

# Nonlinear Charge Movement in Mammalian Cardiac Ventricular Cells

## *Components from Na and Ca Channel Gating*

BRUCE P. BEAN and EDUARDO RIOS

From the Department of Neurobiology, Harvard Medical School, Boston, Massachusetts 02115; and the Department of Physiology, Rush University School of Medicine, Chicago, Illinois 60616

**ABSTRACT** Intramembrane charge movement was recorded in rat and rabbit ventricular cells using the whole-cell voltage clamp technique. Na and K currents were eliminated by using tetraethylammonium as the main cation internally and externally, and Ca channel current was blocked by Cd and La. With steps in the range of  $-110$  to  $-150$  used to define linear capacitance, extra charge moves during steps positive to  $\sim -70$  mV. With holding potentials near  $-100$  mV, the extra charge moving outward on depolarization (ON charge) is roughly equal to the extra charge moving inward on repolarization (OFF charge) after 50–100 ms. Both ON and OFF charge saturate above  $\sim +20$  mV; saturating charge movement is  $\sim 1,100$  fC ( $\sim 11$  nC/ $\mu$ F of linear capacitance). When the holding potential is depolarized to  $-50$  mV, ON charge is reduced by  $\sim 40\%$ , with little change in OFF charge. The reduction of ON charge by holding potential in this range matches inactivation of Na current measured in the same cells, suggesting that this component might arise from Na channel gating. The ON charge remaining at a holding potential of  $-50$  mV has properties expected of Ca channel gating current: it is greatly reduced by application of  $10 \mu$ M D600 when accompanied by long depolarizations and it is reduced at more positive holding potentials with a voltage dependence similar to that of Ca channel inactivation. However, the D600-sensitive charge movement is much larger than the Ca channel gating current that would be expected if the movement of channel gating charge were always accompanied by complete opening of the channel.

### INTRODUCTION

Voltage-dependent processes in membranes, such as the gating of ionic channels, require the movement of charge within the membranes. Such charge movement was first recorded in the sarcolemmal membranes of skeletal muscle (Schneider and Chandler, 1973), where an intramembrane charge movement seems to be closely associated with the coupling of depolarization to release of Ca from the sarcoplas-

Address reprint requests to Dr. Bruce P. Bean, Department of Neurobiology, Harvard Medical School, 220 Longwood Avenue, Boston, MA 02115.

mic reticulum (for discussion of recent work, see Melzer et al., 1986; Lamb, 1987; Brum and Rios, 1987). Probably the best-understood example is the gating current of Na channels in axons (Armstrong and Bezanilla, 1973), where detailed experiments have shown the relation between charge movement and channel activation, as well as between charge immobilization and channel inactivation (see Armstrong, 1981, for review).

Charge movements that are probably associated with the gating of Ca channels have been recorded in molluscan neurons (Adams and Gage, 1976, 1979; Kostyuk et al., 1984) and nifedipine (Lamb, 1986*b*; Rios and Brum, 1987; Lamb and Walsh, 1987). One possibility is that such Ca channel blockers depress only a component of gating currents of calcium channels has come from recent experiments on the intramembrane charge movement in skeletal muscle. It has been found that this charge movement can be partially inhibited by calcium channel blockers such as D600 (Hui et al., 1984) and nifedipine (Lamb, 1986*b*; Rios and Brum, 1987; Lamb and Walsh, 1987). One possibility is that such Ca channel blockers depress only a component of charge movement arising from Ca channel gating, with a distinct component involved with excitation-contraction (E-C) coupling being unaffected (Lamb, 1986*b*; Lamb and Walsh, 1987). However, the blockers can also depress both contraction (Eisenberg et al., 1983; Berwe et al., 1987) and Ca release from the sarcoplasmic reticulum (Rios and Brum, 1987), raising the possibility that the voltage sensors governing E-C coupling are also sensitive to Ca channel blockers. It has been specifically proposed that the high-affinity dihydropyridine binding sites found at high density in skeletal muscle t-tubules are the voltage-sensors governing E-C coupling (Rios et al., 1986; Beam et al., 1986; Rios and Brum, 1987). This idea has been supported by the discovery that injection of the cloned cDNA encoding the skeletal muscle dihydropyridine receptor restores E-C coupling in muscles from mice with the muscular dysgenesis mutation, which normally lack E-C coupling (Tanabe et al., 1988). Moreover, injection of the cDNA of the dihydropyridine receptor also restores slow Ca current to the dysgenic muscle, supporting the idea that the dihydropyridine receptor might actually serve a dual function, serving both as a calcium channel and voltage sensor for E-C coupling (Rios et al., 1986; Beam et al., 1986; Rios and Brum, 1987; Tanabe et al., 1987, 1988).

Comparative studies of charge movement in vertebrate cardiac muscle may be useful in helping to understand the origins of the charge movement in skeletal muscle. Most evidence suggests that E-C coupling in cardiac muscle is quite different from that in skeletal muscle, being triggered by Ca entry through sarcolemmal Ca channels (see Fabiato, 1983, 1985) and not by direct depolarization-induced release of Ca from sarcoplasmic reticulum (see Morad and Goldman, 1973; Chapman, 1979, 1983). In this case, one might guess that cardiac muscle would have nonlinear charge movement from Ca channel gating but not a component associated with Ca release from the sarcoplasmic reticulum. However, a recent study of the voltage dependence of Ca release in cardiac cells (Cannell et al., 1987) has raised anew the possibility of direct voltage control of sarcoplasmic reticulum, which presumably would be accompanied by E-C coupling-related charge movement like that in skeletal muscle. In addition to possibly shedding light on E-C coupling mechanisms, studies of nonlinear charge movement should help clarify gating mechanisms of car-

diac calcium channels and the ways in which they are modulated by drugs and hormones.

We have recorded nonlinear charge movement in single cardiac muscle cells, with the goal of identifying components from Ca channel gating and, perhaps, depolarization-induced Ca release. We have found that nonlinear charge movement can readily be recorded from ventricular cells from rabbit and rat hearts and that the charge movement can be resolved into at least two components by changes in holding potential. One component appears to be Na channel gating current and can be immobilized at depolarized holding potentials. The other has time- and voltage-dependent properties like those expected from a Ca channel gating current and can be inhibited by D600. However, it is much larger than would be expected from Ca channels if movement of gating charge necessarily leads to a high probability of channel openness.

## METHODS

### *Cell Isolation*

Cells were isolated from the ventricles of rabbit or rat hearts using enzymes applied by Langendorff perfusion, using a method similar to those of Isenberg and Klockner (1982) and Mitra and Morad (1985). Hearts were excised from animals that were anesthetized with ether (and in the case of rabbits, injected with 2,000 U of heparin) and mounted on a Langendorff apparatus with solution reservoirs 27 in above the heart. The heart was perfused for 5 min with Ca-free saline (in millimolar, 135 NaCl, 5.4 KCl, 1 MgCl<sub>2</sub>, 0.33 NaHPO<sub>4</sub>, 10 HEPES, pH 7.3, bubbled with O<sub>2</sub>), then for 15–30 min with enzyme-containing solution (1.5 mg/ml collagenase [Type I; Worthington Biochemical Corp., Freehold, NJ], 0.3 mg/ml protease [Type XXIV; Sigma Chemical Co., St. Louis, MO] in the same saline but with 200 μM added CaCl<sub>2</sub>). These perfusates were warmed to 35°C. After the enzyme treatment, the heart was rinsed by perfusion with a K glutamate solution (in millimolar, 140 K glutamate, 5 MgCl<sub>2</sub>, 1 EGTA, 10 glucose, 10 HEPES, pH 7.4) at room temperature. After ~100 ml of this solution had passed through the heart, the heart was cut down into the K glutamate solution, and cells were freed by mincing with scissors and triturating with broken-off Pasteur pipettes (tip diameter ~1 mm). Cells were stored in the K glutamate solution at 5°C and used within 24 h.

### *Electrical Recording*

Cells were voltage-clamped using patch pipettes (Hamill et al., 1981). To eliminate ionic currents, internal and external solutions contained as few permeant ions as possible. The internal solution, which also contained an ATP-regenerating system that slows rundown of Ca currents in these cells (see Forscher and Oxford, 1985), was (in millimolar) 128 tetraethylammonium (TEA) Cl, 4.5 MgCl<sub>2</sub>, 9 EGTA, 9 HEPES, 4 MgATP, 14 creatine phosphate (Tris salt), 0.3 GTP (Tris salt), and 50 U/ml creatine phosphokinase (Type I; Sigma Chemical Co.), pH adjusted to 7.4 with TEA OH. The ATP, GTP, creatine phosphate, and creatine phosphokinase were added from concentrated aliquots frozen at –70°C, and the internal solution was kept on ice until use. Since it was impossible to form gigaseals with pipettes that were dipped into creatine phosphokinase-containing solution, pipettes were filled by sucking a small amount of enzyme-free solution into the tip and then backfilling with enzyme-containing solution.

The external solution for recording charge movement consisted of (in millimolar), 10 BaCl<sub>2</sub>, 6 CdCl<sub>2</sub>, 0.1 LaCl<sub>3</sub>, 154 TEA Cl, 10 HEPES, PH adjusted to 7.4 with TEA OH. To

record Ba currents, Cd and La were omitted from the solution; Na current was recorded by omitting tetrodotoxin (TTX) and adding 10 or 20 mM NaCl.

After establishing a whole-cell clamp, the cell was lifted off the floor of the chamber and moved into the flow of one of a series of 10 microcapillaries mounted parallel to the chamber bottom. Rapid solution changes (<100 ms) were made by moving the cell from in front of one tube to another (see Friel and Bean, 1988).

Experiments were done at 7–16°C, as noted.

