Kinetic and Steady-State Properties of Na\(^+\) Channel and Ca\(^{2+}\) Channel Charge Movements in Ventricular Myocytes of Embryonic Chick Heart

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ABSTRACT Nonlinear or asymmetric charge movement was recorded from single ventricular myocytes cultured from 17-d-old embryonic chick hearts using the whole-cell patch clamp method. The myocytes were exposed to the appropriate intracellular and extracellular solutions designed to block Na\(^+\), Ca\(^{2+}\), and K\(^+\) ionic currents. The linear components of the capacity and leakage currents during test voltage steps were eliminated by adding summed, hyperpolarizing control step currents. Upon depolarization from negative holding potentials the nonlinear charge movement was composed of two distinct and separable kinetic components. An early rapidly decaying component (decay time constant range: 0.12–0.50 ms) was significant at test potentials positive to -70 mV and displayed saturation above 0 mV (midpoint -35 mV; apparent valence 1.6 e\(^-\)). The early ON charge was partially immobilized during brief (5 ms) depolarizing test steps and was more completely immobilized by the application of less negative holding potentials. A second slower-decaying component (decay time constant range: 0.88–3.7 ms) was activated at test potentials positive to -60 mV and showed saturation above +20 mV (midpoint -13 mV, apparent valence 1.9 e\(^-\)). The second component of charge movement was immobilized by long duration (5 s) holding potentials, applied over a more positive voltage range than those that reduced the early component. The voltage dependencies for activation and inactivation of the Na\(^+\) and Ca\(^{2+}\) ionic currents were determined for myocytes in which these currents were not blocked. There was a positive correlation between the voltage dependence of activation and inactivation of the Na\(^+\) and Ca\(^{2+}\) ionic currents and the activation and immobilization of the fast and slow components of charge movement. These complementary kinetic and steady-state properties lead to the conclusion that the two components of charge movement are associated with the voltage-sensitive conformational changes that precede Na\(^+\) and Ca\(^{2+}\) channel openings.

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INTRODUCTION

Asymmetric or nonlinear intramembranous charge movement is widely believed to represent the initial voltage-dependent step(s) governing diverse processes, such as ion channel opening in excitable cell membranes (see Almers, 1978 and Armstrong, 1981 for reviews) and excitation–contraction (E-C) coupling in skeletal muscle (Schneider and Chandler, 1973). A great amount of detailed information concerning the properties of Na⁺ channel charge movement (gating current) was first obtained using the squid giant axon, where the voltage clamp method is most accurate (Armstrong and Bezanilla, 1973, 1974; Keynes and Rojas, 1974). A few studies have been performed to examine the properties of Ca²⁺ channel gating currents in neuronal cell bodies (Adams and Gage, 1979; Kostyuk, Krishtal, and Pidoplichko, 1981). In skeletal muscle cells, intensive efforts have demonstrated that the charge movement arises from a voltage sensor involved in E-C coupling (see Ríos and Pizarro, 1991 for an excellent review of the field).

Recently, several groups have begun to examine the role and properties of the nonlinear charge movement in neonatal (Field, Hill, and Lamb, 1988) and adult cardiac ventricular cells (Bean and Ríos, 1989; Hadley and Lederer, 1989, 1991). In these studies, the charge movement appeared to be correlated, at least in part, with the gating of “fast” Na⁺ and “slow” (L-type) Ca²⁺ channels. One of the fundamental differences between the fast Na current and the slow Ca current of cardiac muscle is that they display dissimilar activation kinetics. However, the identification and separation of cardiac Na⁺ and Ca²⁺ channel charge movements was previously based mainly on differential holding potentials (HPs) and not on their kinetic properties. In addition, the possibility that a significant fraction of the charge movement arose from the voltage sensors involved in E-C coupling may complicate the interpretation of those studies that employed adult ventricular myocytes.

A cultured embryonic ventricular myocyte preparation was, therefore, chosen for the present experiments in order to obtain a kinetic as well as a voltage-dependent separation of Na⁺ and Ca²⁺ channel charge movement (gating currents). This preparation offered several advantages over the adult myocyte preparation for cardiac charge movement experiments. First, the cultured avian embryonic myocytes have no transverse (T) tubules (Moses and Kasten, 1979). This eliminated the possibility that some fraction of the charge movement arose from an internal membrane source. In addition, the simplified membrane geometry improved the spatial and temporal homogeneity of the voltage clamp. The small size of the myocytes (~15 μm diameter) also contributed to a rapid charging of the membrane capacitance during the whole-cell patch voltage clamp. The improved kinetic resolution afforded by this preparation permitted experimentation at higher temperatures, instead of at 5–10°C, so that enzymatic regulation of channel gating could be studied (Josephson and Sperelakis, 1991a). In light of these advantages, the embryonic myocyte preparation was selected to separate and characterize the kinetic and steady-state properties of cardiac ventricular Na⁺ channel and Ca²⁺ channel gating currents.

A preliminary report of this work has been presented (Josephson and Sperelakis, 1991b).
METHODS

Cell Preparation

Single ventricular myocyte cultures were prepared from 17-d-old embryonic chick hearts by a method similar to that described previously (Josephson and Sperelakis, 1982). In brief, 2 dozen fertilized White Leghorn chick embryos were incubated for 17 d at 37.5°C and staged to confirm their degree of development. Hearts are sterilely removed and collected in a balanced salt solution (4°C). Tissue dissociation was accomplished by gentle rotation of the hearts in a Mg2+- and Ca2+-free Ringer solution containing 0.05% trypsin (Sigma Chemical Co., St. Louis, MO). The cell suspensions were harvested at 5-min intervals, pooled, pelleted by centrifugation (85 g), and washed three times. The cell pellet was resuspended in tissue culture medium (M199; Gibco Laboratories, Grand Island, NY) and plated at 10⁶-10⁷ cells/ml. The myocyte cultures were maintained at 37°C and pH 7.4 in a moist-air CO₂ incubator (for 24-72 h) until used for experimentation.

