voltage- and time-dependent conductances in cardiac tissue. The main conclusion from these analyses is that the ionic currents that underlie the action potential in the heart are more numerous and complex than in the classic squid axon (for reviews, see Trautwein, 1973; McAllister et al., 1975; Noma and Irisawa, 1976; Beeler and Reuter, 1977; Trautwein and McDonald, 1978).

A major problem in the quantitative interpretation of voltage clamp data in heart tissue arises out of the multicellular nature of the preparation. Heart muscle is composed of individual cells connected by junctions of low but varying resistance, into fibers of complex geometry (DeHaan and Fozzard, 1975; Kensler et al., 1977). Substantial voltage gradients may exist within such fibers, and currents crossing the membrane of one cell may differ from those flowing in a distant cell. The problem of electrical inhomogeneity of cardiac tissue and the limitations it places on voltage clamp analyses have been documented and discussed at length. It is generally agreed that, because of the multicellular nature of heart muscle, it has not been possible to control ideally the membrane potential of any preparation. Thus, there remain substantial uncertainties.
regarding the absolute magnitude, kinetics, and voltage dependence of the various action currents (Johnson and Lieberman, 1971; Fozzard and Beeler, 1975; Ramón et al., 1975; Attwell and Cohen, 1977).

Nevertheless, an understanding of the ionic currents in cardiac muscle is beginning to emerge from application of the voltage clamp. The action potential shape recorded from mammalian ventricular tissue has been reconstructed on the basis of two inward and two outward current systems for which (with the exception of activation kinetics for the fast inward current) experimental evidence exists (Beeler and Reuter, 1977; Trautwein and McDonald, 1978); that of the rhythmically active Purkinje fiber has been computed using nine distinguishable currents (McAllister et al., 1975). We have recently reviewed the relations between the nine conductances of the McAllister-Noble-Tsien model and the spontaneous oscillatory properties of the heart (DeHaan and DeFelice, 1978).

All of the above analyses of cardiac membrane currents have been performed on adult heart tissue. Until now, voltage clamp data defining current-voltage relationships in embryonic cardiac preparations have not been available. Evidence concerning the identity and kinetics of ionic currents in such preparations has been indirect, based upon action potential parameters and the sensitivity of electrical events to (adult) current inhibitors. For example, tetrodotoxin (TTX), which specifically blocks the fast-transient inward sodium current ($I_{Na}$) in nerve, skeletal muscle, and adult cardiac tissue (reviewed by Narahashi, 1974), has no effect on hearts from embryonic chicks, 2-4 d of age, even at concentrations up to $3 \times 10^{-5}$ M. However, after 7 d of development, spontaneous activity can be inhibited by TTX concentrations as low as $3 \times 10^{-6}$ M (Ishima, 1968; Shigenobu and Sperelakis, 1971; McDonald et al., 1972). On the other hand, the organic compounds, verapamil and D600, which suppress the slow inward current ($I_{Na}$) in adult cardiac tissue (Kohlharm et al., 1972; Kass and Tsien, 1975), can abolish action potentials in hearts from embryos up to 3 d of age, whereas in hearts 7 d or older, these drugs merely reduce the amplitude and duration of the plateau phase; they do not affect action potential generation at these later stages (Shigenobu et al., 1974; McDonald and Sachs, 1975).

In this paper we report on a voltage clamp analysis of a heart tissue model system consisting of a spheroidal aggregate of embryonic ventricular cells in culture (Sachs and DeHaan, 1973). We have shown previously that the cells within such an aggregate are tightly coupled electrically. The entire aggregate membrane appears to be virtually isopotential during the voltage changes produced by injecting small current pulses through an intracellular micropipette (Clay et al., 1978), although it deviates somewhat from uniformity during the fast rise time of an action potential (DeHaan and Fozzard, 1975). Junctional impedance between cells is $<10\%$ of transmembrane impedance and is independent of intercellular voltage gradients in the range of $10^{-5}-10^{-2}$ V, from DC to 160 Hz (DeFelice and DeHaan, 1977). In the present report, we test further the degree of spatial homogeneity in this preparation under voltage clamp conditions, when substantial currents are applied in fast-rising steps. With the aid of an exploring voltage electrode, we show that deviation from voltage homogeneity during a clamp step is comparable to that achieved in adult cardiac
preparations. We also demonstrate two kinetically and pharmacologically distinct components of inward current and a delayed outward current with properties similar to those observed in adult heart tissue. The advantages of spherical geometry and small size for voltage clamp analysis have also been exploited in a cultured preparation of neuroblastoma cells, 60–110 μm in diameter (Moolenaar and Spector, 1978).

METHODS

Tissue Culture

Culture techniques for the preparation of embryonic heart cell aggregates have been described in detail elsewhere (DeHaan, 1970; Sachs and DeHaan, 1973). Briefly, fertilized eggs from White Leghorn chickens were incubated for 7 d at 37°C. Cells were dissociated from fragments of ventricles by a multiple-cycle trypsinization procedure (DeHaan, 1967), and allowed to aggregate in medium 818A (DeHaan, 1970) during a 72-h period on a gyratory shaker (60–67 rpm, 1.25-in stroke).

Aggregates attached to the bottom of a 35-mm tissue culture dish (Falcon Plastics, Oxnard, Calif.) were maintained at 35–37°C on the heated stage of a dissecting microscope. A mixture of 5% CO₂, 10% O₂, and 85% N₂ was passed through a toroidal gassing ring surrounding the dish, and maintained the pH at 7.4. All experiments were performed in 4 ml of a 3:1 mixture of medium 818A (DeHaan, 1970) and medium 21212 (Nathan et al., 1976) with the following final composition (millimolar per liter): NaCl 124; KCl 4.6; CaCl₂ 1.4; MgCl₂ 0.4; MgSO₄ 0.6; NaH₂PO₄ 0.7; Na₂HPO₄ 0.7; NaHCO₃ 17; and dextrose 5.5.

Electrophysiology

Glass micropipettes were prepared by the glass fiber technique (Tasaki et al., 1968) and filled with 3 M KCl. Electrodes with resistances between 10 and 25 MΩ were selected for voltage clamping. Intracellular electrodes were coupled through Ag-AgCl holders to capacitance-compensated, unity-gain electrometer amplifiers, A₂ and A₃ (Fig. 1 F) (Instrumentation Laboratory, Inc., Lexington, Mass., Picometric, model 181). Intracellular potentials were recorded with respect to the bathing medium which was coupled to a virtual ground through two agar-KCl bridges, connected in parallel to reduce series resistance.