Recordings were made using an Axopatch 1A (Axon Instruments, Inc., Burlingame, CA) or a List EPC/7 (Medical Systems Corp., Greenvak, NY) patch clamp. The capacitance compensation was adjusted to cancel the linear capacity transient; for larger cells, compensation was set at 100 pF (the most possible) and the gain of the clamp kept low enough to avoid saturation of amplifiers.

Currents were filtered at 1–3 kHz, digitized at 100–200  $\mu$ s, and analyzed with an LSI 11/73 computer system (Indec Systems, Inc., Sunnyvale, CA).

#### *Voltage Protocols and Analysis*

“Control” voltage pulses used to define linear capacitance and leak currents were given (usually in the range of –100 to –150 mV) in trains of 16–32 pulses and the resulting currents signal averaged. To further smooth the control pulse currents during analysis (especially to remove bit breakup from greatly scaled-up currents) the averaged control pulse currents were sometimes fit by the sum of two exponential terms plus a constant (leaving unaltered early points that were not fit well). After correction of test pulse currents for linear capacity and leak, the resulting current was sometimes refiltered at 0.5–1 kHz.

In the usual voltage protocol, the test pulse was preceded by a short (15–40 ms) prepulse and was also followed by a step to the prepulse potential (see Figs. 2 and 4). The purpose of this “pedestal” was to ensure that when the holding potential was changed, the actual voltage change during and after the test pulse was unaltered, so that nonlinear charge movements from different holding potentials could be compared directly. The usual pre- and postpulse potentials of –80 to –90 mV were chosen as a compromise; with more positive voltages there could be significant nonlinear charge movement during the prepulse, while with more negative voltages there could be recovery of Na channel inactivation during the prepulse. With the values chosen, there was no recovery of the Na current during the prepulse interval (see Fig. 6).

Since there were often slow changes in the time course of the linear capacity current over the course of an experiment, control pulses were given frequently, preceding and following each series of test pulses, and charge movement was analyzed only if bracketing control pulses were nearly identical.

Average values are given as the mean  $\pm$  standard error of the mean.

#### RESULTS

Fig. 1 shows currents in a rat ventricular cell before and after block of Ba currents by Cd and La. Linear capacity and leak currents have been subtracted. In the absence of Cd and La, depolarization of the cell elicited a large inward current, carried by Ba flowing through Ca channels; in this and other rat and rabbit ventricular cells, the Ca channel currents seemed purely of the L type (see Bean, 1989). As in all cells studied with careful subtraction of linear leak and capacitative currents, this inward Ba current was preceded by a small outward current. Addition of Cd and La to the external solution promptly eliminated the inward current at all potentials, but had little effect on the early outward current.

The choice of Ba as the charge carrier and of Cd and La as blockers were the result of trial and error tests in preliminary experiments. We used Ba as charge carrier because leak currents were smaller and more linear over a wider voltage range than with Ca. To eliminate ionic currents, we first tried simply replacing the 10 mM Ba with 10 mM Mg (which is impermeant in cardiac Ca channels) but the leakage current became too large and unstable for satisfactory leak correction. Fortunately, it proved possible to block ionic current completely even in the continued presence of 10 mM Ba by using the combination of 6 mM Cd and 0.1 mM La. Cd at 3–6 mM alone was sufficient to block test pulse currents completely, but block of

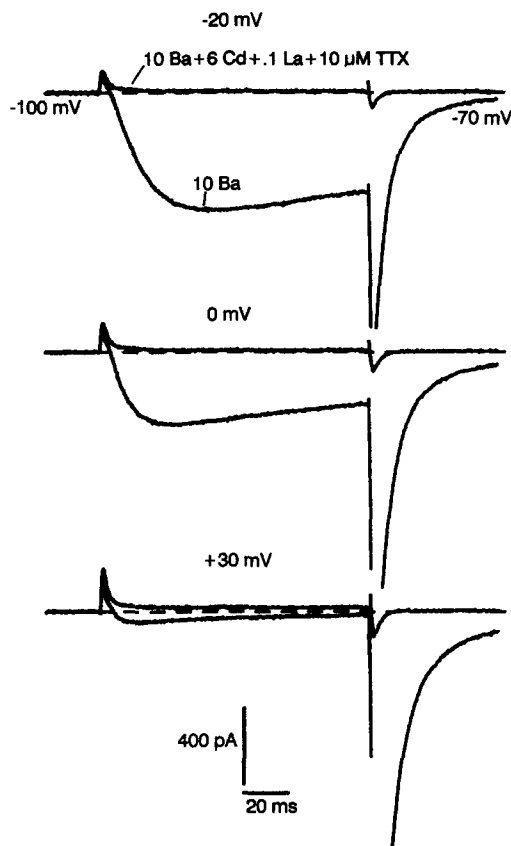


FIGURE 1. Nonlinear charge movement in a rat ventricular cell revealed by blocking ionic current. Currents were elicited by 120-ms pulses from a holding potential of  $-100$  mV to various test potentials (after which the potential was stepped to  $-70$  mV for 60 ms) before and after block of ionic current by Cd and La. Linear capacity and leak currents were subtracted using appropriately scaled currents elicited in response to a step from  $-100$  to  $-116$  mV (no blockers) or  $-100$  to  $-114$  mV (with blockers). Cell W38D, rat ventricular, 71 pF,  $7^{\circ}\text{C}$ .

tail currents at strongly negative potentials was not quite complete (see Brown et al., 1983). La alone blocked test pulse and tail currents equally, but block was not quite complete at 0.1 mM, and we worried that larger concentrations might affect Na or Ca channel gating processes; we found that 0.5 mM La blocked Ba currents irreversibly, and this concentration also blocks K channels (Nathan et al., 1988). The combination of 6 mM Cd and 0.1 mM La seemed to block currents at all potentials completely. In principle, gating current from either Na channels or Ca channels might be inhibited or modified by either Cd or La (0.1 mM La has previously been reported to inhibit Na current in human atrial cells by 30–40% [Bustamante, 1987],

though there was no effect in frog atrial cells [Nathan et al., 1988]). Any inhibition of charge movement must be small, however, since the outward current at large depolarizations, where ionic Ba current was small (e.g., +30 mV in Fig. 1), was not grossly changed on switching to the blocking solution. TTX was routinely used in the blocking solution even though there were no ions in either internal or external solutions expected to be permeant in the Na channel; in agreement with this, charge movement records were no different in a few experiments in which TTX was omitted.

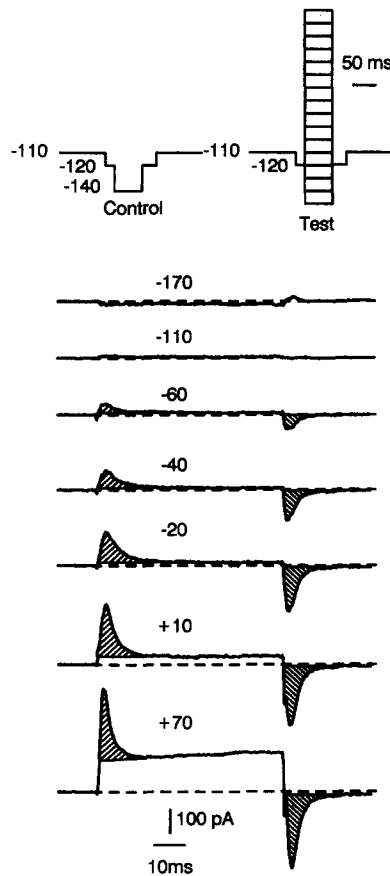


FIGURE 2. Nonlinear charge movement at different test potentials. Traces are single sweeps of current at the indicated voltages, corrected for linear capacity and leak currents by subtraction of appropriately scaled current in response to control pulse shown. Thin solid lines are sloping baselines fit to the final 30 ms of the current during the ON pulse (or the final 7 ms of the OFF current) and extrapolated to the edge of the step (ON or OFF). Shaded areas represent total nonlinear charge movement (plotted in the next figure). Cell W38D.

If the nonlinear currents recorded in the blocking solution reflect intramembrane charge movement and not incompletely blocked ionic currents, they should show saturation at high depolarizations, a feature not likely to be true of outward ionic currents. Figs. 2 and 3 show that the nonlinear currents recorded in the blocker solution do satisfy this requirement. Nonlinear charge movement first became detectable for depolarizations to  $\sim -70$  mV and reached a maximal value for steps to  $\sim +20$  mV; consistently, further depolarizations, to potentials up to +100 mV, moved about the same amount of charge as at +20 mV. Larger depolarizations

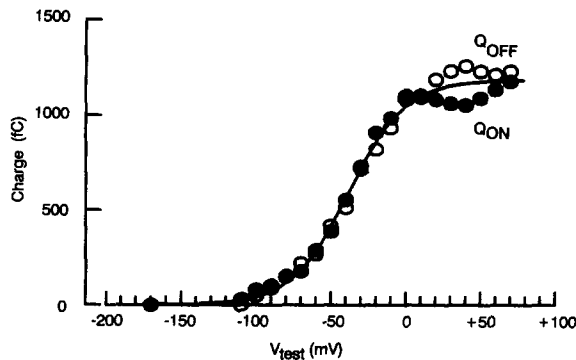


FIGURE 3. Voltage dependence of nonlinear charge movement.  $Q_{ON}$  and  $Q_{OFF}$  were calculated as the integral of the current in Fig. 2. Since the linear capacity of the cell was 71 pF, 1,000 fC on the ordinate corresponds to about 14 nC/ $\mu$ F. Cell W38D.

moved the charge more rapidly. Both the charge movement at the onset of the depolarization (ON charge,  $Q_{ON}$ ) and that moving in the opposite direction on repolarization (OFF charge,  $Q_{OFF}$ ) showed saturation with large depolarizations. In most cells, the saturating values of ON charge and OFF charge were either nearly equal (as for the cell in Figs. 2 and 3) or the OFF charge was slightly less than ON charge (see Table I); in 15 cells from which fairly complete measurements were obtained, the ratio of OFF charge to ON charge was  $0.94 \pm 0.05$ .