Electrical Recording

Single ventricular myocytes were voltage-clamped using the whole-cell configuration of the patch clamp technique (Hamill, Marty, Neher, Sakmann, and Sigworth, 1981). Electrodes were fabricated from thin wall borosilicate glass (TW-150; World Precision Instruments, Sarasota, FL) and filled with the following solution (mM): 120 CsOH, 120 glutamic acid, 2 MgCl₂, 0.2 Na₂GTP, 2 Na₂ATP, 10 EGTA, 10 HEPES buffer. The pH was adjusted with HEPES to 7.25. The extracellular solution contained (mM): 140 NaCl, 5.4 KCl, 1 MgCl₂, 1.8 CaCl₂, 10 glucose, 10 HEPES. The pH was adjusted to 7.4 with HEPES and Trizma base. The electrode resistances ranged from 2 to 5 MΩ when filled with the cesium solution. Junction potentials were nulled before seal formation and no further corrections were made during the experiment. The seal resistances ranged between 10 and 50 GΩ.

In experiments designed to examine nonlinear charge movement, all ionic currents were blocked. In addition to the internal Cs⁺ solution (to block the early outward and delayed outward K⁺ current), 1 mM Cs⁺ was added to the external solution (to block the inward rectifier K⁺ current). 10 μM tetrodotoxin (TTX) was added to block the fast Na⁺ current and 3 mM CoCl₂ was added to block the Ca²⁺ currents. The total extracellular divalent ion concentration was established before the experimental period, and remained constant throughout each experiment. In some experiments, either the Na⁺ or the Ca²⁺ current was not blocked completely so that both the charge movement and the ionic current could be recorded simultaneously. Lanthanum ion (La³⁺) was not used to block Ca²⁺ (Bean and Rios, 1989; Hadley and Lederer, 1989, 1991) because it has been reported to cause large and complex shifts in the voltage dependence of channel gating (Armstrong and Cota, 1990).

Membrane currents were recorded using an Axopatch IB patch clamp (Axon Instruments, Inc., Foster City, CA). Linear capacitive current, due to the charging and discharging of the cell membrane capacitance, was suppressed by analogue capacitance compensation. Careful attention to this procedure was necessary to prevent amplifier saturation due to the large capacitive current signals. Series resistance compensation was used in some experiments.

Data Acquisition and Analysis

Data acquisition and analysis were performed using the PCLAMP programs (Axon Instruments, Inc.) on an IBM AT computer. Membrane currents were filtered at a corner frequency of 10-20 kHz, amplified 10 times with an 8-pole Bessel filter (Frequency Devices Inc., Haverhill, MA), and digitized at 8-30 μs/point using a 12-bit A-D converter (Labmaster; Axon Instruments, Inc.). To remove the residual linear capacitive and leakage current components in the test
current, five scaled, hyperpolarizing control voltage steps (each one-fifth the magnitude of the corresponding test step) were given from control subtracting holding potentials (SHPs) of \(-100\) or \(-120\) mV (P/-5; see inset, Fig. 1), and the resulting summed control currents were added to the test currents. The control voltage region was chosen as a compromise between the need for a region of negligible nonlinear charge movement, and the inability of the myocytes to tolerate more negative potentials. It is estimated that <5% of the total nonlinear charge was moved during the largest control step from a SHP of \(-100\) mV (by comparing the charge movement obtained using more negative SHPs; e.g., \(-150\) mV). The linearity of the entire recording system was checked before experimentation using an RC cell model circuit, and the P/-5 protocol yielded a zero current trace over the range of \(\pm 200\) mV. The myocyte membrane capacity currents elicited during the control steps from a SHP of \(-120\) mV were linear in this voltage region; i.e., when the currents resulting from symmetrical depolarizing and hyperpolarizing steps were added together they yielded a zero current trace.

![Diagram](image)

**Fig. 1.** Time course of the linear capacity current. The inset at the top displays a diagram of the P/-5 voltage protocol (HP, holding potential; test, test voltage steps; SHP, subtracting holding potential applied for the control voltage steps). The currents from the five control steps were summed and added to the test step current to eliminate the linear capacitive and leakage currents. The test step was preceded by the desired HP (-120 to 0 mV for 2 or 5 s duration). A brief (1.2 ms) common potential preceded and followed the test step to allow a comparison of the currents from different HPs. The capacity currents \(I_c\) in response to a voltage step from \(-120\) to \(-140\) mV (negative current) and from \(-140\) to \(-120\) mV (positive current) are superimposed (digitally filtered at 20 kHz). The decay time constant was 32 \(\mu\)s. The charge integral \(Q\) of the capacity current demonstrates the time required to charge the membrane capacitance and to attain the step potential. \(Q\) was 142 fC; input capacitance was 7.1 pF. (Experiment 80805C01)

The time course and the cumulative charge integral \(Q\) of the linear capacity current are displayed in Fig. 1, and they demonstrate that the membrane charging time was rapid in this cardiac myocyte preparation. The rise time of a voltage step (as measured by the 90% decay time of the capacity transient) was between 100 and 200 \(\mu\)s (see Fig. 1). The time course for the decay phase of the hyperpolarizing linear capacity currents was fit by a single exponential; in four cells the time constants ranged from 32 to 75 \(\mu\)s.

The series resistance \(R_s\) estimated from the time constants for decay of the capacity currents and the cell input capacitance (range 5–8 pF) was 4–15 M\(\Omega\). The input capacitance (measured by integration of the linear capacity transient) agreed closely with the predicted input capacitance, assuming a spherical membrane surface area (average diameter 13.3 \(\pm\) 0.9 \(\mu\)m; \(n = 100\) cells) and a membrane capacitance of 1 \(\mu\)F/cm\(^2\).
A HP (ranging from -120 to 0 mV) of 2 or 5 s duration was applied, and ended 1–5 ms before the test step. Sequences of test potentials were repeated and the current signals at each potential were averaged 4–16 times to improve the signal-to-noise ratio. Series of runs were bracketed (i.e., the original protocol was repeated) to insure the stability of the currents over time, and the currents were not analyzed if a significant change (>5%) had occurred in either their magnitude or time course. Measurements of charge movement were made within several minutes of the conductance measurements after blockade of the ionic currents in the paired experiments, and similar results were obtained when the charge movement was measured directly after achieving the whole-cell configuration.

