I_{NaCa} and I_{Cl(Ca)} contribute to isoproterenol-induced delayed afterdepolarizations in midmyocardial cells

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Zygmunt, Andrew C., Robert J. Goodrow, and Charlene M. Weigel. I_{NaCa} and I_{Cl(Ca)} contribute to isoproterenol-induced delayed afterdepolarizations in midmyocardial cells. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H1979–H1992, 1998.—The contributions of electrogenic sodium/calium exchange current (I_{NaCa}), calcium-activated chloride conductance (I_{Cl(Ca)}), and calcium-activated nonselective cation conductance to delayed afterdepolarizations (DAD) were examined. Nonselective cation channels were absent in canine M cells, since inhibition of I_{NaCa} and I_{Cl(Ca)} eliminated all calcium-activated currents without abolishing cell shortening. After the cells were treated with isoproterenol and ouabain to increase calcium loading, I_{NaCa} was 168 ± 30 × 10^{-3} pC/pF and I_{Cl(Ca)} was 114 ± 24 × 10^{-3} pC/pF. Transient overlapping inward and outward currents were evoked positive to the chloride reversal potential (E_{Cl}). Outward current was chloride sensitive, and inward current was blocked by replacement of external sodium with lithium. When E_{Cl} was −50 mV, triggered activity occurred in normal external sodium and persisted after inhibition of I_{NaCa}. Steps to −80 mV revealed oscillating inward currents in normal sodium and chloride, which persisted after inhibition of I_{NaCa}. When E_{Cl} was equal to −113 mV, I_{Cl(Ca)} opposed I_{NaCa} at the resting potential. DAD occurred in normal sodium, and inhibition of outward I_{Cl(Ca)} provoked triggered activity. We conclude that I_{NaCa} represents ~60% of the total calcium-activated current at resting potentials but that both I_{NaCa} and I_{Cl(Ca)} work in concert to cause DAD in calcium-overloaded cells.

Sodium/calcium exchange; calcium-activated chloride conductance; transient inward current; nonselective cation conductance

Interventions that elevate intracellular calcium cause normal repolarization of cardiac action potentials to be followed by oscillations in membrane potential. A calcium-activated transient inward current (I_{TI}) underlies these delayed afterdepolarizations (DAD) and may initiate triggered activity during ventricular tachycardia and ischemia-induced arrhythmias (9, 17, 18, 26, 27).

Kass et al. (19) demonstrated a reversal potential for digitalis-induced I_{TI} in Purkinje fibers and suggested that some combination of electrogenic sodium/calcium exchange current (I_{NaCa}) and calcium-activated cation current could underlie I_{TI}. However, reversal of I_{TI} was not observed in similarly treated muscle preparations, seemingly ruling out a contribution from ion channels while favoring I_{NaCa} (1, 3, 9). The finding that I_{TI} was present after inhibition of I_{NaCa} and discovery of calcium-activated channels equally permeable to sodium and potassium ions and a calcium-activated chloride conductance (I_{Cl(Ca)}) in cardiac myocytes rekindled debate that I_{TI} might be composed of more than one ionic component (4–8, 12, 38). Unfortunately, I_{Cl(Ca)} is often recorded in sodium-free solutions needed to block I_{NaCa}, making it impossible to draw conclusions regarding the relative amplitudes of I_{NaCa}, I_{Cl(Ca)} and nonselective cation conductances within Purkinje fibers as well as ventricular and atrial myocytes.

Acetylstrophanthin-d- and BAY K 8644-induced DAD were elicited in a subset of cells within the midmyocardium with unique electrical characteristics and referred to as M cells (29). I_{Cl(Ca)} in canine M cells contributes to an oscillating inward current in calcium-overloaded cells in the absence of I_{NaCa} (38). The present study was undertaken to determine the importance of I_{Cl(Ca)}, I_{NaCa}, and nonselective cation conductances to the generation of I_{TI} and triggered activity in calcium-overloaded M cells. Cell shortening, ionic currents, and action potentials were recorded after application of isoproterenol and ouabain to load myocytes with calcium. We find no evidence for calcium-activated nonselective cation channels in M cells. Although I_{NaCa} is larger than I_{Cl(Ca)} at the resting potential, either conductance on its own is sufficient to cause DAD and triggered activity. Some of this work has been reported as an abstract (41).