The double-microelectrode voltage clamp technique (Deck et al., 1964; Hecht et al., 1964) was employed with standard circuitry (Cole and Moore, 1960) as illustrated diagrammatically in Fig. 1 F. Potentials up to 140 V were provided by a high-output variable gain operational amplifier A₁ (Analog Devices, Inc., Norwood, Mass., model 171K) in the inverting configuration, and currents were recorded as the voltage drop across the feedback resistor (100 kΩ) of the virtual-ground operational amplifier, A₄ (Analog Devices, Inc., model 48K). Command potentials, consisting of steps provided by a digital pulse generator (Frederick Haer & Co., Ann Arbor, Mich., Pulsar, model 4) and the holding potential, were added to the membrane potential at the summing junction of the control amplifier A₁. Its gain was sometimes reduced to avoid oscillations in current that occasionally followed the on- or off-transients of a large voltage step. These oscillations may have been induced partly by capacitive coupling between the current-passing and voltage-recording electrodes since a grounded shield was not used. Following a command step (Vₐ), the potential recorded by electrode V₁ (in the feedback loop) usually reached its new steady value in about 0.4 ms. This delay was not prolonged by a slight reduction in the gain of amplifier A₁.
Potentials recorded from two or three intracellular microelectrodes and membrane currents were amplified by low-noise operational amplifiers (Analog Devices, Inc., model 504M) and recorded on FM tape (Sangamo Weston, Inc., Springfield, Ill., Sabre, model 2; or Hewlett-Packard Co., Palo Alto, Calif., model 3960) at a speed of 3⅓ in/s (bandwidth, DC to 1.25 kHz). At this speed, the waveform of the fast inward transient was not distorted significantly by the recorder. Control recordings of voltage ramps with

Figure 1. Three-microelectrode voltage clamp tests of spatial homogeneity in aggregates. As illustrated in panel F, $V_1$ is the recorded potential used in the feedback loop; $V_2$ is an exploring intracellular electrode independent of the feedback loop; $V_1 - V_2$ is a measure of the deviation from isopotentiality; $I$ is total membrane current. $V_2$ was placed in line with $V_1$ and the current source as illustrated in (F) for (A) and (C), i.e., 30-50 μm from $V_1$ and about 100 μm from the current source; $V_2$ was placed in line between $V_1$ and the current source i.e., 30-50 μm from each electrode in (B). Panel D is a faster sweep of (C). In (E) trace 1 was obtained with $V_2$ close to the current source, and trace 2 with $V_2$ far from the current source. Holding currents (currents required to maintain the membrane at the holding potential) were -5 nA (A) and -40 nA (B) and (C), at -62 mV and -60 mV, respectively. Aggregate impaled in panel B was exposed to $1.67 \times 10^{-7}$ g/ml cytochalasin B. The records in panels A-E were obtained from four different aggregates, all having diameters of 144 ± 7 μm. Vertical scale: $V_1$ and $V_2$, 40 mV; $V_1 - V_2$, 40 mV in panels A, B, and C; 20 mV in panel D; $I$, 1 μA in A, B, and C; 0.5 μA in D. Horizontal scale: 10 ms in A, B, and C; 4 ms in D and E.
slopes of 0.9-1.5 V/ms were reduced in amplitude by <10%. These were comparable
with the fastest rates we recorded (e.g., Fig. 1 D). On the other hand, the much faster
rise times of square-wave voltage steps and capacitive transients were presumably
distorted by the tape recorder, and were recorded with damped oscillations after their
onsets (see $V_t$, Fig. 1 C) and offsets (Fig. 9).

Data Analysis

All measurements of membrane current magnitudes and kinetics were made directly
from the oscilloscope CRT during replay of the tapes. Because of variation in the
absolute magnitude of currents recorded among different aggregates and for different
impalments within the same aggregate, we were unable to determine a current-voltage
relationship for leakage currents, (Such a correction would have required recording
leakage after blocking the dynamic currents with TTX, D600, Cs, etc., replacing these
drugs with the standard medium, and then recording the time-dependent currents, all
from the same aggregate and ideally during the same impalement.) Rather, both inward
and outward currents were adjusted for leakage only at the “holding potential”; i.e., they
were determined with respect to the “holding current” (current required to maintain the
membrane at the holding potential) preceding each voltage step. Thus, since leakage
currents were not corrected for (and likely increased) during clamp steps, our inward,
and especially our outward currents are probably somewhat overestimated. In preparing
the current-voltage relationship shown (Fig. 6), dynamic currents preceded by large
holding currents were not included.

Time constants for the decay of inward currents were obtained by measuring changes
in total current with time from oscilloscope traces and using these values in an asymptotic
regression analysis of the function $A + Be^{-rt}$. The relatively long duration of the
capacitive transient precluded an analysis of the fast transient inward current’s activation
kinetics.

Aggregates used for this study varied from 120 to 180 $\mu$m in diameter. Net currents
were referred to total cell surface area for calculation of specific currents. Aggregates
were viewed at a magnification of $\times 62$, and their major and minor axes were measured
with an ocular reticle whose smallest division represented 36 $\mu$m. Measurements could
be made accurately to about 0.2 divisions (7 $\mu$m). Total aggregate volumes ($V_a$) were
obtained using the equation for a prolate spheroid, $V_a = \frac{4}{3}\pi ab^2$, where $a$ and $b$
represent the major and minor hemi-axes. The number of cells per aggregate ($N_a$) was
calculated by two different methods. Isolated ventricle cells in suspension were spherical
in shape until they adhered to the surface of the substratum or other cells. The mean cell
diameter measured from 1,545 cells before aggregation was $10.8 \pm 0.9 \mu$m, yielding a
mean cell volume ($V_c$) of 660 $\mu$m$^3$. As confirmed in electron micrographs, this volume
does not change significantly when these cells are closely associated within spheroidal
aggregates. In addition, we have shown that total aggregate volume consists of 80% cells
and 20% extracellular space (McDonald and DeHaan, 1973; Elsas et al., 1975). Thus, the
number of cells per aggregate could be calculated by $N_a = 0.8 \frac{V_a}{V_c}$. $N_a$ was also
estimated by measuring aggregate DNA content using an observed value of 2.8 pg DNA
per cell, measured in single ventricular cell suspensions and chicken erythrocytes
(Santora et al., 1978). Total DNA per aggregate was determined from pooled groups of
counted, sized aggregates by the method of Giles and Meyers (1965) or by a modified
ethidium bromide spectrofluorometric method. The mean cell volume calculated from

1 Atherton, B., and R. L. DeHaan. Unpublished data.
2 Baste, C. A., and R.L. DeHaan. Unpublished data.
3 Kellar, K., and J. M. Kinkade. Unpublished data.
the aggregate DNA assays was 651 μm². Thus, estimates of \( N_a \) derived from the two methods agreed within ± 1%. Total cell surface area per aggregate was calculated as the product of the number of cells per aggregate and the mean surface area per cell (366 μm²). The total membrane area of a spheroidal aggregate 150 μm in diameter is 7.8 × 10^{-3} cm². Aggregates used in voltage clamp experiments ranged in size from 119 × 126 μm (4.0 × 10^{-3} cm²) to 175 × 180 μm (11.9 × 10^{-3} cm²). In calculating the total active membrane area, no correction was made for non-cardiac cells, which probably represented <20% of the aggregate population (Sachs and DeHaan, 1973).