The size of the nonlinear charge movement in Fig. 2 was fairly typical, with about 1,100 fC of charge moving at large depolarizations. As shown in Table I, the charge moved by large depolarizations varied from ~500 to 2,000 fC (mean 1,060 fC) and

TABLE I  
*Cell Capacitance, Charge Movement, and Barium Current in Rabbit and Rat Ventricular Cells*

Cell species	$C_{cell}$	Negative holding potentials			Depolarized holding potentials			$I_{Ba}$
		$Q_{ON}$	$Q_{OFF}$	HP	$Q_{ON}$	$Q_{OFF}$	HP	
	pF	fC	fC	mV	fC	fC	mV	pA
F06D Rabbit	84	1,104	670	-100				530
F07B Rabbit	72	1,011	680	-100	480	680	-50	2,700
F08A Rabbit	91	1,070	934	-110	740	914	-50	20
F08C Rabbit	43	550	470	-110	310	375	-50	
F09A Rabbit	125	2,155	2,443	-100				
F09B Rabbit	91	788	757	-110	450	663	-50	90
F09D Rabbit	131	949	1,272	-110	593	1,389	-50	690
F09F Rabbit	98	1,018	927	-110	623	920	-50	680
F10A Rat	107	800	900	-110	595	770	-50	95
F10E Rat	93	474	568	-110	332	571	-50	169
F10G Rat	155	1,015	940	-110	683	749	-50	500
F10H Rat	65	1,096	835	-110	690	835	-50	510
W37A Rat	196	1,889	1,834	-110				
W38D Rat	71	1,105	1,000	-110	550	880	-40	795
W38F Rat	95	877	759					225
Mean	101	1,060	999		550	795		584
SEM	10	113	133		43	77		207

was correlated with the size of the cell as determined from linear cell capacitance (mean 101 pF). Normalized to linear capacitance, the ON charge was 5–20 fC/pF (nC/ $\mu$ F), with a mean of  $11 \pm 1$  fC/pF. There were no striking differences between rabbit and rat ventricular cells.

In most cells, as in Fig. 2, the “control” records used for linear leak and capacity current correction were obtained from steps in the most negative voltage region that could safely be explored, usually in the range of  $-110$  to  $-140$  mV. It seems reasonable to assume that the control currents in this range are little contaminated with nonlinear charge movement themselves, since there was usually little or no nonlinearity in the range  $-170$  to  $-100$  mV. In some experiments, we followed a different strategy, using control steps in a very positive voltage range, delivered from positive holding potentials (cf. Bezanilla et al., 1982; Brum and Rios, 1987). There should also be little movement of nonlinear charge movement in these steps, since the charge movement is saturated at these potentials (Fig. 3); in agreement with this, there was little or no difference in the corrected records when both methods were used in the same cell. This lends confidence to the idea that the nonlinear currents arise from extra charge moving outward during the test depolarizations, not from charge moving during control pulses.

#### *Dependence on Holding Potential*

Holding the cell at more depolarized potentials than the usual  $-100$  to  $-110$  mV resulted in a decrease in ON charge movement. Fig. 4 shows a typical example. The lower panel of the figure compares four current records. The upper two records are from steps to  $+10$  mV delivered from steady holding potentials of either  $-110$  or  $-40$  mV. In both cases, the step to  $+10$  mV was preceded and followed by a brief time at  $-90$  mV so that the size of the steps, and thus the linear components of capacitative and leak currents, were identical in the two cases; all that differs is the steady holding potential. The ON charge was considerably reduced when the holding potential was depolarized, from 1,050 fC at a holding potential of  $-110$  mV to 600 fC at a holding potential of  $-40$  mV. In contrast, the OFF charge was unaffected by the change in holding potential.

When the holding potential in the experiment in Fig. 4 was depolarized, the decrease in nonlinear charge movement for a step to  $+10$  mV was accompanied by an increase in inward nonlinear charge moved at very negative test potentials. As already discussed, with negative holding potentials there was little nonlinearity in charge movement in the region of  $-100$  to  $-170$  mV or so. The lower current traces in Fig. 4 show the current for a step to  $-160$  mV (from the pedestal of  $-90$  mV), corrected for linear capacitative currents and leak currents obtained from a step from  $-120$  to  $-140$  mV. With a holding potential of  $-110$  mV, the subtraction resulted in a small inward current during the step from  $-90$  to  $-160$  mV; this small nonlinear current could arise from extra charge movement during either the test pulse or the control pulse (or a combination of the two). Changing the holding potential to  $-40$  mV resulted in a substantial increase in this inward nonlinear charge movement; this new charge movement must be mainly extra inward charge moving during the test pulse, since the control pulse was the same as for the more negative holding potential.



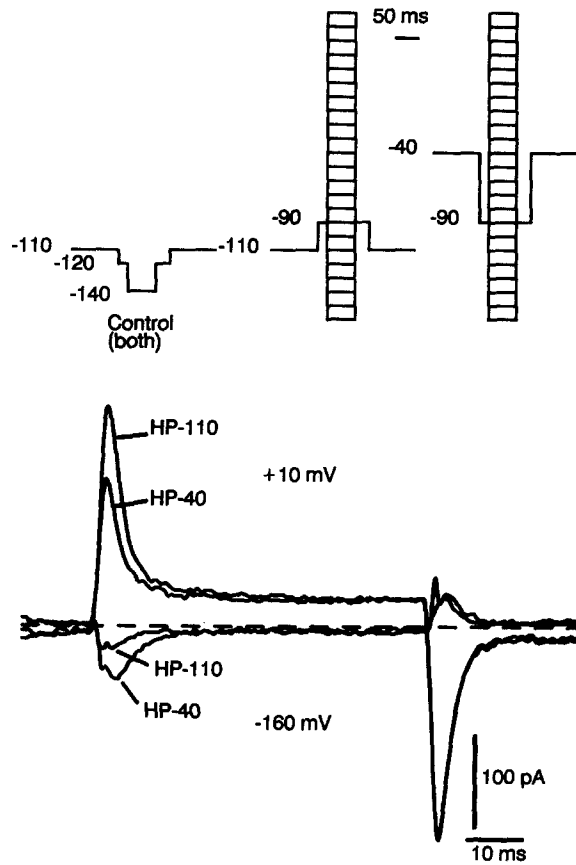


FIGURE 4. Effects of holding potential on nonlinear charge movement. Asymmetry currents elicited for steps to +10 and -160 mV from steady holding potentials (HP) of -110 and -40 mV are shown; the records from -40 mV were obtained about 1 min after changing the holding potential. Cell W38D.

Fig. 5 shows (for the same cell as in Fig. 4) the dependence on holding potential of the nonlinear charge moved as a function of test potential. Depolarizing the holding potential to -40 mV caused a decrease in the outward nonlinear charge moved by test pulses positive to -50 mV and an increase in the inward charge moved by

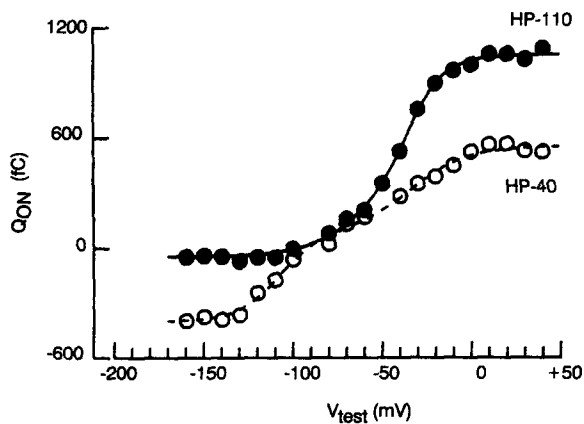


FIGURE 5. Voltage dependence of  $Q_{ON}$  from two different holding potentials. Data are from the same cell as in Fig. 4 and the pulse protocols were as shown there. Solid line through data from -110 mV:  $-49 + 546/[1 + \exp \{-(V + 56)/18\}] + 557/[1 + \exp \{-(V + 34)/9\}]$ . Solid line through data from -40 mV:  $-419 + 507/[1 + \exp \{-(V + 108)/14\}] + 518/[1 + \exp \{-(V + 29)/19\}]$ . Cell W38D.

pulses negative to  $-100$  mV: the effect of holding at  $-40$  mV is to change the voltage dependence with which nonlinear charge moves. With a holding potential of  $-110$  mV, most charge movement is in the range of  $-80$  to  $0$  mV, and the voltage dependence of the nonlinear charge movement can be fit reasonably well by a simple Boltzmann distribution (although the fitted line in Fig. 5 is the sum of two Boltzmann expressions with midpoints of  $-56$  and  $-34$  mV, a single Boltzmann expression with a midpoint of  $-42$  mV would fit almost as well). With a holding potential of  $-40$  mV, there are apparently two components of charge movement with greatly different voltage dependencies; one component moves in the range  $-80$  to  $0$  mV (very similar to that with the holding potential of  $-110$  mV, but only  $\sim 60\%$  as large) and the other component moves in the range  $-100$  to  $-150$  mV, where there is little charge movement with the more negative holding potential. It is as if changing the holding potential to  $-40$  mV converts  $\sim 40\%$  of the charge moving in the range of  $-80$  to  $0$  mV into a charge moving in the range of  $-100$  to  $-150$  (the increase in charge moving in the negative voltage range is just slightly less than the decrease in the charge moving in the positive voltage range).