METHODS

Adult male mongrel dogs were given 200 U/kg heparin (sodium salt) and anesthetized with 35 mg/kg intravenous pentobarbital sodium, and their hearts were quickly removed and placed in Tyrode solution. Single myocytes were obtained by enzymatic dissociation from a wedge-shaped section of the ventricular free wall supplied by the left circumflex coronary artery (38). Cells from the midmyocardial region of the left ventricle were used in this study. All procedures followed were in accordance with guidelines established by the Institutional Animal Care and Use Committee.

Tyrode solution used in the dissociation contained (in mM) 135 NaCl, 5.4 KCl, 1 MgCl_2, 0 or 0.5 CaCl_2, 10 glucose, 0.33 NaH_2PO_4, and 10 HEPES, and pH was adjusted to 7.4 with NaOH. Standard patch-clamp technique was used to record whole-cell currents or action potentials. The composition of the standard external solution was (in mM) 2 CaCl_2, 4 KCl, 1 MgCl_2, 10 glucose, 140 NaCl, and 10 HEPES, and pH was adjusted to 7.4 with NaOH. When required, external sodium was reduced by equimolar substitution with either lithium or N-methyl-D-glucamine (NMG), and external chloride was replaced by gluconate. Sodium/calcium exchange; calcium-activated chloride conductance; transient inward current; nonselective cation conductance

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chloride was altered by equimolar substitution of aspartate for chloride. Potassium-free solutions were made by omitting KCl from the external solution and by substituting cesium for potassium in the pipette solution. When required, internal sodium was reduced by equimolar substitution of cesium. Most experiments in sodium-free solutions were accomplished without the addition of EGTA to the pipette solution. Those instances when EGTA was required are indicated in the text.

Endogenous calcium buffers are dialyzed from cells using standard patch electrodes. The amphotericin B perforated-patch technique was used in a few cells to verify recordings of action potentials and DAD obtained by standard patch technique. Composition of the external solution was (in mM) 2 CaCl2, 4 KC1, 1 MgCl2, 10 glucose, 140 NaCl, and 10 HEPES, and pH was adjusted to 7.4 with NaOH. Pipette solution contained (in mM) 0.00026 amphotericin B, 135 potassium aspartate, 10 NaCl, 10 KCl, 10 HEPES, 1 MgCl2, and 0.01 CaCl2, and pH was adjusted to 7.1 with KOH. When required, internal chloride was altered by equimolar substitution of aspartate for chloride. We have assumed that potassium, sodium, and chloride equilibrate with pipette solution within minutes of incorporation of amphotericin B in the membrane, at rates similar to those suggested for the nystatin perforated-patch technique (16a).

Amphotericin B (Sigma Chemical, St. Louis, MO) was made in dimethyl sulfoxide (60 mg/ml) and diluted 250-fold into pipette solution to a final concentration of 240 µg/ml. Fresh dilutions into pipette solution were made every 1.5 h. CdCl2, ouabain, and isoproterenol were made as concentrated stocks in water and diluted 1,000-fold into external solution. Isoproterenol stock was kept under nitrogen. Final concentrations of 140 mM sodium or 140 mM NMG were used in a darkened room. Amphotericin B, SITS, and ouabain were used in a few cells to verify recordings of action potentials and DAD obtained by standard patch technique. The amphotericin B perforated-patch electrodes were used in a few cells to verify recordings of action potentials and DAD obtained by standard patch technique. The amphotericin B perforated-patch technique was used in those experiments where EGTA was required to determine the effects of this substitution on ground potential, the development of a ground potential when switching to experimental solutions. Protocols required using a sewer pipe to replace sodium surrounding voltage-clamped cells. To evaluate the effects of this substitution on ground potential, the current-voltage relation of the calcium current was determined in the presence of 140 mM sodium or 140 mM NMG applied via sewer pipe. Threshold and peak voltage of the current-voltage relation were unaffected, suggesting that

Fig. 1. Rapid substitution of external sodium using a sewer pipe. Cell was bathed in potassium-free external solution containing 140 mM sodium, and a sewer pipe placed within 100 µm of cell was filled with external solution modified to replace sodium with N-methyl-D-glucamine (NMG). Sodium current (INa) was recorded in normal sodium (top left, 0 ms), and during subsequent depolarizations, effects of turning on sewer pipe before activation of INa were evaluated. Intervals indicated in each panel refer to period sewer pipe was turned on before activation of INa.
ground potential was not altered by sodium substitution (5 cells).