Drugs

Tetrodotoxin (Calbiochem, San Diego, Calif.) and compound D600-hydrochloride (Knoll Pharmaceutical Co., Whippany, N. J.) were dissolved in distilled water to make stock solutions of 1.0 mg/ml and 0.1 mg/ml, respectively. For some experiments, aggregates were incubated overnight in 1.67 × 10^{-7} g/ml cytochalasin B (Calbiochem) to disrupt myofibrils and prevent contraction (Sachs et al., 1974). The cytochalasin B was dissolved in dimethylsulfoxide to make a stock solution of 0.1 mg/ml. All drug concentrations are expressed in units of grams per milliliter in order to compare directly with values used by other investigators.

Voltage Control

The potential difference (\( V_1 - V_2 \)) between different cells in an aggregate may be taken as a direct measure of intracellular homogeneity (excluding external series resistance, discussed below) (New and Trautwein, 1972; Reuter and Scholz, 1977). With an exploring intracellular electrode (\( V_2 \)) in the configuration shown in Fig. 1 F, the degree of voltage homogeneity during voltage clamp steps was generally dependent upon the magnitude of the current passed into the aggregate. For small voltage steps, for example, to −56 mV (Fig. 1 A) where \( I_{Na} \) was only partially activated (−43 nA), \( V_1 - V_2 \) was never >1 mV. For larger depolarizing steps (−30 mV, Fig. 1 B), where \( I_{Na} \) was almost maximal (−1.57 μA), \( V_1 - V_2 \) was −8 mV at the time of apparent peak inward current. For a still larger voltage step to 0 mV (Fig. 1 C, D), \( I_{Na} \) was reduced (−0.72 μA), and \( V_1 - V_2 \) was only 4 mV during peak inward current. Although these measurements were made on several different aggregates, our ability to select preparations of uniform size resulted in data of an acceptable degree of reproducibility. Voltage homogenity was usually better with steps that produced little current (Table I), and better during slow inward transients than during fast. With large clamp steps, reasonable control was reached within 3–5 ms. Deviation from homogeneity was greatest during the capacitive transient; uniformity always improved during the decay of \( I_{Na} \) (Fig. 1 D). Mean deviation from isopotentiality at the time of maximal activation of \( I_{Na} \) was 6.4 ± 0.7 mV (mean ± SE), or 12% in 13 experiments. After the fast inward current had inactivated, \( V_1 - V_2 \) was usually <2 mV.

Fig. 1 E illustrates the effect of electrode position on the potential recorded by the test electrode (\( V_2 \)) during a 50-mV depolarizing clamp step. This experiment was performed (without having to move the electrodes physically) by reversing the \( V_1 \) and \( V_2 \) electrode inputs to their respective amplifiers, \( A_2 \) and \( A_3 \), just before the second of two consecutive steps to 0 mV. During the first step (trace 1), the test electrode was located between the current source and \( V_1 \); the test electrode was then switched to amplifier \( A_3 \), and the most distant electrode to \( A_2 \) (effectively reversing the positions of \( V_1 \) and \( V_2 \)). When \( V_2 \) was far (100 μm) from the current source (trace 2), the development of potential was delayed; when close to the current source (30–50 μm), the potential recorded by \( V_2 \) overshot the command potential by almost 10 mV (trace 1). The rapid initial transient (<0.5 ms) in each trace (Fig. 1 E), due mainly to charging of the membrane capacitance, may have also been influenced by capacitive coupling between the current source and voltage.
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Although the potential seen at $V_1$ did not vary with position since it was controlled directly by the clamp, the shift in $V_2$ indicates that for a 60-mV step, a gradient of current lasting ~3 ms spread outward from the current source. With greater separations between $V_1$ and the current source (>100 μm), slight deviations of $V_1$ from the command potential might be expected.

**Stability of Membrane Currents**

Damped oscillations, possibly due to capacitive coupling between the current and voltage electrodes, were sometimes observed in relation to the capacitive surge following the

**Table I**

| $V_1$ | $I_h$ | $I_{sa}$ | $I_n$ | MDP($I$) | MDP($V_1$) | MDP($V_2$) | $V_1$ | $V_1$-$V_2$ |
|-------|-------|----------|-------|----------|------------|------------|-------|-------------|
| mV    | nA    | nA       | nA    | mV       | mV         | mV         | position | mV          |
| -60*  | -5    | -        | -     | -73      | -76        | -79        | F      | <1          |
| -58*  | -5    | -40      | 0     | -73      | -76        | -79        | F      | <1          |
| -56*  | -5    | -43      | 0     | -73      | -76        | -79        | F      | <1          |
| -54*  | -10   | -56      | 0     | -73      | -76        | -79        | F      | <1          |
| -31‡  | -200  | 2,200    | -176  | -77      | -77        | -75        | F      | -4          |
| -30‡  | -40   | 1,570    | -150  | -72      | -76        | -70        | C      | +8          |
| 0     | -40   | -720     | -110  | -62      | -71        | -71        | F      | -4          |
| 0     | -75   | -325     | -50   | -58      | -60        | -58        | F      | -5          |
| 0‡    | +10   | -822     | -155  | -77      | -77        | -75        | F      | -6          |
| 0     | -25   | -525     | -113  | -69      | -66        | -68        | F      | -12         |
| 0     | -5    | -850     | -90   | -68      | -67        | -64        | F      | -9          |
| 0     | 0     | -950     | -60   | -60      | -60        | -60        | C      | -4          |
| 0     | -10   | -1,550   | -110  | -70      | -70        | c-76       | C      | +4          |
| 0     | -88   | -675     | -100  | -62      | -74        | -75        | S      | +9          |
| 0     | -240  | -1,110   | -100  | -67      | -67        | -70        | S      | +4          |
| 0     | -150  | -940     | -90   | -60      | -65        | -65        | S      | +7          |
| 0     | -25   | -663     | -150  | -73      | -73        | -73        | S      | -7          |

$V_1$, value of step potential recorded by $V_1$ electrode. $I_h$, holding current required to maintain membrane potential at -60 mV. $I_{sa}$, peak fast inward current with respect to the holding current. $I_n$, peak slow inward current with respect to the holding current. MDP, maximum diastolic potential during spontaneous activity recorded just before voltage clamp by current source ($I$) and $V_1$ and $V_2$ electrodes. $V_1$ position, position of the exploring electrode ($V_2$) with respect to the current source ($I$) and voltage electrode ($V_1$). F (far) indicates that $V_1$ was placed in line between $I$ and $V_2$, with a 30-50 μm spacing between each electrode. C (close) indicates that position of $V_1$ and $V_2$ is reversed with respect to $I$. S (symmetric) indicates that $I$, $V_1$, and $V_2$ formed the vertices of an equilateral triangle, approximately 100 μm on a side. $V_1$-$V_2$, deviation from homogeneity determined at the time of peak fast inward current.