The conversion, by a change in holding potential, of charge moving in a positive voltage range to charge moving in a negative range is strongly reminiscent of a result obtained by Bezanilla et al. (1982) with Na channel gating current in squid axons. Earlier experiments (Armstrong and Bezanilla, 1977) established that depolarized holding potentials which inactivate Na channels result in a reduction (by about two-thirds) of the gating current moved by large test depolarizations; Bezanilla et al. (1982) found that this decrease was accompanied by a (nearly equal) increase in charge moving in a very negative voltage range. Apparently, when a Na channel becomes inactivated, gating charge no longer moves in the usual voltage range (where channels no longer open), but can be made to move (in the opposite direction) by application of very negative test pulses.

#### *Charge Immobilization and Na Channel Inactivation*

This parallel raises the obvious possibility that the component of charge in heart cells whose voltage dependence changes with holding potential arises from Na channels. A component of gating charge from Na channels would certainly be expected since these cells have large Na currents (e.g., Brown et al., 1981). We tested this possibility by comparing the voltage dependence of charge immobilization with that of Na current inactivation. Fig. 6 shows an experiment in which the voltage dependences of the two phenomena were determined in the same cell under nearly the same ionic conditions. First, charge movement was measured as usual, using the Na-free external solution (with TEA as the main cation). The dependence of ON gating charge on holding potential was determined using a voltage protocol like that in Fig. 4, but varying holding potential over a wide range ( $-120$  to  $+20$  mV). As in the cell in Figs. 4 and 5, increasingly depolarized holding potentials in the range of  $-110$  to  $-30$  mV resulted in a reduction of ON gating charge (elicited by a step to  $+80$  mV), with no change in the OFF gating charge. Next, the external solution was changed to allow measurement of ionic Na current: the TTX was removed and  $10$  mM NaCl was added to the solution, which was otherwise identical to that used for the charge movement measurements. Since the other constituents ( $154$  mM TEA Cl,

10 mM BaCl<sub>2</sub>, 6 mM CdCl<sub>2</sub>, and 0.1 mM LaCl<sub>3</sub>) are unchanged, the addition of 10 mM NaCl should result in only a minimal change in ionic strength or surface charge screening, so the voltage-dependence of Na channel gating ought to be little affected. The use of 10 mM Na kept the size of the Na current small enough to avoid significant series resistance errors. With Na present, the initial outward charge movement was followed by an inward ionic Na current, as expected. Depolarization

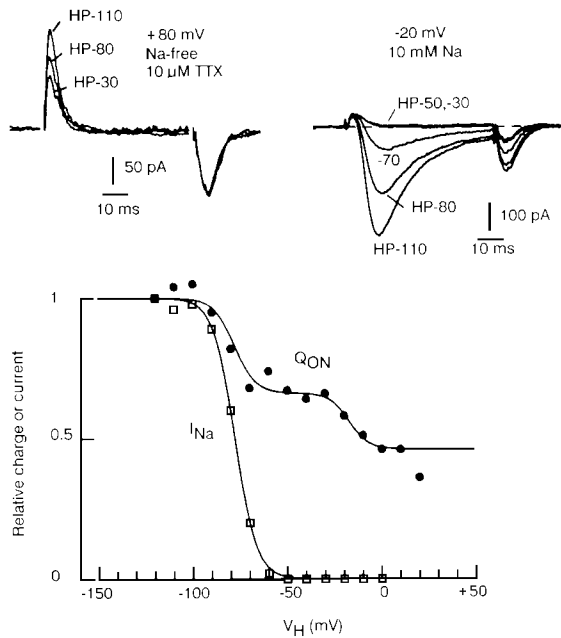


FIGURE 6. Inactivation of sodium current and charge immobilization in the same cell. (*Top left*) Nonlinear charge movement at +80 mV elicited from different holding potentials; external solution (in millimolar) 10 BaCl<sub>2</sub>, 6 CdCl<sub>2</sub>, 0.1 LaCl<sub>3</sub>, 154 TEA Cl, 10 μM TTX, 10 HEPES, pH 7.4. Charge movement was determined using a voltage protocol like that in Fig. 4. From different holding potentials (established for 4 s), the voltage was stepped to -80 mV for 40 ms, then stepped to +80 mV for 60 ms (and then returned to -80 mV for 31 ms before returning to the holding potential). Leak and linear capacitance were determined using steps from +30 to +70

mV, from a steady holding potential of +30 mV. (*Top right*) Na currents recorded in the same cell. Pulse protocol and solutions were identical to those for charge movement except that the test pulse was -20 mV (near the maximum of the peak current-voltage curve for Na current), TTX was omitted, and 10 mM NaCl was added. Leak and linear capacitance correction from steps from +30 to +70 mV. Bottom:  $Q_{ON}$  and peak  $I_{Na}$  as a function of holding potential. Values were normalized to those recorded with a holding potential of -120 mV ( $Q_{ON}$ : 1,119 fC,  $I_{Na}$ : 413 pA). In each case, holding potential was stepped in 10-mV increments from -120 to +20 mV;  $Q_{ON}$  (●) or  $I_{Na}$  (□) was determined 4 s after establishing the new holding potential. Solid line through  $I_{Na}$  points:  $1/[1 + \exp[(V_H + 77)/5.6]]$ . Solid line through charge movement points:  $0.46 + 0.34/[1 + \exp[(V_H + 77)/5.6]] + 0.20/[1 + \exp[(V_H + 17)/5]]$ . Cell F08A, rabbit ventricular, 91 pF, 10°C.

of the holding potential resulted in inactivation of the Na current, which completely disappeared at a holding potential of -50 mV. The voltage dependence of Na channel inactivation could be fit well by a simple Boltzmann curve with a midpoint of -77 mV and a slope factor of 5.6. As shown in the lower part of Fig. 6, the voltage dependence of Na current availability corresponds exactly with the voltage dependence of ON charge availability over the same voltage range; the data points from

ON charge movement are fit by the sum of two Boltzmann expressions (plus a constant), one of which has the same midpoint and slope factor as the Boltzmann expression used to fit the Na current data.

The close similarity in voltage dependence suggests that the portion of gating charge that is reduced by depolarization of holding potential in the range  $-110$  to  $-50$  mV ( $\sim 35\%$  of the total in Fig. 6, similar to the  $40\%$  in Fig. 5) does, in fact, arise from Na channel gating. In 11 cells, the reduction of ON gating charge averaged  $38 \pm 3\%$  when the holding potential was depolarized from  $-110$  or  $-100$  mV to  $-50$  or  $-40$  mV.

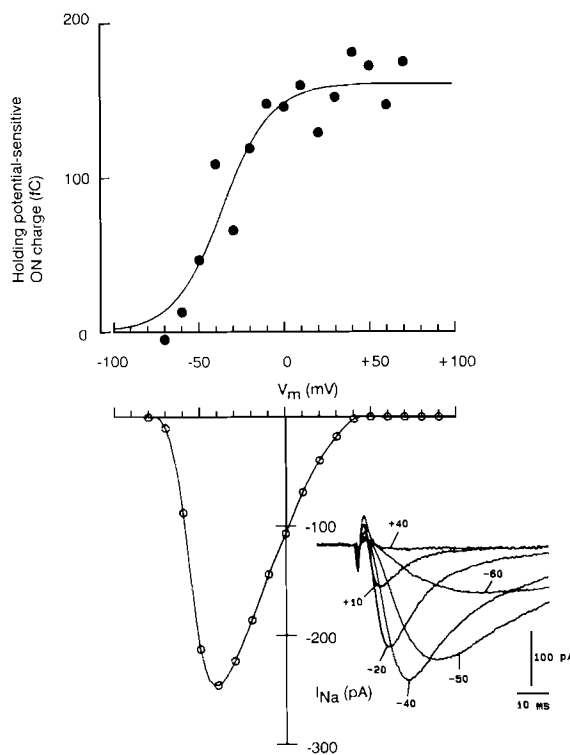


FIGURE 7. The voltage dependence of  $I_{Na}$  activation and holding potential-sensitive charge movement. (*Top*) Holding potential-sensitive charge movement was determined by subtracting (for each test voltage) the nonlinear charge movement elicited from a holding potential of  $-50$  mV from that elicited from  $-110$  mV; pulse protocol was as in Fig. 4, with a prepulse to  $-80$  mV for 40 ms before each test pulse. (*Solid line*)  $161/[1 + \exp[-(V + 36)/14.5]]$ . (*Bottom*) Peak  $I_{Na}$  vs. test pulse potential.  $I_{Na}$  was recorded using a holding potential of  $-100$  mV (and with a 40-ms prepulse to  $-80$  mV), using 10 mM Na, as in Fig. 6. For both charge movement and  $I_{Na}$ , linear leak and capacity components were subtracted using a control pulse from  $+30$  to  $+70$  mV from a holding potential of  $+30$  mV. Cell F08C, rabbit ventricular, 43 pF,  $10^\circ\text{C}$ .

#### Comparison with Na Current Activation

It is important to check that the putative Na channel gating charge moves in a voltage range reasonable for Na channel gating. Fig. 7 shows that this is so. In this cell, the test voltage dependence of the charge immobilized at  $-50$  mV (*top*) was compared with the test voltage dependence of Na current determined after adding 10 mM Na to the external solution (*bottom*). Consistent with the hypothesis that the immobilized charge arises from Na channel gating, there is significant holding potential-sensitive ON charge movement at the potentials (positive to  $\sim -60$  mV)

where Na current is significantly activated. The possible movement of holding potential-sensitive charge at even more negative potentials (as expected from squid axon results, where gating charge moves considerably negative to actual channel opening [see Armstrong, 1981]) would not have been detected in this experiment because the prepulse potential was  $-80$  mV; further experiments will be needed to better define the relation between gating charge movement and Na channel opening. However, the results seem consistent with supposing that most or all of the holding potential-sensitive ON charge arises from Na channel gating.