Whole cell currents and transmembrane potentials were filtered with a four-pole low-pass Bessel filter at 5 kHz, digitized between 0.5 and 2 kHz (Digidata 1200, Axon Instruments), and stored on a computer. Significant differences between means were determined by a paired or unpaired Student’s t-test. Unloaded cell shortening was recorded with a video edge motion detector (model VED 104; Crescent Electronics, Sandy, UT) coupled with a Phillips type FTM800NH/HGI camera operating at a 60-Hz scan rate. The single-ended output of this detector was linear over the range 0–40 μm. Clampex 6 acquisition software (Axon Instruments) was used to concurrently record cell shortening and either transmembrane potential or ionic current.

RESULTS

The capacity to rapidly substitute external sodium to inhibit inward $I_{\text{NaCa}}$ is central to our investigation. We used a sewer pipe to limit the interval spent in sodium-free solutions and thus reduce alterations of intracellular sodium, pH, and calcium that might be expected after blocking $\text{Na}^+$/H$^+$ transport, $I_{\text{Na}}$, and inward $I_{\text{NaCa}}$. In this study, cells were exposed to sodium-free solutions for periods ranging from 1.2 to 2.8 s. We have assumed that the primary effect of eliminating external sodium is to abolish inward $I_{\text{NaCa}}$, although changes in intracellular pH or calcium might not be eliminated even with these brief exposures. To evaluate the time needed to completely substitute sodium surrounding a single myocyte, $I_{\text{Na}}$ was recorded in normal sodium and after substitution with an ion too large to penetrate sodium channels.

Figure 1 shows that a complete exchange of NMG for external sodium and a concomitant reduction in $I_{\text{Na}}$ was achieved within 200 ms of activating a sewer pipe to deliver NMG-containing solution. Patch pipettes were filled with potassium-free solution containing 5 mM EGTA. Cells were bathed in potassium-free solution containing normal sodium, and the sewer pipe was filled with an external solution in which sodium had been completely replaced with NMG. Because inward $I_{\text{Na}}$ is effectively reduced by replacement of external sodium with NMG, we compared $I_{\text{Na}}$ in normal sodium with those currents recorded after successively longer exposures to NMG-containing solution from the sewer pipe. The cell was held at $-80$ mV and repetitively pulsed to $-40$ mV to activate sodium channels at a rate of 0.2 Hz. During the first pulse, the sewer pipe was not turned on, and the current shown in Fig. 1, top left, was recorded. With subsequent pulses, the sewer pipe was activated for progressively longer periods before the step to $-40$ mV and always turned off at the end of the $-40$ mV step. Intervals indicated in each panel of Fig. 1 refer to the period the sewer pipe was activated before the step to $-40$ mV. $I_{\text{Na}}$ was significantly reduced when the sewer pipe was turned on just 100 ms before activation of $I_{\text{Na}}$, and when turned on 200 ms before activation of $I_{\text{Na}}$, a 3 ms pulse to 10 mV did not result from a sewer pipe-induced shift in the ground potential (see METHODS), but from complete elimination of sodium surrounding the cell. In 20 cells, the time required to completely abolish $I_{\text{Na}}$ was $310 \pm 4.2$ ms.

Figure 2 demonstrates the method used to trigger calcium-activated conductances and verifies that deactivation of $I_{\text{Ca}}$ is too rapid to interfere with our measurements of calcium-activated fluxes, which are made starting 5 ms after stepping back to $-80$ mV. Currents and cell shortening were measured in standard external and pipette solutions, and the sewer pipe was filled with external solution containing 300 µM CdCl$_2$. A cell was held at $-80$ mV before evoking a 5 ms pulse to $-50$ mV to inactivate $I_{\text{Na}}$, followed by a 3 ms pulse to 10 mV to expedite release of calcium from the SR, and currents were measured after returning to $-80$ mV. In Fig. 2A, the voltage template, ionic currents, and cell shortening are shown in the top, middle, and bottom panels, respectively. Lowercase letters identify corresponding current traces and contractions. Commencing with a step to $-80$ mV, a slowly decaying inward current
accompanied by a contraction was recorded (a). CdCl₂ abolished this current and contraction (b), and within 15 s of turning off the flow of cadmium-containing solution from the sewer pipe, both current and contraction had nearly recovered their original amplitudes (c). Similar results were obtained in six additional cells.