The voltage dependencies for the activation of the Na\(^+\) channel and Ca\(^{2+}\) channel conductances (\(G_{Na}\) and \(G_{Ca}\)) were estimated in the absence of their respective blockers. An estimate of \(G_{Ca}\) was obtained from the magnitude of the deactivation tail (ionic) currents after the repolarization of brief (2–6 ms) voltage steps (during which time no inactivation had developed). The peak tail currents were measured isochronally (following a 100–200-\(\mu\)s blanking period) and normalized to their maximum values for construction of \(G_{Ca}\) vs. \(V_m\) curves. No correction was made for the contribution of the OFF charge movement to the peak tail current (see Hadley and Lederer, 1991). For the estimation of \(G_{Na}\), depolarizing voltage steps were applied using a HP of -100 mV; conductances were calculated from measurements of the peak \(I_{Na}\) and \(E_{Na}\) and normalized to their maximum values for construction of \(G_{Na}\) vs. \(V_m\) curves. 3 mM cobalt was present in all \(I_{Na}\) experiments, except when noted (i.e., Fig. 2).

When normalized, time integrals of the gating currents (nonlinear charge movement) are expressed in units of nanocoulombs per microfarad of linear capacitance (nC/\(\mu\)F). Steady-state integration measurements were performed only for those charge movement traces that decayed to a zero current baseline during the voltage step for a period of at least several milliseconds; i.e., they did not display pedestals or sloping baselines, which may indicate residual ionic currents. Steady-state \(Q_{ON}\) and \(Q_{OFF}\) curves were best fit (using a nonlinear least-squares program based on the Levendberg-Marquardt algorithm) to a Boltzmann expression \(Q = Q_{max}/[1 + \exp\{-(V - V_{1/2})/k\}]\) to obtain values for the midpoint (\(V_{1/2}\)), slope (\(k\)), and maximal charge movement (\(Q_{max}\)). In some experiments, the first few digitized points (30–150 \(\mu\)s) after the voltage step were imperfectly subtracted and they were blanked to exclude their contribution to the charge integral. The integral was then calculated assuming that a continuous linear function existed during the blanking period (i.e., from 0 at the start of the pulse to the first measured point). The contribution of this integral was always <5% of the total charge measured. Currents are displayed after digital filtering at the frequency noted in each figure legend. All experiments were conducted at a temperature of 20–23°C. Grouped data are presented as means ± SD.

**RESULTS**

**Na\(^+\) and Ca\(^{2+}\) Channel Ionic Currents**

Fig. 2 A shows representative examples of the Na\(^+\) and Ca\(^{2+}\) ionic currents, recorded using the P/-5 protocol, from a 17-d-old cultured embryonic chick ventricular myocyte. The test potential in this experiment was stepped (in 10-mV increments) from HP to -60 through +30 mV and was preceded by a HP of either -90 (upper traces) or -50 mV (lower traces). In this series of experiments, the Na\(^+\) and Ca\(^{2+}\) channel blockers (tetrodotoxin and Co\(^{2+}\)) were omitted from the extracellular solutions, although K\(^+\) channels were blocked as described in Methods. From the HP of -90 mV, the test steps elicited both \(I_{Na}\) and \(I_{Ca}\). After the application of a HP of -50 mV, \(I_{Na}\) was almost completely inactivated, revealing the slower activation of \(I_{Ca}\).
The current–voltage relationships for the peak inward currents recorded from HPs of \(-90\) and \(-50\) mV are plotted in Fig. 2 B. It may be seen that activation of the Na\(^+\) currents was recorded at potentials above \(-60\) mV, and that \(I_{\text{Na}}\) reached a maximum around \(-20\) mV. In comparison, the Ca\(^{2+}\) current was activated above \(-40\) mV and peaked at around \(+10\) mV. The magnitude of the peak Na\(^+\) current was approximately an order of magnitude larger than the Ca\(^{2+}\) current, under these conditions, in a majority of the myocytes (>100 cells). However, \(\sim20\%\) of the cells tested displayed significantly smaller than average Ca\(^{2+}\) currents, presumably resulting from a specific but as yet unknown alteration caused by the culture procedure. Advantage was taken of this condition in experiments designed to characterize the Na\(^+\) channel charge movement, as described in the following section.

\[\text{Figure 2. Na}^+\text{ and Ca}^{2+}\text{ channel ionic currents recorded from an embryonic ventricular myocyte using the protocol described in Fig. 1, in the absence of TTX and Co}^{3+}. (A) \text{Superimposed current traces recorded from HPs of} -90 \text{mV} (I_{\text{Na}} \text{and} I_{\text{Ca}}) \text{and} -50 \text{mV} (I_{\text{Ca}}) \text{to test potentials of} -60 \text{to} +30 \text{mV} (\text{in} 10\text{-mV steps}). \text{Digitally filtered at} 10 \text{kHz.} (B) \text{Current–voltage relationships for the peak inward ionic current during each step, using HPs of} -90 \text{and} -50 \text{mV. (Experiment G0427C28)}\]

**Sodium Channel Gating Currents**

After the complete blockade of \(I_{\text{Na}}\) and \(I_{\text{Ca}}\), an early, brief component (duration 0.5–1.0 ms) of nonlinear charge movement was prominent in recordings from myocytes that were conditioned at more negative holding potentials. Myocytes chosen for this set of experiments displayed a robust \(I_{\text{Na}}\), but little \(I_{\text{Ca}}\). This feature, and the following properties of this component, suggest that this signal reports the gating charge movement associated with the activation of fast Na\(^+\) channels.

Fig. 3 shows the time course for the linear capacity current (recorded during a control step from \(-100\) to \(-120\) mV) and for the early component of nonlinear charge movement (recorded during a test step to \(+20\) mV, \(P/\sim5\) protocol). The peak magnitudes of the currents were scaled to be approximately equal to facilitate a comparison of their time courses. It may be seen that the nonlinear charge movement was recorded during the late falling phase of the linear capacity current, i.e., after a nearly complete charging of the linear membrane capacitance. However,
due to the limited bandwidth of the patch clamp recording system (resulting mainly from the relatively large series resistance to the cell), a rising phase of the early nonlinear charge movement was sometimes recorded. The charge movement during the first 100 µs of the voltage step is undefined; however, it is likely that the rising phase of this component is artifactual and that the signal recorded is an underestimate of the magnitude of the true gating current.

