The Initial Inward Current in Spherical Clusters of Chick Embryonic Heart Cells

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ABSTRACT The rapid inward sodium current in spherical clusters of 11-d-old embryonic chick heart cells, ranging in size between 65 and 90 μm diameter, was studied using the two-microelectrode voltage-clamp technique. Using these preparations, it was possible to resolve the activation phase of the rapid inward current for potentials negative to -25 mV at 37°C. The rapid inward current exhibited a voltage and time dependence similar to that observed in other excitable tissues. It was initiated at potential steps more positive than -45 mV. The magnitude of the current reached its maximum value at a potential of ~ -20 mV. The measured reversal potential was that predicted by the Nernst equation for sodium ions. The falling phase of the current followed a single exponential time-course with a time constant of inactivation, τᵢ, ranging between 2.14 ms at -40 mV and 0.18 ms at -5 mV. The time constant of inactivation, τᵢ, determined by a single voltage-step protocol was compared to the time constant, τᵢ, determined by a double voltage-step protocol and no significant difference between the two constants of inactivation was found. Furthermore, the time constants of inactivation and reactivation at the same potential in the same preparation were similar. The results of this study demonstrate that the sodium current of heart cells recorded at 37°C can be described by Hodgkin-Huxley kinetics with speeds approximately four times faster than the squid giant axon at 15°C.

INTRODUCTION

The voltage-clamp technique, introduced by Cole (1949) and used by Hodgkin and Huxley (as described in their now classic series of papers, 1952 a,b,c,d) to obtain a quantitative description of the voltage and time dependence of the membrane current in the squid giant axon, has been applied to many biological preparations. Extension of the voltage-clamp technique to multicellular tissues, typified by natural cardiac muscle, presents two problems that are not encountered in single cell preparations. The first is the need for low resistance connections between cells so that current can flow with negligible voltage gradients throughout the preparation. The second is that part of the extracellular space has a surface-to-volume ratio several hundred times that of intracellular space. This space, either in the form of narrow clefts between
tightly packed cells or a tortuous and complex transverse tubular system (cf. Sommer and Johnson, 1979), can promote local accumulation and depletion of ions as well as significant drops in extracellular potential during current flow. These two problems have made it impossible to achieve rapid spatial and temporal control of membrane potential in naturally occurring preparations of cardiac muscle and have seriously hindered studies of the sodium-carrying mechanism thought to underlie the generation of the depolarization phase of the action potential (Johnson and Lieberman, 1971; Fozzard and Beeler, 1975).

The aim of this paper is to analyze changes in sodium permeability associated with electrical excitability in small spherical clusters of embryonic chick cardiac cells in tissue culture using a two-intracellular microelectrode technique. The results presented here demonstrate the feasibility of studying the fast sodium current in this preparation and indicate that the sodium carrying mechanism has kinetic properties similar to those observed in nerve.

**METHODS**

**Preparation**

Clusters of cardiac muscle cells were obtained by modifying the culture techniques used to prepare linear arrays of heart cells (Lieberman et al., 1972). The hearts from 11-d-old chick embryos were dissected free in a laminar flow hood (Contamination Control, Inc., Kulpsville, Pa.), minced, rinsed, and gently agitated at 37°C in 10 ml of Ca-Mg-free Hank's balanced salt solution containing 0.5% trypsin. After 8 min, the supernatant fluid was discarded and prewarmed trypsin solution was added to the flask. The heart fragments were then subject to two 8-min cycles of trypsinization to dissociate the tissue completely. Freshly dissociated cells were added immediately to cold (0°C) culture medium and then filtered through multiple layers of gauze. The suspension was centrifuged at 322 g for 6 min and the supernate was discarded. The cell pellet was resuspended in cold culture medium and a 1-ml aliquot was placed in each of four 100-mm culture dishes containing 4 ml of culture medium and incubated at 37°C for 1 h. Cells contained in the muscle-enriched supernatant solutions were then counted and appropriately diluted to a concentration of 1 × 10^7 cells/ml. The cells were seeded in 3 ml of culture medium at known densities (1.0 - 1.5 × 10^6 cells/60-mm plate) on agar-coated culture dishes (3002, Falcon Plastics, Beeton, Dickinson, & Co., Rutherford, N.J.) in which triangular depressions were cut in the agar film by a 27 gauge needle. The cultures were incubated from 3 to 7 d at 37°C in a humidified chamber containing 4% CO₂ and 96% air. The culture medium was devoid of antibiotics and contained 55% Medium 199 (Grand Island Biological Co., Grand Island, N.Y.), 41% K-free Earle's balanced salt solution, 2% fetal calf serum (Grantee Diagnostics, Inc., Burlington, N.C.) and 1% chick embryo extract (Kaighn et al., 1966). The concentrations of Na⁺, K⁺, and Ca²⁺ ions were determined by a computerized sequential multiple analyzer (SMAC, Technicon Instruments Corp., Tarrytown, N.Y.) and the mean values were as follows (milliequivalent/liter): Na⁺, 145; K⁺, 3.5; Ca²⁺, 4.1. Osmolarity of the media was measured (model 5100 B, Wescor, Inc., Logan, Utah), and the mean value of all media was 277 mosM/kg H₂O.