#### *D600 Sensitivity of Charge Movement*

What is the origin of the charge movement that remains at a holding potential of  $-50$  mV? The most obvious possibility is that this charge arises from the gating of Ca channels, which are the most prominent voltage-gated channels in the cells after Na channels. If so, it seemed possible that this component of charge movement would be sensitive to calcium channel blockers. We therefore tested the effects of D600, a phenylalkylamine drug that is a potent blocker of cardiac Ca current (e.g., McDonald et al., 1980; Lee and Tsien, 1984). Block of Ba currents by D600 under our experimental conditions is shown in Fig. 8 A. The cell was held at  $-50$  mV and Ba current was elicited by a step to  $-10$  mV (preceded by a 35-ms step to  $-80$  mV). Application of  $10 \mu\text{M}$  D600 inhibited the Ba current by  $\sim 40\%$ . Then, two long conditioning pulses to  $-10$  mV, lasting 14 and 21 s, were given, and the holding potential was returned to  $-50$  mV. The conditioning pulses to  $-10$  mV greatly enhanced D600 block, so that now the Ba current was almost totally blocked even though the holding potential was returned to  $-50$  mV. The complete block persisted as long as the holding potential remained at  $-50$  mV. In experiments on other cells, it was found that holding for a minute or so at a very negative holding potential ( $-120$  to  $-110$  mV) could partially reverse the enhanced block.

One reason the cell in Fig. 8 A was chosen for the experiment shown was that (for unknown reasons) it had an unusually small Ba current, so that the outward ON charge movement could be seen fairly clearly, preceding the ionic current. The D600 block of the ionic current was accompanied by effects on the charge movement. The initial application of D600 at  $-50$  mV resulted in a very small decrease in the peak of the outward charge movement. However, after the conditioning pulses to  $-10$  mV and complete block of the Ba current, there was a substantial reduction in the peak of the outward charge movement.

Fig. 8 B shows the effects of D600 in another cell in which the ionic current was blocked to allow better resolution of charge movement. The voltage protocol and sequence of holding potential changes were virtually identical to those in Fig. 8 A. Initially, the holding potential was  $-50$  mV and a test pulse to  $+20$  mV (preceded by a 45-ms prepulse at  $-80$  mV) elicited charge movement. D600 ( $10 \mu\text{M}$ ) was applied, and it produced a modest reduction in the ON charge movement (to  $\sim 70\%$  of control). A long conditioning pulse (28 s) was given to  $-10$  mV, after which the holding potential was returned to  $-50$  mV; now, there was much greater inhibition of the charge movement (to  $\sim 15\%$  of control). As with conditioning pulse-enhanced block of ionic current, the charge movement remained at this reduced level as long as the holding potential remained at  $-50$  mV.

Nearly identical results were obtained in repetitions of the experiment in two other cells; the collected results are summarized in Fig. 9. In the absence of D600, there was little effect of a long conditioning pulse to  $-10$  mV (reduction to  $94 \pm 2\%$  of control). Applied at a steady holding potential of  $-50$  mV,  $10 \mu\text{M}$  D600 reduced ON charge to  $69 \pm 7\%$  of control; the reduction was to  $13 \pm 3\%$  after application of the conditioning pulse to  $-10$  mV. The D600/conditioning pulse combination suppressed nearly equal amounts of ON charge ( $498 \pm 133$  fC) and OFF charge ( $473 \pm 86$  fC). After the treatment, the remaining ON charge (from a holding

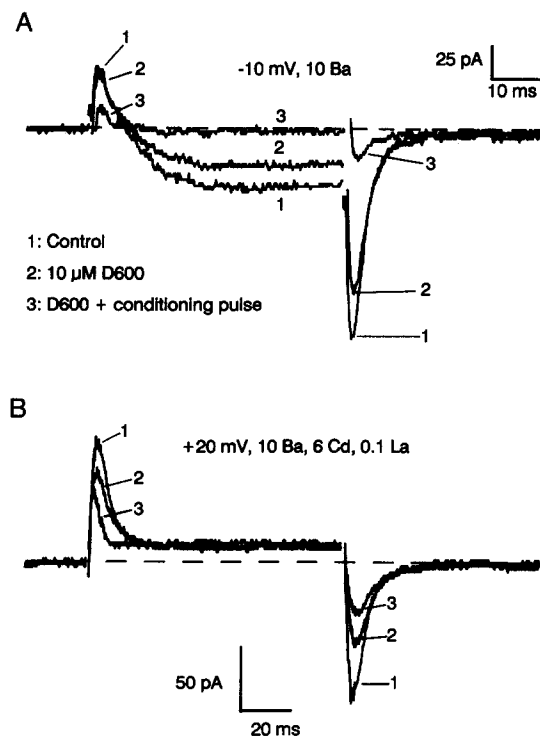


FIGURE 8. Effects of D600 on  $I_{Ba}$  and charge movement. (Top) Effects of  $10 \mu\text{M}$  D600 on current carried by  $10$  mM Ba. Holding potential  $-50$  mV, test pulse to  $-10$  mV (with a pedestal potential of  $-80$  mV preceding [by 25 ms] and following the test pulse). Trace 2 was recorded 80 s after application of D600, trace 3 was recorded 53 s later, 7 s after two conditioning pulses to  $-10$  mV, lasting 14 s and 21 s (and separated by 14 s at  $-50$  mV). Linear capacity and leak corrected using step from  $-110$  to  $-150$ , from a steady holding potential of  $-120$  mV. Cell F10C, rat ventricular, 47 pF,  $11^\circ\text{C}$ . (Bottom) Effects of  $10 \mu\text{M}$  D600 on charge movement. Holding potential  $-50$  mV, test pulse to  $+20$  mV (preceded and followed by 50 ms at  $-80$  mV).

Trace 2 was recorded about 5 min after application of  $10 \mu\text{M}$  D600; trace 3 was recorded 35 sec later, 7 s after a 28-s conditioning pulse to  $-10$  mV. Linear capacity and leak corrected using control step from  $-110$  to  $-140$ , from a steady holding potential of  $-120$  mV. Cell F10H, rat ventricular, 65 pF,  $15^\circ\text{C}$ .

potential of  $-40$  mV) was  $67 \pm 23$  fC while the remaining OFF charge was  $261 \pm 103$  fC.

The effects of D600 on Ca channel current, including the striking long-lasting potentiation of block by a conditioning pulse to  $-10$  mV, are thus mimicked closely by its effects on charge movement. The results seem consistent with the idea that most of the charge movement that is not inactivated by holding at  $-50$  mV could arise from Ca channel gating.

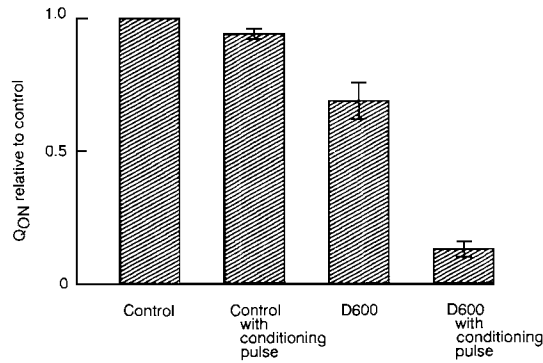


FIGURE 9. Collected results of D600 inhibition of charge movement in three rat ventricular cells. Pulse protocols were as in Fig. 8, with small variations. Control values were  $Q_{ON}$  (determined at test potential of +20 to +50 mV) elicited from a holding potential of -50 mV. In the absence of D600, 24–28-s conditioning pulses to -10 mV had little effect on  $Q_{ON}$  recorded 7–14 s after returning to -50 mV ( $94 \pm 2\%$

of control values). After application of  $10 \mu\text{M}$  D600 at the holding potential of -50 mV,  $Q_{ON}$  was reduced to  $69 \pm 7\%$  of control. After the conditioning pulses to -10 mV,  $Q_{ON}$  was reduced to  $13 \pm 3\%$  of control. Temperature 10–15°C.

#### *Charge Immobilization and Ca Channel Inactivation*

Another way to test this proposition is to examine the effects of Ca channel inactivation. Fig. 10 shows that Ca channel inactivation is accompanied by a reduction in ON charge movement. In this cell, the dependence of Ba current on holding potential was determined before blockade of the current by Cd and La. There was a reduction in the Ba current as the holding potential was depolarized from -40 to

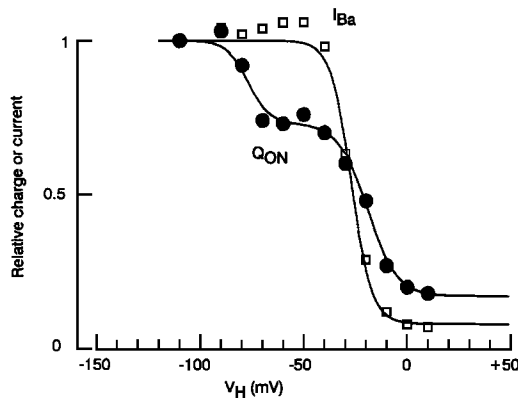


FIGURE 10. Inactivation of  $I_{Ba}$  and charge movement compared in the same cell. ( $\square$ ) Peak  $I_{Ba}$  recorded in an external solution containing 10 mM Ba and no blockers. Test pulse to -10 mV (preceded by 40 ms at -80 mV); holding potential was varied from -110 to +10 mV in 10-mV steps, with test pulse current recorded 7 s after establishment of the new holding potential.  $I_{Ba}$  was normalized with respect to that recorded with a holding potential of -110 mV (361 pA). Linear capacity and leak corrected using control step

from -100 to -130, from steady holding potential of -110 mV. ( $\bullet$ )  $Q_{ON}$  recorded in the same cell after addition of 6 mM Cd and 0.1 mM La. Test pulse to +10 mV (preceded by 40 ms at -80 mV); holding potential was changed in an identical fashion as for  $I_{Ba}$ .  $Q_{ON}$  was normalized with respect to that recorded with a holding potential of -110 mV (1,061 fC). Linear capacity and leak corrected using control step from -100 to -120 from a steady holding potential of -100 mV. Solid line through  $I_{Ba}$  points:  $0.08 + 0.92/[1 + \exp \{(V_H + 27)/5\}]$ . Solid line through  $Q_{ON}$  points:  $0.17 + 0.27/[1 + \exp \{(V_H + 77)/5.2\}] + 0.56/[1 + \exp \{(V_H + 19)/7\}]$ . Cell F09F, rabbit ventricular, 98 pF, 10°C.