**Comparison of \( I_g \) and \( I_{Na} \) Time Course**

The rapid time course for the voltage-dependent activation of the ON charge movement of the early component (\( I_g \)) suggested that it may be associated with \( Na^+ \) channel activation, and it is compared with the time course of \( I_{Na} \) in Fig. 4. The charge integral (\( Q \)) of the gating current is also displayed. In this experiment, the HP was \(-100 \) mV, and test voltage steps were applied from \(-70 \) through \(+20 \) mV, in

![Figure 3](image)

**FIGURE 3.** The time course for the linear capacity current (smooth trace, left scale) and for the nonlinear charge movement (noisy trace, right scale). The voltage step began at the time indicated by the arrow. The linear capacity current was recorded during a hyperpolarizing control step from \(-100 \) to \(-120 \) mV, and was inverted for plotting. (Experiment A1216C01). The peak of the nonlinear charge movement occurred during the late falling phase of the linear capacitive current. HP \(-100 \) mV, test step \(+20 \) mV. Digitally filtered at 10 kHz.

![Figure 4](image)

**FIGURE 4.** Early component of nonlinear charge movement (gating current). The HP was \(-100 \) mV, the SHP was \(-120 \) mV, and test voltage step currents at \(-50, -30, -10, \) and \(+10 \) mV are displayed. At each potential the three traces show the time course of the gating current (\( I_g \)), the charge integral of the gating current (\( Q \)), and the \( Na^+ \) current (\( I_{Na} \)). 10 µM TTX and 3 mM \( Co^{2+} \) were added to the external solution. Digitally filtered at 5 kHz for \( I_{Na} \) and 10 kHz for \( I_g \). (Experiment G9706C17)
10-mV steps. Examples of \( I_g \), \( Q \), and \( I_{Na} \) at test potentials of \(-50\), \(-30\), \(-10\), and \(+10\) mV are shown. Activation of \( I_g \) was first detectable at \(-70\) mV, and it increased in magnitude with further depolarization. It may be seen that \( I_g \) reached its maximum, and began to decay before the peak of \( I_{Na} \). The predominant component of the gating current appeared to decay exponentially, with a voltage-dependent time constant ranging from 0.14 to 0.34 ms. The voltage dependence of the decay time constants of the early component are compared with those of a slower component (associated with Ca channel gating) recorded in six other experiments (see Fig. 10).

**Comparison of \( Q_{Na} \) and \( G_{Na} \)**

The voltage dependence for the steady-state integral of the ON charge movement for the early component (\( Q_{Na} \)) is plotted in Fig. 5. The data points (circles) were normalized to the value of the maximal charge movement (\( Q_{max} \)), and were fit (in this example) with a Boltzmann expression with a midpoint (\( V_{1/2} \)) of \(-36.8\) mV and a slope factor (\( k \)) of 17.1 mV. Also plotted in Fig. 5 is the normalized voltage dependence for activation of the Na conductance (\( G_{Na} \)) before blockade of \( I_{Na} \) by TTX, for comparison with the voltage dependence of the early component of charge movement. It may be seen that the curve describing the activation of the early component of charge movement is less steep and ranges over potentials that are more negative than those describing the activation of the Na\(^+\) conductance (\( V_{1/2} \) of \(-31.6\) mV, \( k \) of 5.2 mV). A similar relationship between Na\(^+\) channel charge movement (\( Q_{Na} \): \( V_{1/2} \) of \(-35.9 \pm 2.8\), \( k \) of 15.7 \pm 3.0) and conductance (\( G_{Na} \): \( V_{1/2} \) of \(-30.4 \pm 1.2\), \( k \) of 5.6 \pm 0.6) was confirmed in other myocytes (see Table I).

**Na\(^+\) Channel Inactivation and Charge Immobilization**

Another line of evidence, which supports the hypothesis that the early component of charge movement is associated with the gating of fast Na\(^+\) channels, comes from inactivation/immobilization studies. The fast Na\(^+\) current becomes inactivated (ren-
TABLE 1
Comparison of the Midpoint ($V_{1/2}$) and Slope ($k$) for $Q_{Na}$ and $G_{Na}$, and for $Q_{Ca}$ and $G_{Ca}$

| Experiment | $Q_{Na}$ | $G_{Na}$ | $Q_{Ca}$ | $G_{Ca}$ |
|------------|---------|---------|---------|---------|
|            | $V_{1/2}$ | $k$ | $V_{1/2}$ | $k$ | $V_{1/2}$ | $k$ | $V_{1/2}$ | $k$ |
| G0427      | -36.8    | 17.1   | -31.6   | 5.2    | G0427    | -14.7   | 11.1   | 1.4    | 14.7    |
| G1727      | -30.6    | 16.0   | -30.8   | 5.6    | G0428    | -12.7   | 13.1   | 2.4    | 8.3     |
| G1725      | -35.2    | 17.8   | -31.8   | 5.7    | G9819    | -14.4   | 14.4   | -2.6   | 14.4    |
| G1808      | -35.1    | 12.7   | -28.9   | 6.2    | G9628    | -15.1   | 14.94  | -2.8   | 13.0    |
| G1517      | -36.9    | 10.8   | -30.4   | 6.4    | G0419    | -16.0   | 11.4   | -12.4  | 11.8    |
| G1829      | -39.8    | 19.8   | -29.4   | 4.5    | NONOS    | -11.4   | 16.2   | -14.6  | 10.8    |
| G0706      | -36.7    | 13.9   |        |       | G0419    | -15.1   | 12.3   |        |       |
|            | -55.9    | 15.7   | -30.4   | 5.6    | G9706    | -12.9   | 13.8   |        |       |

\[ \pm 2.8 \pm 3.0 \pm 1.2 \pm 0.6 \pm 1.5 \pm 1.7 \pm 7.1 \pm 2.4 ± SD \]

ordered unavailable) by the application of depolarizing, conditioning voltage steps, which are of sufficiently long duration to allow the population of channels to reach a steady-state condition. If the early ON charge movement is associated with Na⁺ channel activation, then a fraction of the ON charge movement should be reversibly

![Diagram A](https://via.placeholder.com/150)

**Figure 6.** A comparison of the voltage dependence for Na⁺ current inactivation and for immobilization of the early ON charge. (A) The voltage protocol used to test for inactivation/immobilization. HPs (2 s duration) of −120 to −30 mV were followed (after returning to −120 mV for 1 ms) by a 10-ms test step to +10 mV. The control steps were taken negative to −120 mV (not shown). (B) Superimposed traces of the Na⁺ current available at +10 mV, following application of the HPs indicated in the figure (digitally filtered at 3 kHz). (Experiment G0427C28) (C) Superimposed nonlinear capacitive currents recorded at +10 mV, using the HPs indicated, after complete blockade of the Na⁺ current with 10 μM TTX (digitally filtered at 5 kHz). (Experiment G0706). Note the different current and time calibrations in B and C.