Spontaneously active spherical clusters, ranging from 55 to 90 μm in diameter, were selected for study. Preliminary electron micrographs showed that the preparations consisted almost entirely of muscle cells, which in some cases were surrounded
by a single or double layer of nonmuscle cells.1 Numerous attempts to grow preparations that remained quiescent, using ventricular tissue from embryonic hearts of either 11-d or 16-d culture, were unsuccessful.

**Electrophysiological Recordings**

The medium was overlaid with light mineral oil (Klearol, Witco Chemical Co., New York) to prevent evaporation and the dishes were then transferred to the heated stage (37°C) of an inverted microscope (model M, Nikon, Inc., Instrument Div., Garden City, N.Y.). A gassing ring was positioned around the dish to provide a mixture of 5% CO₂/95% air in order to control the pH at approximately 7.4.

Intracellular microelectrodes (20 - 50 MΩ) were filled with 3 M potassium acetate and positioned at the surface of the preparation by Leitz micromanipulators (E. Leitz, Inc., Rockleigh, N.J.). Two agar-salt bridge macroelectrodes were positioned extracellularly at the periphery of the culture dish. The cells were then impaled using remotely controlled electromagnetic transducers (Chilson et al., 1978) that were mounted on the manipulators and to which the microelectrodes were attached. Fig. 1 shows the diagram of the voltage-clamp circuit. The intracellular and extracellular microelectrodes, M_i and M_o, filled with 3 M potassium acetate, were connected via Ag/AgCl wires to negative-capacitance-compensated headstage amplifiers (A_1 and A_2), which were used to record transmembrane voltage with the differential amplifier, A_3. The output of A_3 formed one input of the summing control amplifier, A_4, the other inputs of which received voltage command signals, V_c and V_e. The output of A_4 was further amplified by two amplifiers in cascade to give a maximum output to the current microelectrode of ± 25 V. Membrane current was measured by opampeter, A_5. Positive current feedback was used in some of the experiments by feeding a fraction of the output of A_4 to the summing junction of A_4 or that of the following amplifier (Hodgkin and Huxley 1952a; Katz and Schwartz, 1974).

The frequency response of the voltage control circuitry was limited by the following three factors depicted by the equivalent circuit diagram in Fig. 1: (a) the stray capacitance to ground of the input voltage circuitry, C_v; (b) the frequency response of the control amplifier, A_4, and subsequent stages; (c) the coupling capacitance, C_c, between the current and voltage electrodes which has a zero frequency of f_c = R_cR C_c.

The coupling capacitance, C_c, was largely eliminated by introducing an aluminum shield between the current and voltage electrodes that was driven by the unity gain output of the headstage voltage amplifier, A_1. In some later experiments, both the current and the intracellular voltage electrodes were shielded by painting them with conductive silver paint no. 5001 (Scientific Products, Inc., Westchester, Pa.) to within 100 μm of the tip and by covering the paint with an insulating layer of lacquer (Helena Rubenstein, New York). All of the cables leading to the current and voltage electrodes were also shielded. The shield of the cable to the current electrode together with its painted silver shield was grounded, whereas the corresponding shields of the voltage electrode were driven from the unity-gain output of the headstage amplifier, A_1. Painting the electrodes had the advantage of partially removing C_v as well as C_c.

The stray capacitance to ground, C_v, was compensated for by adjusting the negative capacitive feedback on the headstage amplifiers, A_1 and A_2.