* Holding potential, -62 mV.

‡ Aggregates incubated overnight in 1.67 × 10⁻⁷ g/ml cytochalasin B, to abolish mechanical contraction (Sachs et al., 1974).

onset (Figs. 1 D and 8 B) or offset (Fig. 9 B) of large voltage steps. The amplitude of these oscillations declined with repeated steps or with increased external series resistance, and could also be reduced by decreasing the gain of the control amplifier without significantly affecting voltage control.

The magnitude and rates of decay of both the capacitive transient and the fast inward current (at potentials positive to -20 mV) sometimes declined a small amount during repeated clamp steps to the same potential. In seven experiments, $I_{sa}$ decreased 13.4 ±
4.9% (mean ± SE) between the first and fourth repeated clamp step. These changes, together with a concomitant rise in holding current, suggested that aggregate series resistance probably increased during repeated passage of significant ionic current (see Discussion). Whenever such distortions were observed, only the first step in a sequence of potentials was used in further analyses.

RESULTS

Series Resistance

Because it is not possible to ground the volume immediately adjacent to the entire aggregate membrane, current flowing across the membrane must pass through an "access resistance" in series with the membrane itself. This includes an external component—mainly the intercellular clefts, the bath volume, and the agar bridges—and the intracellular resistance of the cytoplasm and nexal junctions. To calculate this series resistance \( R_s \), we have neglected "cable" properties and leakage pathways and have assumed an equivalent circuit with lumped resistance \( R_m \) and capacitance \( C_m \) in parallel to represent the cell membrane. A single resistor in series with the membrane is used to represent both intracellular and extracellular series resistance. At the onset of a voltage step, when ideally \( t \rightarrow 0 \), all current flows as charge displacement across the membrane capacitance; thus,

\[
R_s = \frac{\Delta V_c}{I_c}, \tag{1}
\]

where \( I_c \) is the peak capacitive current and \( V_c \) is the command potential. Slightly different calculations of series resistance in multicellular preparations have been made by Attwell and Cohen (1977). From a series of voltage steps in the range of 5–25 mV, where decay of the capacitive current could be fit with a single exponential, the mean values of \( R_s \) (± SE) calculated from Eq. 1 was 22.7 ± 2.2 kΩ. Since two agar-KCl bridges, each with a resistance of about 10 kΩ, were connected in parallel in these experiments, the series resistance presented by the aggregate alone (i.e., intercellular clefts, cytoplasm, and nexal junctions) was about 17 kΩ. This value might be somewhat in error due to the influence of possible capacitive coupling between the current and voltage electrodes (which would tend to increase transiently \( \Delta V_c \) and \( I_c \)), and because of the slow tape recorder response (which would tend to reduce \( I_c \) and therefore increase \( R_s \)). The extent to which these errors would cancel is not clear.

Current Magnitudes and Kinetics

We were not able to separate clearly the capacitive transient, which generally lasted ~ 1.5 ms, from the onset of \( I_{Na} \) (for example, Fig. 1 D). Thus, an accurate analysis of the activation kinetics of \( I_{Na} \) was not possible, and our values for peak \( I_{Na} \) may be somewhat underestimated.

In medium containing 4.6 mM KCl, aggregates beat spontaneously with a mean maximum diastolic potential of -70 mV (Table I) before application of the voltage clamp; nonetheless, a holding potential of -60 mV seemed to
minimize holding currents. From this potential, peak $I_{Na}$ during a step to $-20$ mV was about seven times greater than the peak slow inward current ($I_{st}$) at 0 mV. At $-30$ mV (Fig. 2 A) where $I_{Na}$ approached its maximum value, $I_{st}$ was small; while at 0 mV (Fig. 2 B), $I_{st}$ (arrow) was maximal and $I_{Na}$ was reduced.

The fast and slow components of inward current reached their respective peaks $2.6 \pm 0.3$ ms and $6.8 \pm 0.3$ ms (mean $\pm$ SE, $n = 35$) after the onset of voltage steps, and these times underwent no dramatic changes between $-50$ and $+40$ mV. The decay of each component could be fit by a single exponential (Fig. 3), and time constants for $I_{st}$ were 10-30 times greater than those calculated for $I_{Na}$. The decay kinetics of $I_{st}$ were influenced strongly by membrane potential (Fig. 4) and were most rapid at $-20$ mV. In these experiments, net current was measured at 5-ms intervals between 10 and 40 ms after onset of a step, and no correction was made for the influence of outward currents, which would have tended to shorten time constants at more positive potentials. Most of these results were obtained in the presence of TTX ($\bullet$) to remove the influence of

\[ I_{Na} \]

but the few values obtained in normal media (○) suggested that neither $I_{Na}$ nor TTX itself had a large effect on the decay kinetics of $I_{st}$.

**Current-Voltage Relationships**

Fig. 5 illustrates a series of 400-ms steps to potentials between $-50$ and $-10$ mV. At $-50$ mV fast inward current was only partially activated. At more positive potentials, $I_{Na}$ became maximally activated ($-30$ mV), and the slow component increased (Fig. 5 E). Outward current (measured from the holding current preceding each step) was small for potentials negative to $-20$ mV, but began to increase significantly at more positive potentials. The slow development of a large outward current at $-10$ mV which was not present at $-30$ mV indicates the appearance of delayed rectification. The increasing magnitude of inward-going tails at the end of steps at $-30$ to $-10$ mV may be attributed to a steady-state component of $I_{st}$ (i.e., $I_{st} = f_{o}d_{o}I_{st}$), whose activation increases (inactivation decreases) with more positive potentials (Bassingthwaighte and Reuter, 1972), or may reflect an increased leakage current as a result of myofibrillar contraction during the step.
Fig. 6 illustrates the voltage dependence of inward and outward currents over the range from -82 to +40 mV obtained from 19 aggregates. Peak $I_{Na}$ was maximal at about -20 mV ($-185 \mu A/cm^2$). In TTX, slow inward current (at 10 ms) was maximal near 0 mV and reached a mean value of $-20 \mu A/cm^2$. The threshold for activating $I_h$ was 20-30 mV positive to that for $I_{Na}$. The magnitude of $I_h$ was not corrected for outward-going current and therefore may be underestimated at positive potentials. Thus, the true reversal potential for $I_h$ may be more positive than the value shown. The reversal potential for $I_{Na}$ was calculated to be +38.5 mV on the basis of the intracellular concentration of Na⁺, measured in 7-d chick heart cell aggregates (McDonald and DeHaan, 1973).