(D) The voltage dependence of the normalized Na⁺ current inactivation ($I_{Na}/I_{Na max}$) and of the normalized immobilization of (the integral of) the early nonlinear capacitive current ($Q_{Na}/Q_{Na max}$). The Na⁺ current inactivation was fit to a Boltzmann expression with a $V_{1/2}$ of −73.1 mV and a $k$ of −7.7 mV.
decreased or immobilized after the application of the inactivation protocol (see Almers, 1978 and Armstrong, 1981 for excellent reviews).

A comparison of the voltage dependence of the steady-state inactivation of \( I_{\text{Na}} \) and the immobilization of the early component of \( Q_{\text{ON}} \) is shown in Fig. 6. Part A is a diagram of the test voltage protocol used; the control steps (recorded between \(-120\) and \(-140\) mV) are not shown. Part B shows the superimposed \( \text{Na}^+ \) currents elicited by this protocol; the HP is indicated (in millivolts) for each current. After complete blockade of the \( \text{Na}^+ \) current with \( 10 \mu\text{M TTX} \) the inactivation voltage protocol was applied and the resulting nonlinear charge movement (gating current) was recorded in other myocytes. The gating currents recorded after the application of HPs of \(-120\), \(-70\), and \(-50\) mV are displayed (superimposed) in Fig. 6C. Fig. 6D graphically compares the voltage dependence for the inactivation of the \( \text{Na}^+ \) current (\( I_{\text{Na}}/I_{\text{Namax}} \)) with the voltage dependence for the immobilization of the integral of the early charge movement (\( Q_{\text{Na}}/Q_{\text{Namax}} \)). The charge immobilization curve appeared to be shifted to the right and was less steep than the inactivation curve. Similar results were found in four other myocytes; at a HP of \(-30\) mV (where \( I_{\text{Na}} \) is completely inactivated) the early ON charge was found to be incompletely immobilized (range of 75–90% immobilization).

The ratio of the OFF charge to the ON charge during brief voltage steps was also examined to determine the time dependence for the development of charge immobilization (see Armstrong, 1981 for review). Fig. 7A shows the \( \text{Na}^+ \) channel ON and OFF nonlinear charge movement recorded during 1.0- and 5.2-ms duration test steps to \(-20\) mV (HP, \(-100\) mV; control SHP, \(-120\) mV). Above each current is the charge integral of the ON and OFF response. If there were no development of charge immobilization during the voltage step, then the integral of the ON charge would equal the integral of the OFF charge. It was found that the OFF charge was
approximately equal to the ON charge for short (e.g., 1 ms) duration pulses. With longer duration steps, the OFF charge became increasingly smaller than the ON charge. At 5.2 ms the OFF charge was ~50% of the ON charge. Qualitatively similar results were obtained in three myocytes and the ratios of $Q_{OFF}/Q_{ON}$ as a function of the test step duration are displayed in Fig. 7 B.

**Calcium Channel Charge Movement**

A second, slower-decaying component of charge movement was present in most myocytes and its properties suggested that it was associated with Ca$^{2+}$ channel gating. At more negative HPs the slow component of charge movement was usually preceded by the fast component, associated with Na$^{+}$ channel activation, as described above. The steady-state separation of the two kinetic components of nonlinear charge movement is illustrated in Fig. 8. In this representative experiment the decay phase of the ON charge movement recorded from a HP of $-100$ mV displayed the fast and slow components (Fig. 8 A). After a reduction of the HP to $-50$ mV (for 2 s) an identical test step (from $-100$ to $+30$ mV) produced a reduction in the total charge moved, mainly due to a reduction in the magnitude of the early, faster component. The difference between the currents recorded from HP $-100$ mV (A) and HP $-50$ mV (B) is displayed below (A-B trace). The difference current trace confirms that the additional ON charge movement, which was available from HP $-100$ mV, was contributed mainly by the faster component. The results of this experiment also demonstrate that the slower component was not as drastically changed by the reduction of the HP to $-50$ mV. A small amount of slow OFF charge movement in the difference trace probably results from the additional slow charge movement.

**Figure 8.** Steady-state separation of the fast and slow components of charge movement. (A) Nonlinear charge movement was recorded with a test step of $-100$ to $+30$ mV, using a HP of $-100$ mV (as shown by the dotted line in the voltage protocol). (B) Charge movement recorded with a test step identical to that in A after the application of HP $-50$ for 2 s. (A - B) The difference current resulting from the subtraction of trace B from trace A. (Experiment G1726C20)
available from HP -100 mV. Thus, the fast and slow components of charge movement can be identified and separated on the basis of steady-state HP protocols.

Another example of an experiment in which both kinetic components of charge movement were present is shown in Fig. 9. In part A, the HP was -100 mV and the test potential was +20 mV. The early, fast component (associated with Na\(^+\) channel charge movement) rapidly decayed and was followed by the second, slower-decaying component. The superimposed traces are the charge integrals of the current traces and they clearly show the separation and contribution of the Na\(^+\) (\(Q_{Na}\)) and putative Ca\(^{2+}\) (\(Q_{Ca}\)) channel charge movements. Part B shows the charge movement after changing the HP to -50 mV. As can be seen, the early, fast component was dramatically reduced but the second, slower component was less affected by the reduction of the HP. The total charge moved (\(Q_{\text{max}}\)) from HP -100 mV and HP -50 mV in 13 cells is given in Table II.

| Experiment | \(Q_{\text{max}}\) HP -100 mV | \(Q_{\text{max}}\) HP -50 mV |
|------------|----------------|----------------|
| G1620C04  | 33             | 15             |
| G1621C01  | 31             | 19             |
| G0421C01  | 93             | 34             |
| G9818C01  | 64             | 44             |
| G9820C01  | 130            | 71             |
| G9901C01  | 60             | 42             |
| NON0305   | 46             | 22             |
| G1801C18  | 81             | 41             |
| G1801C06  | 140            | 89             |
| G1801C35  | 39             | 12             |
| G1726C20  | 70             | 54             |
| G1726C40  | 88             | 55             |
| G1726C52  | 65             | 29             |
| G0419C01  | 73             | 40             |

Mean ± SD: 72.5 ± 34.1 40.5 ± 22.6
To determine the relative contribution and the kinetic properties of each component of the total charge movement, the decay phases of the currents were fit with a sum of two exponentials. The time constants (and amplitudes) for the decay of the fast and slow components recorded from HP = -100 mV and HP = -50 mV are presented in Table III. The time constants were not changed by the change in HP; only their amplitudes varied. The ratio of the amplitudes of the time constants at HP = -50 mV as compared with HP = -100 mV was 0.28 ± 0.18 for A1 and 0.78 ± 0.11 for A2. These results confirm that the predominant effect of the reduction of HP to -50 mV was an immobilization of the faster component of the charge movement which is associated with the gating of the Na⁺ channels. However, it should be noted that the slow component was reduced by 22%, thereby suggesting that significant immobilization of QCa also occurred.