It would seem that the ultimate limiting factor governing the stability of the voltage control system would be the finite propagation time between the point of injection of current and the point at which the voltage is measured (Kootsey and Johnson, 1972). In a spherical aggregate preparation such as the one used in the

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1 Adams, W. J., and M. Lieberman. Unpublished observations.
present studies, such a delay or lag would arise from the frequency dependence of the current source. At high frequencies (short times), when the membrane impedance is low, because of the finite resistance to the flow of current within the cytoplasm, current leaves the preparation close to the current source, rather than traverse intracellular space to leave the cell through more distant membrane. As a consequence, the membrane current density at high frequencies or short times is greater close to the current source than it is further away. At lower frequencies, however, the membrane current density might be quite uniform throughout the preparation, especially in a spherical cell or spheroidal aggregate (DeHaan and Fozzard, 1975), such as might be approximated by the cultured cluster. The result is that on injection of, e.g., a step of current, the membrane close to the current source charges, initially, more rapidly than at later times. In a spherical cell, therefore, the single exponential time-course of potential change, characteristic of an internally isopotential cell, is preceded close to the current source by a fast step-like jump in potential. Correspondingly, the membrane at more distant sites, e.g., diametrically opposite the source, charges at first

**Figure 1.** Voltage clamp circuitry. (A1 and A2) negative capacity microelectrode preamplifiers; (A3) unity gain differential amplifier; (A4) cascade of three operational amplifiers—first stage: differential amplifier with variable gain (1–10), second stage: operational amplifier with fixed gain of 10 and variable bandwidth, final stage: high-voltage operational amplifier with fixed gain of 10 and maximum output swing of ±25 V; (A5) unity gain operational amplifier for recording voltage on tip of the current microelectrode; (A6) operational amplifier with macroelectrode input for collecting current. (Inset) Equivalent circuit diagram showing locations of stray capacitances: $C_o$, the coupling capacitance between the current and voltage microelectrodes; $C_w$, the stray capacitance to ground of the input voltage circuitry; $C_l$, the capacitance across the current microelectrode and the bathing solution.
more slowly, that is to say, with a lag than at later times. If the intracellular voltage electrode were situated, for example, opposite the current source, then this initial lag causes the voltage control system to oscillate, unless the response time of the control amplifier is made long in comparison with the lag (Kootsey and Johnson, 1972).

Studies of the input impedance of the preparation\(^2\) show, however, that this frequency dependence of the current source is not in practice the limiting factor governing the stability of the voltage control system. Over the frequency range involved in the experiments presented here, the preparation behaves as a simple lumped resistance and capacitance in parallel, in series with a resistance (see Series Resistance in Results), stability being determined, apparently, by residual unneutralized stray capacitance between the voltage and current electrodes. It was found empirically that stability was improved when a "series resistance" was introduced by inserting the voltage electrode close to the current electrode and then compensating for this resistance with positive current feedback (see Series Resistance).

The frequency response of the current-monitoring circuit was tailored by the capacitor across the feedback resistor of \(A_0\), giving a resultant bandwidth of 10 K Hz. The opammeter, \(A_m\), measures all the current flowing into the bath, including current that does not pass through the preparation, namely the capacitive current through \(C_i\). This introduces error into the current measurement at high frequencies. An improved estimate of current flow can be obtained by using a grounded silver shield over the current microelectrode to within 100 \(\mu\)m of its tip. Using silver-coated microelectrodes, it was possible to show that in practice the current flow through \(C_i\) was negligible at frequencies of interest.

When necessary, the preparation was stimulated by rectangular current pulses (1 ms in duration) applied extracellularly through a bevel-edged glass microelectrode filled with 1.5% agar in Saline G and situated close to the preparation (Lieberman et al., 1973). The maximum rate of depolarization \((dV/dt)_{\text{max}}\) of the action potential was determined by electronic differentiation using an \(R-C\) circuit with a time constant of \(\sim 35\ \mu\)s.

Experimental Control and Data Collection

Because of the difficulty in maintaining impalements for the length of time needed to change command potential or times manually, the speed of data acquisition was increased by using a PDP 11/40 computer (Digital Equipment Corp., Marlboro, Mass.) as an on-line experimental control and data collection system. The analog signal from the output of the current monitoring circuit was sampled and digitized every 43 \(\mu\)s for the first 20 ms of each voltage-clamp step. This data was stored on magnetic disk (3M Company, St. Paul, Minn.). Sequences of voltage-clamp steps were applied to the summing junction of amplifier, \(A_n\), with the use of a D/A converter (LPS, Digital Equipment Corp.). This arrangement permitted the pulse sequences to be preprogrammed before the beginning of an experiment. The software for this system was written in macroassembler language to provide the speed necessary for real-time data acquisition and processing.