**Figure 3.** Logarithmic plots of the exponential decay of fast and slow components of inward current in Fig. 2. Experimental values of total current (O) were obtained at 0.4-ms ($\tau_1$) or 4-ms ($\tau_2$) intervals, beginning just after each peak and were fit by eye (solid lines).

**Pharmacological Effects**

Fig. 7 illustrates the effects of TTX and D600 on action potentials recorded from 7-d heart cell aggregates. TTX in concentrations as low as $10^{-6}$ g/ml blocked spontaneous activity in 7-d aggregates. However, when action potentials were stimulated electrically in the presence of TTX, their maximum upstroke velocities were reduced from > 120 V/s (Fig. 7 B, trace 1) to < 20 V/s (trace 2). Note that the plateau phase of the action potential was not affected significantly (Fig. 7 A, trace 2). D600, at a concentration of $5 \times 10^{-7}$ g/ml, reduced the amplitude and duration of the plateau dramatically (Fig. 7 C, trace 2) without affecting spontaneous activity, the overshoot potential or upstroke velocity (Fig. 7 D, trace 2). However, the combination of TTX and D600 (not illustrated) blocked generation of the action potential, even with intense electrical stimulation. The effects of TTX could be reversed by changing the bathing medium, but this was not the case with
D600. A complete reversal of its action took place only gradually over a period of 1 h or more.

The effects of TTX and D600 on membrane currents were consistent with their influence on the action potential. Fast and slow sweeps of control currents generated

Figure 4. Voltage dependence of slow inward current decay kinetics. Time constants were calculated from measured values of total current in the presence (●) or absence (○) of TTX (1.25 × 10⁻⁶ g/ml) which were used in a regression analysis of the function $A + Be^{-ut}$. The solid line is drawn through the mean value at each potential.

Figure 5. Membrane currents during consecutive 400-ms steps in a 135-µm diameter aggregate. Holding potential, -60 mV; holding currents, 0 nA (A), -5 nA (B), -2 nA (C), -70 nA (D) and -75 nA (E). Horizontal scale: 100 ms. Vertical scale: 80 mV, 0.5 µA.
during a 400-ms step from −60 mV to 0 mV are illustrated in Figs. 8 A and 9 A. D600 (5 \times 10^{-7}\,\text{g/ml}) blocked the slow inward current (Fig. 8 B), reduced outward current activation and increased net outward, time-independent current (Fig. 9 B). The upward deflection which follows the fast inward transient (Fig. 8 B) is most likely part of the damped oscillation generated by capacitive coupling (see Methods; see also the oscillation following the offset of the step, Fig. 9 B). TTX (1.25 \times 10^{-6}\,\text{g/ml}) blocked the fast component of inward current but did not affect the slow component or outward-going current (Figs. 8 C and 9 C). In Figs. 8 D and 9 D, TTX (1.25 \times 10^{-6}\,\text{g/ml}) was added to an aggregate already affected by D600 (5 \times 10^{-7}\,\text{g/ml}). The combination of the two drugs blocked both components of inward current leaving only time-independent outward current (Fig. 9 D). Prolongation of the capacitive transient in TTX (Fig. 8 C) and in D600 + TTX (Fig. 8 D) may have resulted from increased membrane input resistance in the presence of these drugs, or may represent its true waveform, which had been shortened in the control experiment (Fig. 8 A) by the rapid onset of the fast inward current.
FIGURE 7. Action potentials recorded during continuous impalement of two untreated aggregates (traces 1) and after exposure (traces 2) to TTX (10^{-5} \text{g/ml}) (A) and (B), and to D600 (5 \times 10^{-7} \text{g/ml}) (C) and (D). Maximum upstroke velocities of the controls were 153 V/s (B, trace 1) and 137 V/s (D, trace 1). TTX slowed the upstroke velocity to 12 V/s (B, trace 2) 2 min after its addition. Note that the plateau was not affected significantly (A). D600 reduced the amplitude and duration of the plateau (C, trace 2) but did not affect the upstroke velocity (D, trace 2). Horizontal scale: 100 ms, (A); 40 ms, (C); 1 ms, (B) and (D). Vertical scale: 50 mV. Horizontal lines represent 0 mV.

FIGURE 8. Effects of drugs on membrane currents during voltage clamp steps to 0 mV. A, Control. B, D600 (5 \times 10^{-7} \text{g/ml}). C, TTX (1.25 \times 10^{-6} \text{g/ml}). D, D600 (5 \times 10^{-7} \text{g/ml}) and TTX (1.25 \times 10^{-6} \text{g/ml}). Holding potential, −60 mV; holding currents, 0 nA (A), −10 nA (B), −4 nA (C), and −30 nA (D). (A) and (C) were recorded from one aggregate, diameter 140 \mu\text{m}; (B) and (D) were from a different aggregate, diameter 151 \mu\text{m}. Horizontal scale: 10 ms. Vertical scale: 80 mV, 0.5 \mu\text{A}.
DISCUSSION

Voltage Control

The most severe limitation on the voltage clamp technique as applied to cardiac tissue has been lack of voltage control, i.e., spatial inhomogeneity (Johnson and Lieberman, 1971; Fozzard and Beeler, 1975). With an exploring intracellular electrode independent of the clamp feedback circuit, several investigators have demonstrated uncontrolled voltage changes in ostensibly "clamped" regions of the tissue, even when the command voltage electrode exhibited virtually perfect control (Beeler and Reuter, 1970a; New and Trautwein, 1972; McGuigan, 1974; Tarr and Tank, 1974; DeHemptinne, 1976). Nonetheless, technical improvements have been made in clamp circuitry and in the preparation itself to minimize deviations from voltage homogeneity and permit meaningful analyses of the $I_t(V)$ relations (New and Trautwein, 1972; DeHemptinne, 1976; Goldman and Morad, 1977).