The decay phases of the ON gating currents (charge movement) were fit with a sum of two exponentials: \( A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2} \). The test potential was +20 mV.

The voltage dependence for the predominant time constant of decay of the second, slow component of charge movement (Ig-Ca) (circles) is compared with the time constants for decay of the early component (Ig-Na) (triangles) in Fig. 10. A bell-shaped voltage dependence is obtained for the time constants for decay of both Ig-Na and Ig-Ca, with a decreasing time constant at potentials positive to -30 or -20 mV (maximum and minimum values: 1.40 and 0.88 ms for Ig-Ca; 0.34 and 0.14 ms for Ig-Na). Thus, the major kinetic components of Na and Ca channel charge movement were clearly separable by the 5- to 10-fold difference in their decay time constants.
**Q_{Ca} Does Not Immobilize during Brief Steps**

Multiple additional lines of evidence associate the second, slowly decaying component of charge movement with the gating of calcium channels. Fig. 11A shows examples at various test potentials of the ON and OFF charge movement recorded using a HP of −50 mV. Superimposed on each current trace is the integral of the charge movement. It is apparent that at each potential the magnitude of the slow OFF charge is equal to the slow ON charge. The observation that charge is conserved helps to substantiate that the signal is capacitive in nature, as would be expected for membrane-bound gating charges. Furthermore, the absence of charge immobilization of this component is consistent with the slower development of the inactivation of I_{Ca}. In addition, as shown in Fig. 11B, both the ON and OFF charge movement saturated with strong depolarization, a property that is also consistent with gating charge movement. Moreover, the activation of the slow component of charge movement ranged over more positive potentials than that measured for the fast component. The slow charge movement component was first detected at −60 mV, and approached saturation at +30 mV. The data are fit with a Boltzmann expression, yielding a midpoint of −14.9 mV, a slope factor of 17.6 mV, and a Q_{max} of 24.1 fC.

**Comparison of Q_{Ca} and G_{Ca}**

If the second, slower component of nonlinear charge movement governs the activation of the calcium channel conductance (G_{Ca}), then the charge movement (Q_{Ca}) would be expected to occur over a similar voltage range. Fig. 12 demonstrates that this was so. At the start of this experiment, I_{Ca} was not blocked. The voltage dependence for the activation of the calcium conductance (G_{Ca}) was determined by a tail current method (as shown in the inset), using a HP of −50 mV. The normalized activation curve for G_{Ca} begins at potentials positive to −40 mV and reaches saturation above +20 to +30 mV. The Boltzmann fit to the data gives a midpoint of −2.7 mV and a slope factor of 12.9 mV. After complete blockade of I_{Ca} (with 3 mM CoCl₂) the same voltage protocol was used to record the charge movement from the
same myocyte. The ON charge movement \( (Q_{Ca}) \) was integrated (see inset), normalized, and plotted as a function of the test potential. It may be seen that the \( Q_{Ca} \) curve (midpoint \(-14.4\) mV, slope factor \(14.5\) mV) ranges over a voltage range similar to, but more negative than, \( I_{Ca} \) activation. The single Boltzmann relation provided a

\[
A \quad -30 \quad 10
\]

\[
-20 \quad 20
\]

\[
0 \quad 30
\]

**FIGURE 11.** The second, slower component of nonlinear charge movement. (A) Nonlinear charge movement, recorded using a HP of \(-50\) mV. The potential was returned to \(-100\) mV for \(1.2\) ms before the test step. \(10\) \(\mu M\) TTX and \(3\) mM CoCl\(_2\) were present. The numbers above each current trace are the test potentials, in millivolts. Digitally filtered at \(5\) kHz. Superimposed on each trace is the charge integral of the current. The current, charge, and time calibrations are \(20\) pA, \(2\) nC/\(\mu F\), and \(1.5\) ms. (B) The voltage dependence of \(Q_{ON} \) (open circles) and \(Q_{OFF} \) (closed circles). The curve plotted through the data is the best-fit to a Boltzmann expression, with a \(V_{1/2}\) of \(-14.9\) mV, a \(\kappa\) of \(17.6\) mV, and a \(Q_{max}\) of \(24.1\) fC. (Experiment N0N05C05)

good fit to the data, which is consistent with the notion that it is derived from a single channel population. A summary of the results comparing the voltage dependence for the activation of \(G_{Ca} \) and for the second component of nonlinear charge movement \( (Q_{Ca}) \) is given in Table I.
Steady-State Immobilization of $Q_{ca}$

Another characteristic feature of the cardiac L-type Ca$^{2+}$ current is the development of voltage-dependent inactivation over a range of potentials more positive than those that result in the inactivation of the fast Na$^+$ current. Therefore, the inactivation of $I_{ca}$ might be expected to be correlated with a reduction (immobilization) of the second component of charge movement. Fig. 13 shows the voltage dependence for the immobilization of the total charge movement ($Q$) in recordings that displayed an early component (Na channel charge movement) and a second component (Ca channel charge movement). The data points describing the immobilization of $Q$ ($Q/Q_{max}$) yielded a double-component relationship, which is consistent with the hypothesis that two different channel populations may be contributing to the charge movement. Indeed, the midpoints of the two components differed by 60 mV (Fig. 13 $B$). The data were fit by a sum of two Boltzmann expressions yielding the midpoints of $-77.5$ mV (slope $-7.0$ mV) and $-17.9$ mV (slope $-7.8$ mV).
following values for the percent of $Q_{\text{max}}$ and midpoint for each component: 41.7%, -77.5 mV and 58.3%, -17.9 mV. The relative contribution of each component to the total charge movement was variable; in 11 myocytes a range of 23–69% of the total charge movement was immobilized at a HP of -50 mV. The more negative phase of immobilization of the charge movement (-120 to -50 mV) has a voltage dependence similar to that of Na$^+$ channel immobilization (Fig. 6 D), which again suggests that the faster component of the total charge movement may represent the gating of Na$^+$ channels. The less negative phase of immobilization is correlated predominantly with the reduction of the second, slow component associated with Ca$^{2+}$ channel gating.