Experimental Protocol

After successfully penetrating the preparation with two microelectrodes, one for recording voltage and one for passing current, the voltage response of the system to a depolarizing command pulse was monitored on a storage oscilloscope and the gain, frequency response, positive current feedback, and capacitive feedback compensation

\(^2\) Mathias, R. T., L. Ebihara, M. Lieberman, and E. A. Johnson. Unpublished results.
of A1 (less so of A2) were adjusted until the best response was achieved, i.e., the establishment of a new membrane potential in as short a time as possible without overshoot or oscillation. The relay connecting the voltage-clamp electronics to the computer was then closed, triggering a series of preprogrammed command pulse sequences. The current and voltage records in response to these pulse sequences were recorded on oscillograph film (C-5, Grass Instrument Co., Quincy, Mass.). The current records were also stored on magnetic disk as described above. The data used here was obtained within the first 6 min after successful penetration with the microelectrodes, the first 3 min being used to adjust the parameters of the pulse sequence and the remainder to execute the complete series of pulse sequences. Each pulse sequence was separated from the next by 700 ms during which time the membrane potential was held at −60 mV, a potential at which the holding current was minimal.

Data Analysis
The data was smoothed using a three-point polynomial method to remove high frequency noise. Correction for leakage was made by fitting the traces to a flat or sloping base line by linear regression analysis and then by redrawing the curves relative to the fitted base line. Single exponentials were then fitted by linear regression analysis, whereas double exponentials were fitted by minimizing the least square error function using PRAXIS, an algorithm for finding zeros and extrema of functions without calculating derivatives (Brent, 1973).

The sodium current was separated from the total current by assuming that during times of interest any changes in membrane conductance that occurred involved only one ionic conductance, namely that of a fast sodium channel. In some experiments D600 was used to block time-dependent late currents.

Pharmacological Agents
Tetrodotoxin (Calbiochem, San Diego, Calif.) and compound D600-hydrochloride (Knoll, Ludwigshafen am Rhein, W. Germany) were dissolved in distilled water to make up stock solutions of 400 and 100 µg/ml, respectively, and administered via a lambda micropipette under mineral oil directly into the culture medium at the edge of the culture dish. Final concentrations of tetrodotoxin (TTX) and D600 in the culture dish were 3.2 × 10⁻⁵ g/ml and 1.25 µg/ml, respectively.

RESULTS
After impalement of two microelectrodes, all preparations ceased beating spontaneously, at least momentarily. Some preparations remained quiescent with a stable resting potential of between −20 and −40 mV. In others, spontaneous activity returned, action potentials being recorded for periods of up to several hours. Experiments were terminated prematurely by loss of one or both impalements, usually as a consequence of large depolarizing voltage-clamp steps.

Fig. 2 illustrates a typical cardiac action potential recorded from a cultured cluster of 75 µm in diameter. The rapid phase of depolarization reached a maximum value of +20 mV and had a maximum rate of depolarization of ca. 130 V/s. This phase was followed by one of prolonged repolarization with a plateau of 50-100 ms duration terminated by a relatively rapid repolarization to a maximum diastolic potential of between −75 and −80 mV.
Capacitive Transient

Fig. 3 shows the family of membrane currents recorded during a sequence of depolarizing and hyperpolarizing voltage-clamp steps. For small depolarizing and hyperpolarizing pulses, the current decayed from an initial maximum as a single exponential with a time constant between 50 and 150 μs. The voltage step rose to 90% of its final value within 50-80 μs. Total input capacitance of the preparation was estimated by integrating the area under this capacitive transient, yielding values that ranged between 1 and 2 nF. Assuming a value of 1 μF cm⁻² for the specific membrane capacitance, the preparations had an average surface membrane area equal to that of a smooth spherical cell of ~ 200 μm in diameter. Total capacitance can also be estimated by knowing the diameter and general morphology of the preparation. The preparations used in our experiments had an average diameter of ~ 75 μm. Electron microscope studies of cultured clusters of cardiac muscle have shown that the cell volume to surface area ratio is 1.28 × 10⁻⁴ cm and that ~ 15% of the preparation consists of extracellular space.¹ Using these values and neglecting the contribution of nonmuscle cells, we calculated an average surface membrane area of 1.47 × 10⁻³ cm² and a total membrane capacitance of 1.47 nF. These values are consistent with those obtained by integrating the area under the capacitive transient.

Series Resistance

The majority of the series resistance arises from the point source effect of the current microelectrode. This resistance is independent of frequency but does
depend on the radial position of the current and voltage electrodes, their angular separation, and on the internal resistivity. The differential equations describing the potential distribution induced within a spherical multicellular syncytium by injecting a sinusoidal current through an intracellular microelectrode were derived by Eisenberg et al. (1979). In their solution the intracellular potential is described by the equation:

\[ V_i(r, R, \theta; j\omega) = V_i^{(0)}(j\omega) + \epsilon V_i^{(1)}(r, R, \theta; j\omega), \]

where \( \epsilon \) equals \( R_i/(R_i + R_e) \), \( r \) is the radial coordinate at which potential is measured, \( R \) is the radial coordinate of the source, and \( \theta \) is the angular separation of source point and observation point.