-10 mV, and the voltage dependence could be fit reasonably well by a Boltzmann expression with a midpoint of  $\sim -25$  mV. About 10% of the current was apparently resistant to inactivation even at very positive potentials; inactivation was incomplete in all cells studied and, in fact, inactivation was usually less complete than in the cell shown in Fig. 10. There was little inactivation over the range from -100 to -40 mV, supporting the idea that the current was purely L-type current. After block of the ionic current, the effects of holding potential on ON charge movement were determined using the same voltage protocol (except with a more positive test pulse in order to maximize ON charge). As in the similar experiment already shown in Fig. 6, reduction of ON charge movement by holding potential occurred in two distinct phases. About 30% of the charge is lost over the range of -90 to -50 mV, corresponding to the range of Na channel inactivation. Another 50% or so is lost over the range of -50 to +10 mV. As Fig. 10 shows, the voltage dependence of this second phase of reduction is similar to that of Ba current inactivation. A Boltzmann expression corresponding to this second phase of charge movement reduction has a midpoint of -19 mV, somewhat more positive than the inactivation curve midpoint of -27 mV. The difference might be expected if the two curves reflected the same process, since the charge movement was determined after addition of 6 mM Cd (and 0.1 mM La) to the external solution, which (by more effective screening of negative surface charge) would tend to shift the voltage dependence of intramembrane processes in a positive direction. It is interesting that less of the nonlinear charge was lost in this voltage range in the experiment in Fig. 6; this may fit with the observation that inactivation of Ba current is sometimes only 60–80% complete even at 0 mV or so.

#### *Comparison of Charge Movement and Ca Channel Current*

Figs. 11 and 12 show a comparison of the nonlinear charge movement elicited from -40 mV with the Ba current recorded in the same cell. Fig. 11 shows the voltage dependence of the Ba current; significant activation is first detectable at  $\sim -50$  mV, and the peak of the current-voltage relation is at  $\sim -20$  mV. As judged by its inactivation kinetics and voltage dependence, the current was purely L-type current. L-type currents in rat heart cells differ from those in other species (for example, rabbit and frog) in having an unusually slowly decaying component of tail current (Fig. 11). The slowly decaying component of tail current is preferentially activated by large depolarizations; both slow and fast components of tail current are blocked by nimodipine (Bean, B. P., unpublished observations) implying that both arise from L-type channels. The lower curve in the bottom panel shows an activation curve for the Ca channels, constructed by plotting the size of the tail current (measured at -70 mV) as a function of test pulse size. (The two-component appearance of the activation curve could, in principle, reflect two different populations of channels, but it could equally well arise from a single-channel type having a complex voltage dependence, perhaps related to the slow and fast components of tail current.) After block of the ionic current by Cd and La, the voltage dependence of nonlinear charge movement was determined in the same cell. Fig. 12 plots the nonlinear charge movement measured (using a holding potential of -40 mV so that the supposed Na channel component is lacking) and compares it with the activation curve



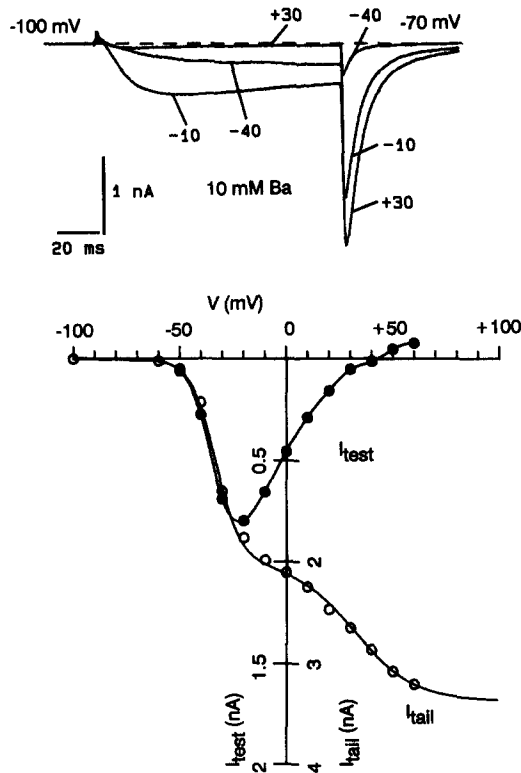


FIGURE 11. Voltage dependence of  $I_{Ba}$  activation. (Top) Currents during and after 120-ms test pulses to various potentials from  $-100$  mV holding potential; tail currents at  $-70$  mV. Leak corrected using step from  $-100$  to  $-114$  mV. (Bottom) Peak test pulse and tail currents vs. test pulse potential. Cell W38D, rat ventricular, 71 pF,  $7^\circ\text{C}$ .

for Ba conductance. There is some charge movement negative to the voltages at which Ba current first begins to activate, and there is more charge movement over the voltage range at which Ca channels are opened ( $-50$  to  $\sim +30$  mV), as must be true if some of the charge movement does actually arise from Ca channel gating.

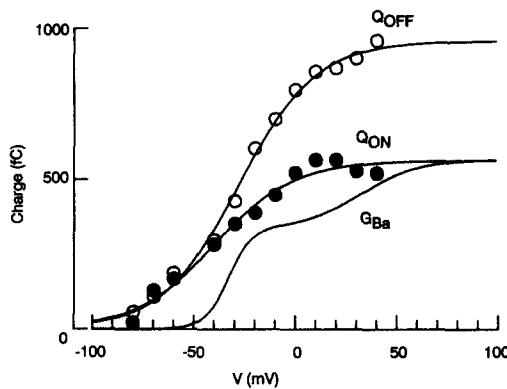


FIGURE 12. Comparison of  $G_{Ba}$  and charge movement elicited from  $-40$  mV. ON charge movement was determined during 120-ms test pulses to various potentials delivered from a holding potential of  $-40$  mV (and preceded by a 16-ms step to  $-90$  mV); OFF charge movement was determined during a 60-ms step to  $-80$  mV after each test pulse. Linear capacitance and leak corrected using step from  $-120$  to  $-140$  from steady holding potential

of  $-100$  mV.  $G_{Ba}$  curve is from fit to tail current data in Fig. 11. Solid line through  $Q_{ON}$  points:  $563/[1 + \exp [-(V + 40)/19]]$ . Solid line through  $Q_{OFF}$  points:  $959/[1 + \exp [-(V + 28)/19]]$ . Cell W38D, rat ventricular, 71 pF,  $7^\circ\text{C}$ .

Fig. 12 also shows that the saturating OFF charge is considerably larger than the ON charge when a holding potential of  $-40$  mV was used. This was a consistent observation (Table I), with the ratio of  $Q_{\text{OFF}}/Q_{\text{ON}}$  averaging  $1.46 \pm 0.11$  when studied in 11 cells using holding potentials of  $-50$  to  $-40$  mV. As will be discussed, this observation may be at odds with the idea that all of the nonlinear charge movement arises from Na channels and Ca channels and may suggest contributions from other sources as well. Quantitatively, the extra OFF charge ( $\sim 250$  fC) corresponds well with the component of OFF charge that is not suppressed by the D600/conditioning pulse combination ( $\sim 260$  fC).

#### DISCUSSION

As shown already by the work of Beam and Knudson (1988) on dissociated skeletal muscle cells, the whole-cell recording technique is well suited for recording intramembrane charge movement in single cells. The signal/noise ratio is high compared with that of other voltage clamp techniques; background noise is low due to the high resistance of both the electrode seals and the cells, which can be efficiently dialyzed with impermeant ions. No signal averaging was necessary to obtain records of nonlinear charge movement with reasonably low noise. In any cell with a reasonable density of voltage-dependent channels, it should be quite feasible to combine recordings of macroscopic current, single channels, and gating current in the same preparation.

##### *Components of Nonlinear Charge Movement*

Our results suggest that most of the nonlinear charge movement in mammalian ventricular cells arises from Na channel and Ca channel gating. The identification of Na channel gating charge rests on the finding that  $\sim 40\%$  of the total nonlinear charge movement is immobilized (or more precisely, shifted in its voltage dependence) with the same dependence on holding potential as Na channel inactivation. If, as in squid axons, only two-thirds of the Na channel gating charge becomes immobilized, then  $\sim 60\%$  of the total nonlinear charge is Na channel gating current.

The identification of most of the charge moving from holding potentials near  $-50$  mV as Ca channel gating current is suggested by the immobilization of much of this charge with a voltage dependence similar to that of Ca channel inactivation and by the inhibition of most of this charge by D600, with a phenomenology similar to that of Ca channel block. However, as noted below, another possibility is that some of the D600-sensitive charge is not from Ca channel gating but from another voltage-dependent process governing Ca release from internal stores. It should be noted that D600 is not perfectly selective, since it can also block Na current under some conditions (e.g., Bustamante, 1985); however, with our protocol it seems unlikely that the D600-sensitive charge included much Na channel gating charge since (a) D600 was applied at holding potentials where Na channels were already completely inactivated and (b) the inhibition was weak until conditioning pulses were given that put Ca channels in the inactivated state. There is no reason to think that these conditioning pulses would affect D600 interaction with the Na channels, which would be in the inactivated state throughout; in contrast, it was shown

directly that inhibition of charge and inhibition of Ca current were enhanced in parallel by the pulses (Fig. 8).