The requirements for an optimal cardiac preparation appear to be a low intercellular resistance, a minimal ratio of length to diameter and an overall small size to minimize the total membrane to be controlled. Although the embryonic heart cell aggregates analyzed in the present study meet the criterion of overall small size (120–180 μm in diameter), the small diameter of individual myocytes, 10–12 μm (and therefore the large number of cells per aggregate) dictates a significant total membrane surface area and series resistance. Our results with an exploring voltage electrode have shown that such aggregates deviate from isopotentiality by $6.4 \pm 0.7$ mV (mean ± SE), or 12%, during the first 3-5 ms of clamp steps, when the fast transient inward current was maximally activated, and by < 2 mV (~ 3%) thereafter. These values are comparable with those measured in adult cardiac tissue during peak $I_{Na}$ (Fig. 6, New and
Trautwein, 1972; Fig. 7, DeHemptinne, 1976) and during \( I_d \) (Fig. 4, Reuter and Scholz, 1977). Haas et al., (1971) reported (but did not illustrate) deviations of only 2 mV during the flow of fast inward current. However, the “fast” inward currents they recorded were much slower (20-ms duration) than in other investigations (see Fig. 1, Haas et al., 1971). We have also seen maximal deviations of 2–3 mV during some clamp steps, but only when \( I_{Na} \) was slowed greatly would there be increased series resistance.

Potential gradients of several mV during the first 3–5 ms of a voltage clamp step would result in a spatial distribution of magnitudes and kinetics of recorded currents. For \( I_{Na} \) this spread of magnitudes would be greatest at the point of maximal negative slope conductance (about \(-40\) mV, Fig. 6). For example, a 10-mV spread about \(-40\) mV would yield a range of currents from \(-50\) to \(-145\) \( \mu \)A/cm². The total inward current recorded in the bath for a step to \(-40\) mV would be a weighted sum of values between these two extremes and would also include values generated (at other potentials) in other regions of the aggregate. The weighting function would be the spatial distribution of current emanating from a point source within the aggregate. It is probably safe to assume that the cells that would deviate most from the command potential would lie between the current source and the potential-sensing electrode (\( V_i \)). Because of the decay of potential radially from the point source, these cells would be more positive than \( V_i \) during a depolarizing clamp step. This is exactly what was observed with the exploring electrode (trace 1, Fig. 1 E). Thus, inward-going currents are likely to be overestimated in the negative-slope regions of our current-voltage relationships (Fig. 6) and underestimated in the positive-slope regions. Such a shift of these curves toward more negative values is also expected as a result of the direct effects of series resistance. Delayed outward currents would not be greatly affected inasmuch as spatial homogeneity was better than 97% after the first 5 ms of a voltage step.

**Series Resistance**

The fact that aggregates contain a large extracellular volume suggests that the intercellular cleft spaces may be in good electrical contact with the external bathing medium. The value of 20% extracellular space is similar to that for adult ventricle but larger than for cardiac Purkinje fiber preparations (Hellam and Studt, 1974). Moreover, embryonic heart cells lack a transverse tubular system (Hirakow and Gotoh, 1975), and the aggregates are not surrounded by an appreciable endothelial layer that adds a substantial component of access resistance to adult preparations (Attwell and Cohen, 1977).

Our mean value for the aggregate series resistance (17 k\( \Omega \)) is comparable to that reported by DeHemptinne (1976) for frog atrial trabeculae in a sucrose gap preparation. Other measurements on the same preparation have led to widely-varying values, from 2 k\( \Omega \) (Goldman and Morad, 1977) to 99 k\( \Omega \) (Connor et al., 1975) depending on the fiber diameter, node width, and other variables. In dog, cat, and cow ventricular trabeculae, values for series resistance as low as 0.3–0.6 k\( \Omega \) have been reported (Beeler and Reuter, 1970a; New and Trautwein, 1972; Reuter and Scholz, 1977).

Because the series resistance prevents measurement of potentials directly
across heart cell membranes, any demonstration of spatial homogeneity, including our own, must be viewed with caution. For example, a negligible difference in the intracellular potentials recorded from two different cells (\(V_1\) and \(V_2\)) within an aggregate might also be interpreted in terms of four different potentials which tend to cancel

\[
V_1 = V_{m1} + V_{s1} = V_{m2} + V_{s2} = V_2, \tag{2}
\]

where \(V_m\) is the voltage drop across the membrane and \(V_s\) is the drop across the series resistance. The actual membrane potentials would be equal only when \(R_s\) at the two points of measurement were equal, or negligibly small with respect to membrane resistance. The latter condition was probably very nearly approximated in our experiments at potentials near rest and in steady states near the plateau range (from \(-20\) to \(+20\) mV). At both voltage levels, \(R_m\) is high (Goldman and Morad, 1977; Noma and Irisawa, 1976; DeHaan and DeFelice, 1978). Even in the active state, since both electrodes usually penetrated cells deep within the aggregate and therefore "saw" approximately the same intercellular cleft resistance, \(V_{ml}\) would equal \(V_{m2}\) if \(V_1\) were equal to \(V_2\).

In the steady state (as \(t \to \infty\)), the current generated by a small voltage step can be expressed as

\[
I_m = \frac{V_c}{R_m + R_s}. \tag{3}
\]

The voltage drop across the membrane is

\[
V_m = I_mR_m. \tag{4}
\]

Substituting

\[
V_m = V_c \frac{R_m}{R_m + R_s}. \tag{5}
\]

Thus, the true membrane potential deviates from the command potential by the ratio of membrane resistance to total input resistance. When the membrane potential is near the resting level, \(R_m \gg R_s\) and \(V_m\) is nearly equal to \(V_c\). From the slope of the current-voltage relation for outward current at \(-60\) mV (Fig. 6), we estimate the specific input resistance at rest to be about 15 k\(\Omega\) \cdot cm\(^2\). The clamp steps at that potential could be made with very small holding currents (11.2 \pm 6.6 nA) which resulted in negligible leakage errors. This result agrees well with those from current pulse experiments in which the access resistance was referred to total cell surface area (DeHaan and Fozzard, 1975). In more recent experiments (Clay et al., 1978), currents of 0.1-1.0 nA were used to shift the membrane potential only 1-2 mV from known rest potentials; aggregate specific input resistance was measured at potentials between \(-66\) and \(-70\) mV to be \(18 \pm 2.5\) k\(\Omega\) \cdot cm\(^2\). Thus, near rest and for low frequency (i.e., steady state) conditions (Eq. 3), input resistance for a total cell surface area of \(7.8 \times 10^{-3}\) cm\(^2\) can be approximated by \((R_m + R_s) = 2-3 \times 10^8\) \(\Omega\). Since \(R_s\) (including bridges) was \(2.5 \times 10^8\) \(\Omega\) (see Results), \(R_m/(R_m + R_s) = 0.99\), and \(V_m\) differed from \(V_c\) by only 1\%.
In the active state, voltage-dependent sodium channels open and membrane resistance collapses. Using the positive slope of the $I_{Na}$ current-voltage relation at 0 mV (Fig. 6), we calculate a total apparent "controlled" resistance $(R_m + R_s)$ of $\sim 50$ kΩ. In this case, $R_m/(R_m + R_s) = 0.54$. That is, membrane potential calculated from Eq. 5 is only 54% of clamp potential during peak $I_{Na}$. Similar large "error factors" during the fast transient due to series resistance have been acknowledged by DeHemptinne (1976).