The first term of this equation describes an "isopotential cell" in which there are no current or voltage gradients. The second term describes the voltage gradients that result from the three-dimensional spread of current away from the point source, as well as those resulting from the time (frequency)-dependent variation in membrane current density that are associated with an off-center source (see earlier discussion of the frequency dependency of the current source).

Noise analysis of the input impedance of preparations similar to those used in the present study shows that at frequencies corresponding to the times of our voltage-clamp analysis, the frequency dependence of the second term is negligible. The preparation behaves electrically as a single \( R \) and \( C \) in parallel, i.e., there is no spatial variation in transmembrane potential. The only variation in potential is within the cytoplasm as the voltage electrode approaches the current source. This variation in potential is described by a series resistance, the magnitude of which increases as the voltage electrode approaches the source. In our preparations, when the two electrodes were distant from one another, the resistance became negligible. Moreover, as the size of the preparation decreases, the magnitude of the current needed to produce a given displacement in transmembrane potential also decreases. As a consequence, the second term becomes smaller relative to that of the first term, resulting in a reduction of the effective series resistance. With the two electrodes close to one another (10 \( \mu \)m at the surface of the preparation), our estimates of the series resistance from the clamp current transient by the method of Hodgkin and Huxley (1952a) or from the instantaneous jump in voltage under constant current conditions (Beeler and Reuter, 1970) increased markedly with the size of the preparation. Preparations 60–80 \( \mu \)m in diameter had series resistances ranging between 30 and 200 k\( \Omega \) as calculated from the transient. Only those preparations having a measured value of series resistance less than 100 k\( \Omega \) were selected for study. For currents <20 nA, this would result in a maximum voltage deviation of <2 mV. For currents >20 nA, compensated feedback was used. In the present study, it was impossible to achieve adequate voltage control in preparations >100 \( \mu \)m in diameter. Similar findings have been reported by Nathan and DeHaan (1979).

For the case of a multicellular aggregate, in which the total membrane area in the preparation is divided into two components, one facing bulk extracel-
lular space, the other facing narrow clefts separating the membrane of closely apposed cells, there will be, in addition to the lumped series resistance, $R_s$, a distributed series resistance term, $R_e$ which represents the resistance of such clefts. From the impedance analyses, the value of this series resistance was negligible, in preparations similar to those analyzed here, a preparation behaving as a single, lumped, parallel $R-C$ network, up to at least 3 kHz.

**Inward Sodium Current**

A transient inward current was initiated at potential steps more positive than $-45 \text{ mV}$. The magnitude of this current reached its maximum value at a potential of about $-20 \text{ mV}$, as illustrated in Fig. 3. The current reversed in sign, becoming outward at a potential of about $+30 \text{ mV}$.

![Figure 3](image)

**Figure 3.** Oscillographic record of current and voltage traces recorded during a series of depolarizing and hyperpolarizing voltage-clamp steps. Holding potential was $-60 \text{ mV}$ and the holding current was $-5 \text{ nA}$. Temperature = $37^\circ \text{C}$. Calibration = 50 nA, 20 mV, 200 μs.

Peak currents from a typical family of current and voltage records are plotted in Fig. 4 with and without correction for leakage. Inasmuch as the capacitive transient in this preparation decayed with an estimated time constant of 50 μs, the capacitive component should have little or no effect on the magnitude of the peak current and was neglected in the analysis. The current displayed a voltage dependence similar to that found in other excitable membranes (Cole, 1968). The curve in the positive slope region is linear at least up to the reversal potential. The curve in the negative slope region is not too steep, having several data points, indicating adequate control of membrane potential. The reversal potential obtained from this analysis is in agreement with the reversal potential calculated from the Nernst equation using the known internal (40 mM) and external (145 mM) sodium concentrations.
for this preparation,\(^3\,^4\) indicating that sodium was the principal charge carrier of this current. At potentials positive to the reversal potential, the current turned on too rapidly for the peak current to be resolved from the capacitive transient.