Although we were tempted to identify all of the cadmium-sensitive current measured at −80 mV as activated by a rise in cytosolic calcium, deactivation of I\textsubscript{Ca} might contribute a short-lived element to this cadmium-sensitive inward current. To determine the time course of tail currents attributable to I\textsubscript{Ca}, currents were recorded in sodium- and potassium-free solutions. I\textsubscript{Cl(Ca)} was inhibited by reducing external chloride to 6 mM and internal chloride to 2 mM. Pipettes contained 250 µM EGTA to compensate for an increase in cytosolic calcium due to inhibition of I\textsubscript{NaCa}. Cells continued to contract under these conditions. A cell was held at −50 mV. Currents were measured at −80 mV immediately after either a 300-ms pulse to 10 mV, or a 3-ms pulse to 10 mV. In Fig. 2B, the voltage template and superimposed currents during long and short steps to 10 mV are shown in the top and middle panels, respectively. I\textsubscript{Ca} completely inactivated within 75 ms of the beginning of the long pulse but does not have this same opportunity to inactivate during a 3-ms pulse. For this reason, I\textsubscript{Ca} tail currents can only be present after a 3-ms pulse, and subtraction of currents immediately after a 300-ms pulse from those currents immediately after a 3-ms pulse will reveal the time course of I\textsubscript{Ca} deactivation. Shown in Fig. 2B, bottom panel, are the subtracted currents commencing with the step back to −80 mV. The amplifier was overloaded for a period of 2 ms during which time we can say nothing about deactivation. After this initial period, a rapidly decaying tail was observed, which normally would be partially hidden by decay of capacitive current. Although the inward current might be a capacitance artifact, in six cells subtractions always yielded a rapidly decaying inward deflection. We conclude that inward currents shown in Fig. 2A largely represent conductances activated by a rise in intracellular calcium. To allow for complete decay of capacitive current and I\textsubscript{Ca} deactivation, we have been conservative and quantified calcium-activated currents as beginning 5 ms after stepping back to −80 mV. These conductances underlie DAD in calcium-overloaded cells, and their identification is the focus of this study.

Calcium-activated conductances were investigated after first inhibiting I\textsubscript{Cl(Ca)} by reducing external chloride to 6 mM and internal chloride to 2 mM. We asked whether both inward I\textsubscript{NaCa} and nonselective cation conductance could be evoked by a rise in intracellular calcium, using the protocol first illustrated in Fig. 2A. Currents recorded in normal external sodium were compared with those remaining after complete substitution of external sodium and inhibition of inward I\textsubscript{NaCa} (20). Figure 3 illustrates that the calcium-activated nonselective cation conductance is absent from the canine midmyocardium. In Fig. 3A, the external solution was potassium free and contained 140 mM sodium. A sewer pipe was filled with a modified external solution in which all sodium was replaced by NMG. Patch pipettes were filled with an internal solution that was potassium free, contained 10 mM sodium, and contained no EGTA. Shown in Fig. 3A, middle panel,