Because the voltage drop across $R_s$ is negative during the flow of inward current, an equal and opposite change is required across $R_m$ to maintain the recorded potential ($V_p$) constant (Ramón, et al., 1975). This implies that relatively small depolarizing command steps ($V_c$) are seen by the membrane as much larger ones, resulting in a significant magnitude of inward current. For example, given an error factor of 54%, a step from -60 mV to -20 mV would result in a voltage drop of -18.4 mV across $R_s$ and an excess of +18.4 mV across $R_m$. Thus, series resistance tends to increase the steepness of the negative resistance branch and shift the peak of our fast inward current-voltage relationship to the left, perhaps as much as 20 mV. Similar shifts have been predicted in computer simulations of multicellular preparations in voltage clamp (Ramón et al., 1975). Ideally, the reversal potential of $I_{Na}$ would not be affected since zero current requires no voltage drop across $R_s$; however, the presence of $I_n$ and outward currents at that potential negate this argument. The magnitude of the error will depend upon the magnitude of membrane conductance for these other currents (Attwell and Cohen, 1977). The curve for slow inward current would be shifted in the same direction, but to a much smaller extent because of the smaller current densities (Fig. 6; see also Connor et al., 1975). The delayed outward current-voltage relationship would be shifted to more positive values, but also would not be affected greatly at potentials more negative than +20 mV.

The changes in membrane currents during repeated clamp steps to the same potential (Methods), which suggested an increasing series resistance, are consistent with these ideas. An increase in $R_s$ would: (a) shift the inward current-voltage relationships to the left, thereby reducing the fast inward current at potentials positive to -20 mV (Fig. 6); (b) reduce the magnitude and kinetics of the capacitive transient, (Eq. 1); and (c) reduce the space constant and uniformity of potential. Similar effects have been reported in the squid giant axon as a result of uncompensated series resistance (Bezanilla et al., 1970), and have been predicted in calculations (Attwell and Cohen, 1977) and computer simulations (Ramón et al., 1975) of multicellular preparations.

The decline of damped current oscillations with repeated steps might also have been due to a progressive rise in $R_s$. For example, the oscillation following the fast inward transient in Fig. 1 B ($R_s = 16$ kΩ) may be compared with the smooth decay of $I_{Na}$ in Fig. 2 A where series resistance was increased ($R_s = 34$ kΩ). These results support the hypothesis that larger values of series resistance tend to stabilize voltage-clamped preparations (Connor et al., 1975; Poincressault et al., 1976). Finally, the increase in holding current which accompanied the changes described above might have resulted not only from a progressive decline in the resistance of a leakage pathway (about the current-passing electrode) possibly aggravated by contraction, but also from an increasing
demand for current to offset the voltage drop across a rising intracellular core series resistance. In heart tissue, core resistance due to decoupling of cells undoubtedly increases as the current driven through nexal junctions exceeds a certain level (New and Trautwein, 1972). Such a reduction or block of gap junction permeability could result from leakage of extracellular calcium into the cytoplasm. This ion has been shown to increase junctional resistance between cells (Rose and Loewenstein, 1975; DeMello, 1975; Rose et al., 1977).

**Current Magnitudes and Kinetics**

Because it was not technically feasible to correct completely for leakage currents, most of the values we obtained for inward and outward currents are probably overestimates. Although these magnitudes were corrected for leakage at the holding potential, they may still be in error since such currents likely varied with absolute potential and may have increased during the course of voltage steps. The latter would have been true particularly of outward currents which were measured at the end of 400-ms steps.

In contrast, other factors tended to reduce the magnitudes of these currents: (a) the slow decay of the capacitive transient affected $I_{Na}$; (b) the activation of outward currents affected $I_{n}$; and (c) the slow inactivation of $I_{n}$ affected outward currents. In addition, previous experiments (Nathan et al., 1976) suggest that $I_{Na}$ is only partially activated at a holding potential of $-60 \text{ mV}$. When membrane potential was varied with injected dc current, the maximum upstroke velocity of an action potential with maximum diastolic potential (MDP) of $-60 \text{ mV}$ was only 50% of its peak value at an MDP of $-92 \text{ mV}$. If the maximum rate of rise is taken as a qualitative measure of availability of fast inward current (Weidmann, 1955), the maximum current density recorded for $I_{Na}, -185 \mu A/cm^2$ (Fig. 6) obtained from a holding potential of $-60 \text{ mV}$, would be increased to $-370 \mu A/cm^2$ with steps from $-92 \text{ mV}$. These values are comparable to those obtained in adult cardiac muscle preparations where membrane surface areas have been estimated. Connor et al. (1975) recorded a peak $I_{Na}$ in frog atrial trabeculae (from a holding potential at rest, $-60$ to $-70 \text{ mV}$) of about $-100 \mu A/cm^2$, based upon electron micrographs of equivalent bundle cross sections and measurements of sucrose gap test node widths. Dudel and Rüdel (1970) obtained a maximum value of $-600 \mu A/cm^2$ (from a holding potential of $-100 \text{ mV}$) for cooled sheep Purkinje fibers, based upon estimates of total membrane surface area from their measurements of capacitive current and an assumed value of 12 $\mu F/cm^2$ for membrane specific capacitance.

Reuter (1973) and others (Trautwein et al., 1975) have shown that in sheep ventricular trabeculae, the slow inward current is negligibly inactivated (i.e., $f_n = 1.0$) at $-60 \text{ mV}$, and the threshold for measureable slow inward current is about $-35 \text{ mV}$. Our current-voltage relationship for $I_{n}$ (Fig. 6) is similar in this respect (see Fig. 7, Reuter, 1973). If we assume that $I_{n}$ in our preparation is also negligibly inactivated at the holding potential of $-60 \text{ mV}$, then this, in addition to the fact that delayed outward-going current was small (Fig. 2 B and Fig. 5) at the time of $I_{n}$ measurement (10 ms), suggests that the maximum value of $I_{n}$ (about $-25 \mu A/cm^2$) should be relatively accurate. Thus, the ratio of $I_{Na}/I_{n}$ is between 7:1 and 14:1, depending upon the holding potential ($-60 \text{ mV}$ or $-92 \text{ mV}$).
mV). New and Trautwein (1972) used a holding potential of −58 mV and observed a difference of ~ 10:1 for fast and slow inward currents in cat ventricular trabeculae. Chesnais et al. (1975) obtained a ratio between 8:1 and 14:1 for frog atrial trabeculae.