The falling or inactivation phase of the sodium current followed a single

\[^3\] Horres, C. R., J. F. Aiton, and M. Lieberman. Unpublished data.

\[^4\] Values reported for intracellular sodium concentration in chick embryo heart and tissue cultured cells were 40 and 34 mM, respectively (MacDonald and DeHaan, 1973).
exponential time-course, as shown in Fig. 5. A second slow inward component was observed for depolarizing voltage-clamp steps more positive than \(-20\) mV. This secondary component was typically at least an order of magnitude smaller than the peak inward sodium current and declined by about 5–10 nA over a 15-ms time interval. In large preparations with poor spatial and temporal control of membrane potential, the time constant of inactivation, \(\tau_h\) appeared to be almost independent of voltage. When the spatial nonuniformity within the preparation was reduced to an acceptable level by using only small preparations, 50–80 \(\mu\)m in diameter, the resistance in series with the membrane was greatly reduced, as was the magnitude of the inward current, the time constant, \(\tau_h\), becoming steeply voltage dependent as shown in Fig. 6. At \(-40\) mV the time constant was 2.14 ms, whereas at \(-5\) mV the time constant was 0.18 ms.

The effect of a conditioning prepulse (300 ms duration) on the peak magnitude of the sodium current in response to a subsequent test pulse is illustrated in Fig. 7. The membrane potential was stepped to a fixed value of \(-20\) mV, after a 300-ms conditioning prepulse to voltages over the range of \(-100\) to \(-40\) mV. The peak current during the test pulse was normalized with respect to the maximum peak current and plotted against voltage during the conditioning prepulse. The peak current displayed a sigmoidal voltage dependence similar to the steady-state inactivation process, \(h_a\), seen in squid giant axon and other excitable cells (Cole, 1968). The current saturated at potentials more negative than \(-90\) mV and appeared to be completely inactivated at potentials more positive than \(-50\) mV. It can be fitted to the function \(h_a = 1/(1 + \exp([V - V_h]/K_h))\). In a total of six experiments, mean values of \(K_h\) and \(V_h\) of 6.3 and \(-69\) mV were calculated, respectively.

The time-course of activation was too fast to be accurately separated from the initial capacitive transient for potentials more positive than \(-10\) mV.
Instantaneous I-V Relationships

We attempted to determine the instantaneous I-V relationship by repolarizing the membrane at the peak of the inward sodium current. This approach was not pursued because the time constant of activation and the time constant of the capacitive transient were of the same order of magnitude, thus making it impossible to accurately separate the two currents.

Time-Course of Inactivation as Determined by Double-Pulse Experiments

In these experiments, the duration of a conditioning prepulse was varied while the potential of a subsequent test pulse was held constant at -20 mV. The peak current during the test pulse, \( I_p \), was plotted as a function of the duration of the conditioning pulse for potentials ranging from -80 to -30 mV, as shown in Fig. 8 a. The peak current inactivated with an approximately exponential time-course. Fig. 8 b summarizes the results obtained from 11 experiments in which the time constant of 31.5 ms at -55 mV is shown to decrease to 2.4 ms at -40 mV.

Over the voltage range between -40 and -25 mV, the time constants of inactivation as determined by the one-step and the two-step method could both be reliably determined (Fig. 9). Within this potential range, no significant difference between two time constants of inactivation, \( \tau_a \) and \( \tau_c \), was observed.
Time-Course of Reactivation

The time-course of removal of inactivation was studied by means of two moderate-sized depolarizing pulses applied in succession. The duration of the first pulse was long enough to inactivate the sodium current completely. The pulses were separated by a step to a less depolarized potential for a variable interval of time. Over a narrow range of voltages between -55 and -70 mV bounded by 0.2 < \( h_\infty \) < 0.8, it was possible to study the time-course of reactivation and inactivation simultaneously at the same potential in the same preparation. In these experiments, the preparation was held at its normal resting potential (-60 mV) between pulse sequences in order to minimize the holding current. The first pulse sequence consisted of a depolarizing step to -20 mV for 65 ms to inactivate the sodium current completely. This pulse was then followed by a hyperpolarizing pulse of variable duration to a conditioning potential, \( V_c \), ranging from -55 to -70 mV. This pulse was, in turn, followed by a fixed test pulse to -20 mV. A 300-ms hyperpolarization to -75 mV was applied before the second pulse sequence in order to reactivate the sodium conductance. The remainder of the pulse sequence consisted of the conditioning prepulse, \( V_c \), and test pulse, \( V_t \) with values identical to those used in the first pulse sequence. In these experiments, the time constants of reactivation were similar to the inactivation time constants obtained at the same potential, \( V_c \).