![Fig. 3. Nonselective cation current is absent from canine midmyocardial myocytes. Calcium-activated currents were investigated after first inhibiting calcium-activated chloride conductance [I\textsubscript{Cl(Ca)}] by reducing external and internal chloride. Currents in normal external sodium were compared with those remaining after inhibition of inward sodium/calcium exchange current [I\textsubscript{NaCa}]. In A, middle panel shows currents in normal sodium (trace marked Na\textsuperscript{+}) and after replacement of sodium with NMG (trace marked NMG\textsuperscript{+}). Bottom panel shows that contraction was increased after application of NMG. In B, pipette solution was sodium free to inhibit reverse-mode I\textsubscript{NaCa}, isoproterenol (1 µM) and ouabain (5 µM) were added to external solutions to increase intracellular calcium. Solutions were potassium free, and as before, I\textsubscript{Cl(Ca)} was inhibited by reduction of chloride. Middle panel, currents in normal external sodium (Na\textsuperscript{+}) after replacement of all sodium with lithium (Li\textsuperscript{+}); bottom panel, superimposed recordings of cell shortening in normal sodium and after replacement with lithium.](Image)
are superimposed currents recorded in normal sodium (trace marked Na⁺), and after replacement of sodium with NMG (trace marked NMG⁺). NMG-containing solution was applied 600 ms before a step to −50 mV for a total period of 1.5 s. Replacement of sodium caused an outward shift in currents at all potentials, abolished I\textsubscript{NaCa} during the brief pulse to −50 mV, and eliminated calcium-activated current at −80 mV. Figure 3A, bottom panel, shows that contraction was increased after application of NMG, so it is unlikely that elimination of calcium-activated currents was precipitated by loss of the underlying calcium transient. If canine myocytes possessed calcium-activated nonselective cation channels, we expected that sodium substitution with a large cation like NMG and inhibition of I\textsubscript{NaCa} should have resulted in a transient outward current at −80 mV carried by cesium. We propose that the steady outward current at −80 mV results from reverse-mode I\textsubscript{NaCa} after replacement of all external sodium, consistent with a larger contraction after sodium substitution.

DAD and triggered activity are typically recorded under conditions in which cells become overloaded with calcium. To examine whether a greater calcium load could evoke a nonselective cation conductance, protocols were repeated in the presence of both 1 µM isoproterenol and 5 µM ouabain to duplicate conditions later used to evoke DAD. Solutions were potassium free, and as before, I\textsubscript{Cl(Ca)} was inhibited by drastic reduction of chloride. A sewer pipe was filled with a modified external solution in which all sodium was replaced by lithium. Pipette solution was sodium free to inhibit reverse-mode I\textsubscript{NaCa}. Shown in Fig. 3B, middle panel, are superimposed currents in normal external sodium and after replacement of all sodium with lithium. Lithium inhibited inward I\textsubscript{NaCa} and abolished all calcium-activated current without causing an outward shift in currents or affecting cell shortening. If canine myocytes possessed calcium-activated nonselective cation channels, we expected that a I\textsubscript{Cl(Ca)} carried by lithium would remain after blocking I\textsubscript{NaCa}. We conclude that under conditions in which I\textsubscript{Cl(Ca)} has been blocked, I\textsubscript{NaCa} will be the only remaining calcium-activated conductance in these cells. The outward shift in currents that occurred when internal solution contained sodium most likely results from reverse-mode I\textsubscript{NaCa}, since it was absent when internal sodium was replaced by cesium.

In succeeding experiments, we have used chloride-containing solutions to determine the relative amplitudes of I\textsubscript{Cl(Ca)} and I\textsubscript{NaCa} and their contributions to DAD and triggered activity. Figure 4 shows the amplitudes of I\textsubscript{Cl(Ca)} and I\textsubscript{NaCa} for controls (A) and after isoproterenol and ouabain were applied to increase intracellular calcium loading (B). Solutions were potassium free and contained normal external and internal sodium. External chloride was 146 mM, internal chloride was 22 mM, and the calculated chloride reversal potential (E\textsubscript{Cl}) was −49 mV. In Fig. 4A, the top panel shows currents in normal sodium and after replacement with lithium to block inward I\textsubscript{NaCa}. Lithium caused an outward shift in current at −80 mV and substantially reduced the calcium-activated current even though the cell shortened to a greater degree. The lithium-insensitive current is I\textsubscript{Cl(Ca)}, since it was absent when chloride was drastically reduced (Fig. 3) and could be blocked by 2 mM SITS. The sewer pipe was turned off and currents recovered completely before 1 µM isoproterenol and 5 µM ouabain were applied and protocols repeated in same cell. Currents and contractions are shown in top and bottom panels, respectively.