The kinetics of the fast inward current we recorded in embryonic heart cell aggregates, like those reported for adult cardiac muscle, were substantially slower than the analogous currents in nerve or skeletal muscle. The times at which \( I_{\text{Na}} \) and \( I_d \) reached their respective peaks (2.6 and 6.8 ms) as well as time constants for their decay (Fig. 3) are subject to error not only because of the slowly decaying capacitive transient and the influences of \( I_d \) on \( I_{\text{Na}} \), and outward current on \( I_{\text{Na}} \), but also due to artificial shortening from a lack of complete voltage control. The fact that the times to peak current did not vary with potential further supports the latter conclusion (Connor et al., 1975). Nevertheless, time constants for the decay of \( I_{\text{Na}} \) and \( I_d \) (Fig. 3) are comparable to those calculated by Rougier et al. (1969) for inactivation of inward currents in frog atrial trabeculae, 1.2 ms for \( I_{\text{Na}} \) and 12 ms for \( I_d \), both determined at ~ 0 mV.

The voltage dependence of time constants for slow inward current decay (Fig. 4) is similar to results obtained by New and Trautwein (1972), but their values ranged from ~ 60 ms at potentials between −25 and +10 mV to ~ 150 ms at −35 and at +40 mV. Our time constants at potentials more positive than −10 mV may have been reduced by the delayed increase in outward current at these potentials (Fig. 5 E). In addition, the values at potentials negative to −20 mV may also be in error if activation of \( I_d \) is slow and inactivation fast (Trautwein et al., 1975). These investigators have corrected their original curve (Trautwein et al., 1975) based upon the separation of \( I_d \) kinetics into activation and inactivation variables (Bassingthwaighte and Reuter, 1972). The less rapid decay of slow inward current at potentials positive to −20 mV (Fig. 4) is in qualitative agreement with results obtained from dog (Beeler and Reuter, 1970 b) and cow ventricular trabeculae (Reuter and Scholz, 1977).

**Current-Voltage Relationships**

Despite differences in current magnitudes, the shapes and thresholds of our current-voltage relationships (Fig. 6) agree relatively well with most of those determined for adult cardiac muscle (Rougier et al., 1969; Ochi, 1970; New and Trautwein, 1972; Noma and Irisawa, 1976). Nevertheless, our calculations of the effects of series resistance on \( I_{\text{Na}} \) suggest that the actual curve should be shifted to more positive potentials, possibly by as much as 20 mV. Connor et al. (1975) have demonstrated such a shift after decreasing the voltage drop across \( R_s \) by reducing the amplitude of the fast transient with TTX. Measurements from fast sweep traces of aggregate action potentials (Fig. 7 B, D), recorded just before or after voltage clamp steps, indicate that the voltage at which \( dV/dt \) of the upstroke is maximal lies between −8 and −13 mV. This agrees well with records from the dog Purkinje fiber (Draper and Weidmann, 1951) and with the reconstructed Purkinje fiber action potential (McAllister et al., 1975). To the extent that the aggregate action potential approaches a true simultaneous membrane potential (DeHaan and Fozzard, 1975), the voltage of \( dV/dt_{\text{max}} \) will roughly approximate the potential at which peak \( I_{\text{Na}} \) is achieved on the \( I_{\text{Na}} \) (V)
curve. This is another reason for suggesting that the actual value of peak $I_{Na}$ should be somewhere between the recorded value ($-20$ mV, Fig. 6) and the membrane potential value ($-8$ to $-13$ mV).

Our estimates of the magnitudes and voltage dependence of $I_{sl}$ are probably fairly accurate as represented (Fig. 6). Deviation from voltage homogeneity during this slow event was $<2$ mV, and the magnitude of this current, and therefore, the voltage drop across $R_s$ was small. Thus, the existence of a slow inward current in embryonic heart cell aggregates which is independent of $I_{Na}$ cannot be questioned on the basis of distortions of fast current due to the lack of control (Johnson and Lieberman, 1971). The following observations lend further support to this argument: (a) The threshold for activation of $I_{sl}$ was about $-35$ mV whereas that for $I_{Na}$ was $-55$ mV. The uncertainty in the true membrane potential for these low current densities was $<10$ mV. (b) The kinetics of the two currents differed by a factor between 10 and 30. (c) TTX selectively blocked the fast component of inward current but not the slow component (Fig. 8 C). (d) D600 blocked the slow component but not the fast component (Fig. 8 B). Interestingly, Lieberman et al. (1975) recorded only a single component of inward current from synthetic strands of cardiac tissue which were prepared from 11- to 13-d embryonic chick hearts. This current had a duration of about 100 ms and a voltage dependence (Fig. 4, Lieberman et al., 1975) similar to $I_{sl}$ in our 7-d aggregates. The apparent lack of a fast sodium transient in their preparation is not readily explainable.

Pharmacological Effects

Our results with TTX and D600 (Fig. 7) confirm those of McDonald and Sachs (1975) who recorded action potentials in aggregates similar to ours. The effects of these drugs upon membrane inward currents (Figs. 8 and 9) are also consistent with findings on adult cardiac tissue (Rougier et al., 1969; Besseau and Gargouil, 1969; Kohlhardt et al., 1972; Kass and Tsien, 1975). In addition, the absence of delayed rectification in embryonic chick ventricular aggregates exposed to D600 and TTX (Fig. 9 D) agrees with results obtained in adult rat ventricular tissue (Besseau and Gargouil, 1969). Thus, our data suggest that electrogenesis, in 7-d embryonic chick ventricle as in adult heart tissue, is based upon the sequence of a TTX-sensitive, fast transient inward current followed by a slower phase of inward current and a delayed outward current.

Summary

We have shown that with the present experimental design, spheroidal aggregates of 7-d chick ventricle cells deviate from isopotentiality, on the average, by $\sim 12\%$ during the first 3-5 ms of a voltage clamp step but approach a relatively high degree of control thereafter. Two components of inward current, and a delayed outward current, with kinetic and pharmacologic properties similar to those observed in adult cardiac tissue were recorded. With a $12\%$ mean spatial distribution of membrane potentials and up to $50\%$ error in the true membrane potential during the peak fast inward current due to series resistance, the actual current-voltage relationship for the fast transient would be shifted as much as
20 mV toward more positive potentials. The curves for slow inward current and for outward current are relatively accurate as illustrated.

This study confirms that spheroidal aggregates of embryonic heart cells can be used for further analyses of developmental changes in pacemaker properties and other relatively slow conductance mechanisms (Nathan and DeHaan, 1978); however, additional technical improvements will be necessary before a more accurate description of $I_{Na}$ can be obtained.

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