With depolarizing test pulses given from holding potentials near  $-50$  mV, OFF charge is  $\sim 45\%$  larger than ON charge. The origin of this extra component of OFF charge, which is not inhibited by D600, is unknown. One possibility is that cardiac Na channels behave differently from those in squid axon and when inactivated produce a detectable OFF current when the membrane is repolarized to  $-80$  mV or so. In both squid axons (Armstrong and Bezanilla, 1977) and frog skeletal muscle (Campbell, 1983), OFF charge from inactivated channels is fast enough to be detectable at very negative potentials ( $-120$  mV) or so; perhaps the return of charge at  $-80$  mV is faster in cardiac muscle or is simply more easily resolved because of better signal to noise. A second possibility is that the OFF charge arises from gating of a channel which is activated (rapidly) by the hyperpolarization following the test pulse, giving an OFF charge movement, but which does not produce an ON charge movement; as will be discussed, a possible source is inward rectifier channels.

#### *Contributions from Other Channels*

Delayed rectifier channels seem unlikely to contribute significantly to the nonlinear charge movements. The channels both activate and deactivate very slowly, with time constants of hundreds of milliseconds (e.g., McDonald and Trautwein, 1978; Bennett and Begenisich, 1987; Matsuura et al., 1987; Giles and Imaizumi, 1988). If the gating charge moved with a similar time course it would be lost in the baseline unless it were enormous. In any case, delayed rectifier current is very small in rabbit ventricular cells (Giles and Imaizumi, 1988).

Channels producing the transient outward current observed in rat (Josephson et al., 1984) and rabbit (Giles and Imaizumi, 1988) also probably generate little charge movement. With macroscopic currents of  $2\text{--}6$  nA at  $0$  mV (Josephson et al., 1984), generated by single channels of  $\sim 2$  pA with a 20% probability of being open (Bennendorf et al., 1987), there would be  $\sim 5,000\text{--}15,000$  channels in a rat ventricular cell. Assuming six electron charges per channel, this would correspond to a maximum of  $\sim 15$  fC,  $< 2\%$  of the nonlinear charge we measured. Moreover, transient outward currents in rabbit ventricular cells are far smaller than in rat cells (Giles and Imaizumi, 1988), yet the charge movement was quantitatively very similar in the two cell types.

Inwardly rectifying potassium channels are probably present at a high density in mammalian ventricular cells since they underlie the large background potassium conductance. They gate in a voltage-dependent manner, being opened by hyperpolarization and closed by depolarization (Kurachi, 1985; Sakmann and Trube, 1984; Harvey and TenEick, 1988); in addition to intrinsic voltage-dependent gating, there is voltage-dependent ionic block by internal Mg ions which is enhanced at depolarized potentials and relieved at hyperpolarized potentials (see Matsuda, 1988). Both processes could produce nonlinear charge movement. Opening of the channels could produce the D600-resistant component of OFF charge we saw at depolarized holding potentials. Since the channels become partially inactivated at negative potentials (Sakmann and Trube, 1984; Biermans et al., 1987; Harvey and TenEick,

1988) charge might become immobilized and then move too slowly during depolarization (as channels recover from inactivation) to be easily detectable; this would explain why there is no equivalent contribution to the ON charge. Harvey and Ten-Eick (1988) found that channels recover from inactivation with a time constant of  $\sim 4$  ms at room temperature; under our conditions ( $10^{\circ}\text{C}$ ) recovery would be slower and quite possibly the charge movement during reactivation would be too slow to measure. After being reactivated during the depolarization (typically lasting 60 ms or so) the gating charge would be available to move rapidly again as channels were opened by the repolarization of the cell.

#### *Na Channel Gating Current*

The component of ON charge movement that is eliminated by a holding potential of  $-50$  mV varied from  $\sim 150$  to  $550$  fC (Table I), with a mean of  $347 \pm 40$  fC per cell, or, normalized to cell capacitance,  $4.2 \pm 0.9$  fC/pF.

How much gating charge would be expected from Na channels? The density of Na channels can be estimated by comparing macroscopic currents in single cells with single channel currents, using the equation  $I = N \cdot p \cdot i$ , where  $I$  is the peak macroscopic current at a given voltage,  $i$  is the single channel conductance,  $N$  is the number of channels, and  $p$  is the probability that a channel is open at the peak of the macroscopic current. Macroscopic Na currents in rat ventricular cells are so large that in most studies they have been deliberately reduced by using low Na concentrations, partial inactivation, or tetrodotoxin. Probably the best estimate comes from the work of Brown et al. (1981) who used a two-suction pipette technique to clamp rat ventricular cells. They recorded peak Na currents of  $70$ – $140$  nA in  $145$  Na at room temperature, with peak Na currents occurring near  $-25$  mV. Single cardiac Na channels have a current of  $\sim 1$  pA near  $-25$  mV (Cachelin et al., 1983*b*; Kunze et al., 1985; Patlak and Ortiz, 1985; Nilius, 1988; Sheets et al., 1987), and a reasonable estimate of  $p$  would be  $\sim 0.4$ – $0.5$  at this potential (Cachelin et al., 1983*b*). These numbers lead to estimates of  $240,000$ – $600,000$  Na channels per cell (normalized to surface area estimated from capacitance using  $1 \mu\text{F}/\text{cm}^2$ , this corresponds to  $24$ – $60$  channels per square micrometer). Since the gating behavior of cardiac Na channels is very similar to that of axonal channels, it seems reasonable to estimate a minimum of six electron charges per channel (see Armstrong, 1981). Using these numbers, charge movement from Na channels would be expected to be from  $230$  to  $580$  fC, very close to the holding potential-sensitive component that we measured.

Recently, Hanck et al. (1988) have recorded Na channel gating currents in canine Purkinje cells. They recorded even larger charge movements ( $\sim 2,000$  fC) in cells of similar size, suggesting that the Na channel density may be higher in Purkinje cells. This is supported by an independent estimate of channel density made in the same cell type by Makielski et al. (1987); by comparing whole-cell and single-channel currents, they estimated a density of  $260$  channels per square micrometer, very close to the density of  $200$  channels per square micrometer predicted from the gating charge measurements of Hanck et al. If possible species differences are ignored, comparison with our results suggests that the sodium channel density is about four times lower in mammalian ventricular cells than in Purkinje cells.

*Ca Channel Gating Current*

How much charge movement would be expected from Ca channels in ventricular cells? Two quantities are needed: the gating charge per channel and the number of channels. In cardiac cells with the best resolution of currents at small depolarizations, cardiac calcium current increases 8–10-fold per 10 mV (Bean, B. P., unpublished data). For a simple two-state Boltzmann distribution, this would result if each channel had five to six elementary charges that moved across the membrane when a channel opens; more realistic multistate gating schemes could give more charge movement for a similar conductance–voltage relation (see Armstrong, 1981; Almers, 1978).

The number of channels has been estimated by comparing macroscopic and single-channel currents in guinea pig ventricular cells (McDonald et al., 1986). They arrived at a value of three to five channels per square micrometers. For a cell of 100 pF (assuming  $1 \mu\text{F}/\text{cm}^2$ ), this would be 30,000–50,000 channels per cell. With six electron charges per channel, this would produce 29–48 fC. This is an order of magnitude lower than the experimental value of 550 fC of ON charge from a holding potential of  $-50$  mV.

However, there is reason to believe that the density of Ca channels may often be much higher than the estimate by McDonald et al. In the single-channel recordings they used for the calculation, they found that the peak probability of being open near the peak of the conductance–voltage curve was  $\sim 0.8$ . Yet, the value of  $p_0$  must be much less than this in many cases. Peak macroscopic current in guinea pig ventricular cells is enhanced threefold by the dihydropyridine agonist BAY K 8644 (Hess et al., 1984), and increases of more than 10-fold can be seen in rabbit atrial cells (B.P.B., unpublished). Since BAY K 8644 acts by increasing the open channel probability (see Hess et al., 1984; Kokubun and Reuter, 1984; Tsien et al., 1986), such increases imply that the value of  $p_0$  for unmodified channels must sometimes be  $< 0.1$ . In that case, the density of channels calculated from macroscopic currents would be  $\sim 10$ -fold higher than estimated by McDonald et al. and would lead to estimates of Ca channel–associated gating charge that would be in line with the D600-sensitive charge that we measured. If this were the case, it would mean that movement of the entire gating charge of a channel would not be accompanied by the channel being open with a probability of 1; instead, large depolarizations might result in the intramembrane movement of all of the channel-associated gating charge but with the “activated” channel spending only a small fraction of the time in the open state (at least in the absence of BAY K 8644).

Another way of looking at the comparison is to note that in the cells we studied, the size of the peak Ba current varied widely, from being barely measurable ( $\sim 20$  pA) to  $> 2$  nA. (We do not know whether the smaller Ba currents reflect some run-down or damage during the isolation procedure or cell storage, but most of the cells with small currents were relaxed, striated, and appeared perfectly healthy.) Yet, there was surprisingly little variability in the magnitude of the charge movement elicited from  $-50$  mV in the different cells (Table I). One possible explanation would be that the charge movement does not in fact arise from Ca channels. However, another possibility is that cells with small Ba currents have just as high a den-

sity of Ca channels but that for some reason channel opening is very inefficient, and the channels spend very little time open after the gating charge has moved. It would be interesting to know whether run-down of Ca currents during long recordings is accompanied by loss of charge movement. We saw no decline in nonlinear charge movement even over 1–2 h of recording; we suspect that there would be some run-down of Ca current over this time but have not made long-term recordings of Ca current under the same ionic conditions and temperature.