Figure 7. Steady-state inactivation curve \( h_\infty (V) \) of the sodium current. Abscissa: conditioning prepulse potential; ordinate: peak inward current (normalized with respect to the maximum peak current) during the test pulse. Sequence of membrane potential changes shown in inset. Holding potential was -60 mV. Temperature = 37°C.
The results of a typical experiment are plotted in Fig. 10 for a conditioning prepulse potential, $V_c$, of $-60$ mV. The peak inward current at short times (0–5 ms), when compared with values obtained at long times (5–100 ms),

![Graph showing current as a function of time](image)

**Figure 8.** (a) $I_{Na}$ as a function of duration of conditioning pulse. Sequence of membrane potential changes shown in inset. Abscissa: duration of prepulse potential; ordinate: peak inward current during test pulse to $-20$ mV. (b) Exponential time constant, $\tau_e$, as a function of membrane potential. $\tau_e$ was determined from least squares fit of data obtained from 11 preparations using the experimental method shown in Fig. 8 A. Holding potential was $-60$ mV. Temperature $= 37^\circ$C.

differs less than would be expected for a single exponential decay of current. A similar lag in inactivation of the sodium channel has been reported in other types of preparations, such as the squid giant axon (Bezanilla and Armstrong,
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1977), and it has been suggested that the activation and inactivation gates may be coupled in some way (Hoyt, 1968) rather than being completely independent as in the Hodgkin and Huxley formulation. However, no conclusions can be drawn from the present set of experiments because this delay in inactivation was not consistently seen in all preparations.

The time-course of reactivation was also determined by using \( (dV/dt)_{\text{max}} \) as a measure of the sodium current (Wiedmann, 1955). Inasmuch as the stimulus artifact was superimposable with the upstroke of the action potential recorded from the small spherical cluster, this measurement was made using the

![Graph showing time constants of inactivation](image)

**Figure 9.** Time constants of inactivation as obtained by a one-step and a two-step method plotted as a function of membrane potential. (+) Time constant of inactivation, \( \tau_a \), using a single-pulse arrangement obtained from four preparations. (□) Time constant of inactivation, \( \tau_e \), using a double-pulse arrangement. \( \tau_e \) was obtained from least squares analysis of data from two preparations. Holding potential was -60 mV. Temperature = 37°C.

synthetic strand (Lieberman et al., 1975). In Fig. 11, premature action potentials were initiated at similar values of membrane potential, but at progressively smaller test intervals where the test interval is defined here as the time interval between the upstroke of the conditioning action potential and the upstroke of the test action potential. The maximum velocity of rise of the upstroke of the premature action potential was plotted as a function of test interval in the inset in Fig. 11. The curve follows an approximately exponential time-course with a time constant of 21 ms at a resting membrane potential of -70 mV. This value agrees with the value of \( \tau_R \) determined from the voltage-clamp data and is consistent with data recently reported from intact embryonic chick hearts (Iijima and Pappano, 1979).
**Pharmacologic Interventions**

Because of the difficulties associated with changes in ionic composition of the culture medium without dislodging the preparation or disturbing the impale-

![Figure 10](image-url)  
**Figure 10.** Comparison of time-course of inactivation and reactivation as obtained simultaneously in the same preparation at a conditioning prepulse potential of -60 mV. $\tau_R = 47$ ms; $\tau_c = 33$ ms. Abscissa: duration of prepulse; ordinate: peak inward current during test pulse to -20 mV. Holding potential was -60 mV. Temperature = 37$^o$C.

![Figure 11](image-url)  
**Figure 11.** Time and voltage dependence of $(dV/dt)_{max}$ of a premature action potential recorded from a synthetic strand. Conditioning and test action potentials recorded at progressively decreasing test intervals as shown together with the corresponding differentiated upstrokes. Resting membrane potential was -67 mV. Temperature = 37$^o$C. Inset: $(dV/dt)_{max}$ plotted as a function of test interval.

ments, we decided to test whether the preparation would be responsive to compounds, such as TTX and D600, known to affect the inward currents. Results obtained from these preparations are shown in Fig. 12 and are
basically in accord with those findings described by Nathan and DeHaan (1979). In essence, tetrodotoxin (3.2 × 10^{-5} g/ml) markedly reduced, but did not totally abolish the inward current; the action potentials, albeit with a much reduced rate of depolarization (>100 V/s reduced to ~1 V/s) could still be initiated in the presence of TTX (Fig. 12 B). The effect of D600 (1.25 μg/ml) was to accelerate repolarization without affecting the maximum rate of rise of the action potential, as illustrated in Fig. 12 C. After 10–15 min, the preparations stopped beating spontaneously.