Fig. 4. Relative amplitudes of I\textsubscript{Cl(Ca)} and I\textsubscript{NaCa}. In A, top panel shows currents in normal sodium (Na⁺) and after replacement with lithium to block inward I\textsubscript{NaCa} (Li⁺). Lithium was applied 600 ms before stepping to −50 mV for a total period of 1.5 s. Contraction is shown in bottom panel. In B, sewer pipe was turned off and currents recovered completely before 1 µM isoproterenol and 5 µM ouabain were applied and protocols repeated in same cell. Currents and contractions are shown in top and bottom panels, respectively.
Whole cell currents were recorded using standard patch-clamp technique. External solution was potassium free, with greatly reduced chloride (extracellular chloride concentration = 6 mM), but contained normal sodium, 1 µM isoproterenol, 5 µM ouabain, and 2 mM CaCl₂. Pipette solutions were potassium free and included no EGTA. The sewer pipe was filled with modified external solution in which sodium was replaced with either lithium (4 cells) or NMG (6 cells). Cells were held at –80 mV, and reverse-mode $I_{\text{NaCa}}$ was elicited by turning on the sewer pipe and replacing external sodium. To favor loading of the subsarcolemmal space with sodium, a train of 15 20-ms pulses to –40 mV at a rate of 2 Hz was delivered 1 s before turning on the sewer pipe. Figure 5 shows superimposed traces from two cells of similar size. The larger $I_{\text{NaCa}}$ was recorded when the pipette contained 10 mM sodium, and the smaller $I_{\text{NaCa}}$ was recorded when pipette solution was sodium free. Amplitudes are reported as the difference between the current recorded 1.2 s after switching to lithium and the baseline current in normal sodium. For five cells in which pipettes contained sodium, the outward current evoked in response to substitution of external sodium was $1.25 \pm 0.02 \text{ pA/pF}$ at –80 mV and $0.056 \pm 0.0012 \text{ pA/pF}$ when pipettes contained no sodium (5 cells). When pipettes contained sodium, substitution of external sodium resulted in spontaneous contractions after ~1.5 s. Conversely, when cells were dialyzed by sodium-free pipette solution, spontaneous contractions were still not evident 30 s after activating the sewer pipe.

If $I_{\text{Cl(Ca)}}$ and $I_{\text{NaCa}}$ both contribute to currents in calcium-overloaded cells, we should be able to separate them based on their reversal potentials. In particular, when $E_{\text{Cl}}$ is –49 mV, activation of cyclic release of 

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**Fig. 5.** Reverse-mode $I_{\text{NaCa}}$ is significantly reduced when using sodium-free pipette solution. External solution was potassium free, with greatly reduced chloride (extracellular chloride concentration = 6 mM) but contained normal sodium, 1 µM isoproterenol, 5 µM ouabain, and 2 mM CaCl₂. Pipette solutions were potassium free and included no EGTA. The sewer pipe was filled with modified external solution in which sodium was replaced with lithium. Cells were held at –80 mV, and reverse-mode $I_{\text{NaCa}}$ was elicited by turning on the sewer pipe and replacing external sodium, indicated by bar labeled Na⁺-free. To favor loading of subsarcolemmal space with sodium, a train of 15 20-ms pulses to –40 mV at a rate of 2 Hz was delivered 1 s before turning on the sewer pipe. Larger lithium-induced outward current was recorded when pipette contained 10 mM sodium; cell capacitance was 178 pF. Smaller lithium-induced current was recorded when pipette solution was sodium free; cell capacitance was 201 pF.
calcium from the SR and a step to 50 mV should result in an oscillating outward \( I_{\text{NaCa}} \) superimposed on an oscillating inward \( I_{\text{Cl(Ca)}} \). Substitution of sodium with lithium should block all inward \( I_{\text{NaCa}} \), leaving only an oscillating outward \( I_{\text{Cl(Ca)}} \). Figure 6 shows that a lithium-sensitive inward current and a chloride-sensitive outward current both contribute to oscillating currents at 50 mV. Calcium overload was induced by the addition of 1 \( \mu \)M isoproterenol and 5 \( \mu \)M ouabain to all external solutions. Oscillating currents were evoked by a 3-ms step to 10 mV and measured after stepping to 50 mV. In Fig. 6A, currents were recorded in potassium-free solution, with \( E_{\text{Cl}} \) equal to \(-49 \) mV. During the step to 50 mV, currents in normal sodium are outward and show evidence of overlapping inward and outward currents. Inhibition of \( I_{\text{NaCa}} \) shifts the current in an outward direction and abolished inward oscillations, leaving much larger transient outward currents. Similar results were obtained in eight cells. In Fig. 6B, currents were recorded in potassium-free solution, and \( E_{\text{Cl}} \) was adjusted to 50 mV in a second cell. During the step to 50 mV, currents in normal sodium are outward. Inhibition of \( I_{\text{NaCa}} \) shifts the current in an outward direction and left small transient outward currents. In six cells in which \( E_{\text{Cl}} \) was 50 mV, oscillating outward transients in lithium were \(-100 \) pA. This compares with oscillating outward currents \( >1 \) nA when \( E_{\text{Cl}} \) was \(-49 \) mV. Taken together, these results suggest that \( I_{\text{NaCa}} \) and \( I_{\text{Cl(Ca)}} \) contribute to oscillating currents in calcium-overloaded cells.