**DISCUSSION**

The results of this paper are the first to demonstrate that two kinetic components of nonlinear charge movement can be recorded from cardiac ventricular myocytes and that their properties are strongly correlated with the activation gating of sarcolemmal Na$^+$ channels and Ca$^{2+}$ channels. Many lines of evidence support the conclusion that the nonlinear charge movement signals are capacitive in nature and, furthermore, that they are closely associated with the voltage-dependent gating of Na$^+$ and Ca$^{2+}$ channels:

(a) All ionic currents were blocked by the appropriate solutions. (b) For brief voltage steps (i.e., 1 ms for Na$^+$ and 10 msec for Ca$^{2+}$ charge movement) the amount of ON charge was the same as the OFF charge over the entire voltage range examined. The equality and therefore conservation of the ON and OFF charges is consistent with a capacitive current of intramembranous origin and not an ionic current. (c) Both the Na$^+$ and Ca$^{2+}$ channel charge movements saturated with strong depolarization, also consistent with the expected behavior of membrane-bound charges. (d) The respective component of charge movement was always present when the Na$^+$ or Ca$^{2+}$ current was recorded, and preceded its activation in time. (e) The voltage dependencies of the two components of charge movement were similar to those for activation of the Na$^+$ and Ca$^{2+}$ conductances. (f) The Na$^+$ and Ca$^{2+}$ channel charge movement could be differentially and reversibly immobilized by holding the membrane potential at less negative values, over voltage ranges similar to those producing the inactivation of $I_{\text{Na}}$ and $I_{\text{Ca}}$.

There are several arguments that support the idea that the second component of charge movement was generated by the gating of Ca$^{2+}$ channels and not Na$^+$ channels. The first is based on the expected kinetics of the charge movement as a function of HP. If there was a significant component of Na$^+$ channel charge movement present in the total charge movement at more negative HPs, and little Na$^+$ channel charge movement at a HP of -50 mV, then one would expect the signal at HP $-100$ mV to be composed of two components: a fast-decaying component due to Na$^+$ channel gating, and a slower one due to Ca$^{2+}$ channel gating. At a HP of $-50$ mV, Na$^+$ channel charge movement is mostly immobilized, and there should be only one predominant contribution from Ca$^{2+}$ channel gating. In fact, in these experiments there were two components with dissimilar decay rates which were also separable on the basis of different HPs. It would seem unlikely that the slowly
decaying, second component of charge movement \((Q_{Ca})\) is involved in the gating of fast Na\(^+\) channels, which activate earlier in time.

Another line of evidence against a significant contribution of Na\(^+\) channel gating to the second component of charge movement is based on the knowledge that the Na\(^+\) channel ON charge immobilized with time, leaving a smaller OFF charge upon repolarization. However, the ON and the OFF charges of the second slow component were nearly equal at a HP of -50 mV during brief steps. Any contribution of nonimmobilizable Na\(^+\) channel charge movement at a HP of -50 mV would be expected to be relatively small and to decay rapidly, and could not account for the larger, slower Ca channel OFF charge movement.

Further support for the identification and separation of charge movement from Na\(^+\) and Ca\(^{2+}\) channels is derived from the results of the steady-state activation experiments. The clearly different values for the Boltzmann expression fits to \(Q_N\) and \(Q_{Ca}\) suggest that they represent two distinct processes, which closely correlate with the voltage dependence for the activation of \(G_N\) and \(G_{Ca}\). The fast component \((Q_N)\), which was only available from more negative HPs, displayed a more negative midpoint than the slowly decaying component \((Q_{Ca})\). Additionally, the \(Q_{Ca}\) curve cannot be explained as a positive shift in the \(Q_N\) curve after immobilization, since immobilization would be expected to produce a negative shift in the \(Q_N\cdot V\) curve (Bezanilla, Taylor, and Fernandez, 1982).

The values obtained in the present study for the maximal Na\(^+\) and Ca\(^{2+}\) channel ON charge movement may be compared with those predicted from estimates of channel density and gating charge. Channel densities were estimated from the macroscopic current/(probability of opening × single channel current) \((I/P_{o})\) and from the maximal superimposition of single channel currents in patch clamp experiments (Josephson, I. R., unpublished results). If the average Na\(^+\) channel density in the sarcolemma of the embryonic myocyte is 10/\(\mu\)m\(^2\), and the effective gating charge per channel is 6 e\(^-\), then the estimated Na\(^+\) channel \(Q_{max}\) would be 2.25 nC/\(\mu\)F. This estimate may be compared with the value of 5.5 nC/\(\mu\)F obtained experimentally for Na\(^+\) channel charge movement. If the average Ca\(^{2+}\) channel density in the sarcolemma is 5/\(\mu\)m\(^2\), and the effective gating charge per channel is 4 e\(^-\), then the estimated Ca\(^{2+}\) channel \(Q_{max}\) would be 0.8 nC/\(\mu\)F. This predicted value is somewhat less than that obtained experimentally for Ca\(^{2+}\) channel charge movement (6.75 nC/\(\mu\)F). In these calculations the effective valence was obtained from the slope of the \(Q\cdot V\) relationships for the Na\(^+\) and Ca\(^{2+}\) charge movement (values of \(~2\) e\(^-\) for Na\(^+\) channels and \(~2.0\) e\(^-\) for Ca\(^{2+}\) channels) and 3 Na\(^+\) channel (m\(^3\) activation kinetics) and 2 Ca\(^{2+}\) channel (m\(^2\) activation kinetics) gating particles were assumed. It may be kept in mind, however, that the channel density used in the calculations is an estimate. In addition, the effective valence obtained from the Boltzmann relationship assumes a two-state system of identical, independent, and equivalent particles (Stühmer, Conti, Suzuki, Wang, Noda, Yahagi, Kubo, and Numa, 1989; but see Ruben, Starkus, and Rayner, 1990). Given the assumptions and estimations used in the calculations, the approximate agreement with the experimental findings is satisfactory.
Recently, Na⁺ channel gating currents (Iₙg) recorded from cardiac Purkinje cells have been described (Hanck, Sheets, and Fozzard, 1990). It was found that Iₙg decayed with two time constants, the slower of which was voltage dependent in a manner similar to τₚ for a Hodgkin and Huxley model of Na⁺ current activation. In addition, they reported that both the conductance–voltage, and the charge–voltage relationships have the same voltage midpoints in Purkinje cells. In contrast, the results from cultured chick ventricular cells (10–20 channels/µm²) show that the midpoint of the Na⁺ channel charge–voltage relationship occurs at a more negative potential than the conductance–voltage relationship. The difference in midpoint and slope of Gₐₙ and Qₐₙ in the present study would be consistent with a four-state linear kinetic model (three closed and one open state) for Na⁺ current activation (Armstrong, 1981). It should be noted, however, that the method used for estimation of Gₐₙ (with no correction for inactivation) may explain the presence of the crossover of the Gₐₙ and Qₐₙ curves (see Stimers, Bezanilla, and Taylor, 1985), reported in the Purkinje cell study and in the present results.