The results of voltage-clamp experiments were consistent with the action potential data. TTX suppressed the initial inward transient without affecting the slow component as illustrated in Fig. 13 A. D600 blocked the slow inward current and the delayed outward current but did not suppress the rapid inward current (Fig. 13 B). It should be noted that these experiments were conducted in preparations >100 μm in diameter in order to obtain a measurable slow current. A combination of D600 and TTX abolished all of the time-dependent currents leaving a time-independent outward current which is a linear function of membrane potential.

These findings indicate the existence of a clearly separable slow inward current similar to that reported in other cardiac preparations. The properties of this current will be the subject of a separate investigation.

DISCUSSION

This study demonstrates the feasibility of using standard microelectrode voltage-clamp techniques to analyze the early inward current in small spher-
ical clusters of embryonic chick cardiac muscle cells. The results show that the kinetic properties of this current and its response to tetrodotoxin are similar to those of the sodium current in other excitable tissues such as the squid giant axon (Cole, 1968). Its measured reversal potential agreed with the value calculated from the Nernst equation using known intra- and extracellular sodium concentrations. When the time-course of inactivation of this current was studied by one-step and two-step pulse sequences, no significant difference between the two time constants, $\tau_h$ and $\tau_c$, was found. Our results also show that over the voltage range of $-55$ to $-70$ mV, the time constant of reactivation, $\tau_R$, is similar to the time constant of inactivation, $\tau_c$.

These results are consistent with Hodgkin-Huxley kinetics in which $g_{Na}$ is assumed to be equal to the product of $h$ and some power of $m$, where $h$ and $m$ are each independent first order variables. The results are in sharp contrast to those obtained from frog atrial muscle by Haas et al. (1971) using the sucrose gap technique in which the ratio between the time constants of inactivation, $\tau_h$, and reactivation at the same potential was approximately 1:50. This discrepancy can be explained by the severe limitations inherent in the sucrose gap technique which lead to distortions in the time-course of the rapid inward current during depolarizing voltage-clamp steps (Tarr and Trank, 1974; Reuter, 1979). Gettes and Reuter (1974) came to the same conclusion as Haas et al. (1970) on the basis of reactivation experiments using the maximum velocity of the action potential as an index of the sodium current, but no attempt was made to compare inactivation and reactivation times at the same potential in this study.

Analysis of the kinetic properties of the sodium current is often complicated by a resistance in series with the membrane. Series resistance determines the charging time constant of the membrane capacitance, and it also causes distortion of the time course of the inward current. Computer simulations of the inward transient with and without series resistance, as computed using the Hodgkin and Huxley equations, show that one effect of series resistance is to shorten the time constant of inactivation as determined from the falling phase of the inward current. For large values of series resistance, the transmembrane potential may approach that of an unclamped action potential (Ramon et al., 1975). Another sign pathognomonic of space clamp failure is the appearance of a “notch” on the early inward current trace in the negative resistance region of the peak current-voltage relationship. This notch has been systematically investigated by Taylor et al. (1960) in the squid giant axon. They found that notches were always associated with nonconformities of the potential and current distributions. However, even in current records with no obvious notching, the membrane potential was not necessarily uniform.

It was possible to reduce the series resistance by using small preparations 50–80 $\mu$m in diameter and by partially compensating for the remaining series resistance electronically (Hodgkin and Huxley, 1952 a). Under optimal conditions, the temporal resolution of the voltage-clamp system was fast enough to resolve the activation time-course of the sodium current for potentials negative to $-25$ mV at 37°C. As the series resistance was reduced, the time constant of inactivation, $\tau_h$, tended to converge with the time constant, $\tau_c$, obtained with a two-pulse arrangement.
Our findings are in basic agreement with those recently obtained from rabbit Purkinje fibers (Colatsky and Tsien, 1979) and single adult rat heart muscle cells (Lee et al., 1979). One advantage of our approach is that we are able to study the sodium current at 37°C in a preparation of cardiac cells which has fully recovered from enzymatic treatment, is free from pharmacological modification, and is contained in a standard culture medium. In contrast, by using preparations similar to but larger than those used in the present study, Lieberman et al. (1976) and Nathan and DeHaan (1979) were unable to voltage-clamp adequately the rapid sodium current. The success of the present experiments can be largely attributed to the smaller size of our preparations, to scrupulous shielding of the current and voltage microelectrodes to eliminate interelectrode coupling capacitance, and to positive current feedback.

Our findings are consistent with the idea that cardiac muscle and the squid giant axon share the same kind of sodium permeability mechanism.

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