It would be informative to perform the comparison of single channel and macroscopic current made by McDonald et al. (1986) in cells in which Ca channel current was augmented by BAY K 8644. It will be especially interesting to see what effect BAY K 8644 has on the putative charge movement from Ca channels. Also,  $\beta$ -adrenergic stimulation enhances cardiac Ca current by a different mechanism than Bay K 8644, apparently involving an increase in long-term availability of channels, which is manifested at the single-channel level by a decrease in the number of "null" sweeps during which a channel fails to open in response to depolarization (Cachelin et al., 1983a; Brum et al., 1984) and in macroscopic currents as an increase in the number of functional channels detected by noise analysis (Bean et al., 1984; see Tsien et al., 1986).  $\beta$ -Adrenergic stimulation can produce further increases in macroscopic current in the presence of BAY K 8644 (Tsien et al., 1986). An especially interesting possibility is that channels that are unavailable in the absence of  $\beta$ -adrenergic stimulation nevertheless have their gating charge moved by depolarization. Perhaps the most appropriate comparison between gating charge and Ca channel current would be in cells in which Ca current was enhanced by both  $\beta$  stimulation and BAY K 8644.

To summarize: the D600-sensitive charge movement is about 10 times more than would be expected if Ca channels have a high probability of being open when their gating charge has moved. Either a large part of the D600-sensitive charge movement is not from Ca channels, or Ca channels open very inefficiently when activated by depolarization. Another way to express this is that there may be a voltage-independent gate in addition to the voltage-dependent gating processes, so that movement of gating charge is necessary but not sufficient for channel opening.

#### *Comparison with Ca Channel Gating Current in Other Cells*

The charge movement that we suggest may accompany Ca channel gating (i.e., that remaining at a holding potential of  $-50$  mV) corresponds to about 330 electron charges per square micrometer. This is considerably lower than the estimates of 1,500–2,000 and 800 electron charges per square micrometer estimated for Ca channel gating charge in snail neurons (Kostyuk et al., 1981) and scorpion skeletal muscle (Scheuer and Gilly, 1986) (assuming  $1 \mu\text{F}/\text{cm}^2$ ). It is also less than the value of 700 electron charges per square micrometer that can be calculated from the data of Lamb and Walsh (1987) on the nifedipine-sensitive charge in rabbit fast-twitch muscle fibers, which they suggest represents a Ca channel gating current, but close to their value of  $\sim 200$  electron charges per square micrometer for slow-twitch fibers. As for our calculations, the amount of putative Ca channel gating charge in skeletal muscle corresponds to the Ca channel density estimated from macroscopic

currents only if the probability of a channel being open is quite small at the peak of the conductance–voltage curve (Lamb and Walsh, 1987).

We found that the supposed Ca channel gating charge moves at negative potentials relative to the Ca conductance–voltage relation. The same is true for a comparison of the Ca channel-associated charge movement and Ca current in rabbit skeletal muscle (Lamb and Walsh, 1987) and snail neurons (Kostyuk et al., 1981), though the difference is smaller in scorpion skeletal muscle (Scheuer and Gilly, 1986).

A particularly intriguing parallel between our results and those of Kostyuk et al. (1981) concerns the run-down of the Ca current. They found that in snail neurons, the ionic Ca current became progressively smaller during the experiment, but the decline was not accompanied by an equivalent diminution of the charge movement. Similarly, in our experiments the amount of charge movement was much more constant from cell to cell than was the size of the ionic current (Table I), and the charge movement showed little or no run-down. The hypothesis of Kostyuk et al. that the gating mechanism continues to function in channels that have run-down may also apply to cardiac Ca channels. In mechanistic terms, one might say that the voltage-independent gating process that may coexist with the voltage-dependent one is more susceptible to run-down.

#### *Comparison with Nonlinear Charge Movement in Skeletal Muscle*

The total nonlinear charge movement of 11 nC/ $\mu$ F in rat and rabbit heart cells is less than the 20–50 nC/ $\mu$ F seen in frog (e.g., Brum et al., 1988; Melzer et al., 1986) and rat fast-twitch skeletal muscle fibers (Hollingworth and Marshall, 1981; Dulhunty and Gage, 1983; Simon and Beam, 1985) or the 15 nC/ $\mu$ F seen in rabbit fast-twitch fibers (Lamb, 1986a). The difference is greater if one takes into account that ~60% of the charge movement in heart cells seems to be from Na channels, while the Na channel component may not be measured with the usual protocols for measuring slow charge movement in skeletal muscle (see Campbell, 1983; Almers, 1978). It may be more appropriate to compare the charge elicited from a holding potential of –50 mV; this was 6 nC/ $\mu$ F in our experiments, which is 2–10 times smaller than the slow charge in skeletal muscle.

Regardless of the difference in magnitude, the charge movements in the two muscle types have many similarities: when the charge versus voltage data are fit by Boltzmann curves (Fig. 12), the transition voltage of –30 to –40 mV and slope factor of ~20 are very similar to values from skeletal muscle fibers (see, for example, Simon and Beam, 1983; Lamb, 1986a; Melzer et al., 1986).

The most striking similarity is in the phenomenology of D600 block. We designed our D600 experiments based on the protocols used in frog skeletal muscle by Hui et al. (1984), who found that charge movement was eliminated only if application of D600 was accompanied by a long conditioning depolarization, after which charge remained blocked even at holding potentials where there had been little block before the conditioning depolarization. In addition, our experiments show that cardiac Ca channel current is blocked by D600 with the same phenomenology (the block increases and remains high after a long depolarizing pulse). The same use-dependent block of Ca currents by D600 was demonstrated by Fill et al. (1988) in skeletal muscle (see also Pizarro et al., 1988a).

The comparison suggests that the molecules responsible for slow charge movement in skeletal muscle are similar to cardiac Ca channels of the L type. This concept is strengthened by the observation (Brum et al., 1987; Pizarro et al., 1988*b*) that metal ions support E–C coupling in skeletal muscle with a similar selectivity sequence as the affinity of the ions for intrapore sites of cardiac Ca channels (Hess et al., 1986). More recently, Tanabe et al. (1988) demonstrated that both E–C coupling and slow Ca current are restored in dysgenic muscle when cDNA for the skeletal muscle dihydropyridine receptor is injected, indicating that the same gene product responsible for carrying Ca current has an essential role in E–C coupling, most likely that of voltage sensor.

In skeletal muscle, it seems unlikely that these dihydropyridine receptors are functioning simultaneously as Ca channels and voltage sensors in E–C coupling (Pizarro et al., 1988*a*; Feldmeyer and Luttgau, 1988); even if they had the same primary structure, the molecules could take on different roles depending on the environment or modulatory influences. In that sense, part of the charge movement in skeletal muscle could be due to voltage sensors of E–C coupling and part to functional Ca channels (see also Lamb and Walsh, 1987). All of it would be blocked by D600.

The possibility that the D600-sensitive charge in heart cells is from two sources must also be considered. As already discussed, only a fraction of the measured D600-sensitive charge is absolutely required to arise from Ca channels. It remains possible that part of the D600 charge in heart cells, perhaps even most of it, is generated by other molecules, like the E–C coupling voltage sensors in skeletal muscle.

A direct mechanism of control of Ca release by voltage is not generally accepted in heart muscle (see Morad and Goldman, 1973; Chapman, 1979, 1983). Consistent with this view, recent measurements on voltage clamped cells have shown that both contraction (London and Krueger, 1986) and intracellular Ca transients (Barceñas-Ruiz and Wier, 1987; Cannell et al., 1987) become very small at very positive test potentials, where Ca influx through Ca channels becomes negligible. Nevertheless, detailed comparison of Ca current and Ca transients have been taken as indicating that direct control of Ca release may operate in addition to Ca-induced Ca release (Cannell et al., 1987). An interesting variant of this hypothesis is a recent model (Cohen and Lederer, 1988) in which the L-type Ca channel is connected directly, mechanically, to the gating mechanism of the sarcoplasmic reticulum release channel and that both functions, Ca transport and voltage sensing for Ca release, are performed by the same molecule. If so, the mechanisms of E–C coupling in cardiac and skeletal muscle may be more similar than has generally been believed.

Our present results leave open the question of whether the D600-sensitive charge movement that we have recorded is simply a Ca channel gating current or partly arises from voltage sensors forming part of a direct sarcolemma-to-sarcoplasmic reticulum linkage (or is both at the same time). The similarity between skeletal muscle charge movement and the D600-sensitive component in heart cells is intriguing but, by itself, not especially helpful in clarifying the origins of the charge in either muscle type. In future studies, it may be informative to compare the charge movement in mammalian ventricular cells with that in sinoatrial and atrioventricular cells



or frog ventricular cells, which possess Ca channels but have much sparser sarcoplasmic reticulum (e.g., Staley and Benson, 1968; Sommer and Johnson, 1969; Page and Niedgerkerke, 1972) and many fewer couplings between sarcolemma and sarcoplasmic reticulum (Anderson et al., 1976).

While this paper was under review, Field et al. (1988) reported measurements of nonlinear charge movement in neonatal rat ventricular cells. Normalized to linear capacitance, the charge in neonatal cells is about 40% of that we recorded in adult cells; the major difference is that there is apparently very little Na channel-associated charge in neonatal cells, in line with their much smaller Na currents (compare Cachelin et al., 1983b and Brown et al., 1981). It is interesting that the charge movement tentatively assigned to Ca channel gating in neonatal cells (~4 fC/pF) is not much smaller than that in adult cells (~5 fC/pF); the Ca current densities are similar in cells of different aged animals (being slightly higher in neonatal cells [Cohen and Lederer, 1988]) but the sarcoplasmic reticulum and sarcolemma-to-sarcoplasmic reticulum couplings are much more developed in adult muscle (Page and Buecker, 1981). The comparison suggests that the development of these couplings is not accompanied by a major increase in nonlinear charge movement.

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*Note Added in Proof:* R. W. Hadley and W. J. Lederer have also reported measurements of intramembrane charge movement in adult mammalian myocytes (1989, *Journal of Physiology*, in press). Although made under slightly different ionic conditions, their measurements of the magnitude and voltage dependence of charge movement in rat and guinea pig ventricular cells agree closely with our measurements in rat and rabbit cells.

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