The Qₘₐₓ for the Na⁺ channel gating charge in Purkinje cells was found to be ~ 0.2 fC/µm² or 20 nC/µF, assuming a channel density of 167 channels/µm² and a gating charge/channel of 4–6 e⁻ (Hanck et al., 1990). This value for Na⁺ channel charge movement may be compared with the present findings for embryonic ventricular myocytes. The Na⁺ channel density in the 17-d-old embryonic ventricular cells (10–20 channels/µm²) is ~10 times less than the above estimate for the Purkinje cells. The lower channel density would yield an estimated Qₘₐₓ of ~2–4 nC/µF for the embryonic ventricular cells, which agrees with the experimental values of ~5 nC/µF. A component of the charge movement related to Ca²⁺ channel gating was not observed in the Purkinje cell study, however, perhaps because of the large Na⁺/Ca²⁺ channel ratio in that cell type, and/or because internal fluoride ion (used in the pipette solution) may have blocked Ca²⁺ channel charge movement (Kostyuk et al., 1981).

In previous studies examining Ca²⁺ channel charge movement in cardiac ventricular myocytes, a separation of the Na⁺ and Ca²⁺ channel ON charge movement from a single component signal was performed on the basis of differential HP (Field et al., 1988; Bean and Ríos, 1989; Hadley and Lederer, 1989, 1991). The validity of this procedure rests on the following assumptions: (a) the single component signal recorded in the previous studies reflects Na⁺ and Ca²⁺ charge movements; (b) a HP of ~50 or ~40 mV immobilizes all of the Na⁺ channel charge movement; (c) Ca²⁺ channel charge movement is not immobilized (i.e., is fully available) at a HP of ~50 mV. The present results show that there may be some overlap in the voltage dependence of immobilization of Qₐₙ and Qₖₑₐ and therefore advocate the use of additional kinetic information in separating Na⁺ and Ca²⁺ channel charge movement. A comparison of the maximum nonlinear charge movement from the cardiac cell studies cited above is presented in Table IV.

In addition to their different voltage-dependent steady-state and kinetic properties, Na⁺ and Ca²⁺ channel charge movement may be further identified by the
differential effects of certain pharmacological agents. Ca$^{2+}$ channel antagonists from the dihydropyridine (nifedipine) and phenylalkylamine (D600) classes have been tested on the charge movement of cardiac cells to help identify the nature of the charge movement, as well as to elucidate their mechanism of action in blocking Ca$^{2+}$ channels (Field et al., 1988; Bean and Rios, 1989; Hadley and Lederer, 1991).

Several agents known to enhance the calcium current have also been tested for their ability to alter the Ca$^{2+}$ channel gating charge movement (Josephson and Sperelakis, 1990, 1991a). Both isoproterenol (via cAMP-dependent phosphorylation) and BAY K 8644 (a dihydropyridine Ca$^{2+}$ channel agonist) have been shown to shift the activation of $i_{Ca}$ to more negative potentials, and to increase $i_{Ca}$ by increasing the probability of opening of single Ca channels. Isoproterenol and BAY K 8644 also altered the kinetics of Ca$^{2+}$ channel ON charge in a voltage-dependent fashion. The resulting earlier distribution of the gating charge movement during exposure to these agents is consistent with, and may be at least partially responsible for, the

| TABLE IV |
| --- |

| Comparison of the Maximum Nonlinear Charge Movement from Cardiac Cell Preparations |
| --- |

| Cell type/species | $C_{input}$ | HP | $Q_{ON}$ |
| --- | --- | --- | --- |
| Ventricular myocyte rat/rabbit (adult) (Bean and Rios, 1989) | 101 | -110 | 1,100 | 11 |
| Ventricular myocyte guinea pig/rat (adult) (Hadley and Lederer, 1989) | 60 | -100 | 650 | 11 |
| Ventricular myocyte neonatal rat (Field et al., 1988) | 13 | -100 | 50 | 3.9 |
| Purkinje myocyte canine (adult) (Hanck et al., 1990) | 80 | -150 | 1,520 | 20 |
| Ventricular myocyte 17-d-old embryonic chick (present results) | 6 | -100 | 72 | 12 |

The decrease in the latency to first opening (observed in single Ca$^{2+}$ channel recordings), the decrease in the time to peak of $i_{Ca}$, and the voltage-dependent shift of $i_{Ca}$. In the future, it will be of great interest to determine whether other modulators of Na$^{+}$ and Ca$^{2+}$ channel function also exert their effects through alterations of the gating charge movement.

**Summary**

In conclusion, this study demonstrates that both kinetic and steady-state properties may be used to facilitate the identification and separation of cardiac Na$^{+}$ and Ca$^{2+}$ channel charge movement. These results, obtained from an embryonic myocyte preparation, complement and extend the recent findings obtained from mature cardiac ventricular cells (Bean and Rios, 1989; Hadley and Lederer, 1989, 1991) and demonstrate the usefulness of this preparation for studying cardiac ion channel charge movement.
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