The most convincing demonstration that these two currents contribute to triggered activity in midmyocardial cells is to choose conditions that eliminate one or the other current and test whether triggered activity can still be evoked. In the absence of EGTA in the pipette solution, cells will not survive elimination of \( I_{\text{NaCa}} \) except for brief periods. When pipettes contained 10 mM sodium, lithium applied for 15 s caused hypercontraction of cells, from which there was no recovery. Instead, we have recorded action potentials in calcium-overloaded cells in the absence of inward \( I_{\text{Cl(Ca)}} \) to see if \( I_{\text{NaCa}} \) is sufficient to support triggered activity. The perforated-patch technique was used to record action potentials in standard external solution to which 1 \( \mu \)M isoproterenol and 5 \( \mu \)M ouabain were added. Standard internal solution was modified to reduce internal chloride to 2 \( \mu \)M, with \( E_{\text{Cl}} \) equal to \(-113 \) mV. In A, last stimulated beat is shown at left. In B, last stimulated beat is shown at left. \( I_{\text{Cl(Ca)}} \) was reduced by application of 0.5 mM SITS, and protocol was repeated in same cell.

**Fig. 6.** \( I_{\text{NaCa}} \) and \( I_{\text{Cl(Ca)}} \) both contribute to oscillating currents in isoproterenol- and ouabain-treated cells. In A, currents were recorded in potassium-free solution, with chloride reversal potential (\( E_{\text{Cl}} \)) equal to \(-50 \) mV. Shown are currents recorded at 50 mV in normal sodium (\( Na^+ \)) and after substitution with lithium (\( Li^+ \)). In B, currents were recorded in potassium-free solution, but chloride was adjusted such that \( E_{\text{Cl}} \) was equal to step potential. Again, currents were recorded at 50 mV in normal sodium (\( Na^+ \)) and after substitution with lithium (\( Li^+ \)). In both cells, lithium was applied 800 ms before step to 50 mV for a total period of 2.5 s.
stance and an enhanced ability of $I_{NaCa}$ to elicit action potentials.

To differentiate between these two alternatives, we used the perforated-patch technique to record inwardly rectifying potassium current and tested the effects of 0.5 mM SITS on this current. Whole cell currents were recorded in normal external and pipette solutions containing 1 µM isoproterenol and 5 µM ouabain. Cells were held at −80 mV and pulsed to −90 mV for 300 ms at a rate of 2 Hz. SITS was applied, and the protocol was repeated. Current density was 0.61 ± 0.07 pA/pF (n = 10) at the holding potential of −80 mV and 4.83 ± 0.12 pA/pF at the end of a 300 ms step to −90 mV and was unaffected by SITS.

Taken together, these results are consistent with our characterization of calcium-activated currents in mid-myocardial cells and suggest that $I_{NaCa}$ has a major role in generating DAD in calcium-overloaded cells. Because our characterization of currents also suggests a role for $I_{Cl(Ca)}$, we next focused on the effects of transiently abolishing inward $I_{NaCa}$ while recording triggered activity with normal chloride in the pipette.

Figure 8 shows the effects of inhibiting $I_{NaCa}$ on DAD and triggered activity. Standard patch-clamp technique was used to record action potentials and cell shortening in standard external and internal solutions, with $E_{Cl}$ equal to −50 mV. Triggered activity and DAD were evoked after a train of 15 stimulated beats at a 500-ms BCL in the presence of 1 µM isoproterenol and 5 µM ouabain. In Fig. 8A, the last stimulated beat is shown at the left, followed by three triggered beats. The second triggered beat was elicited from a slowly rising DAD, and each beat was associated with a contraction (Fig. 8A, bottom trace). In Fig. 8B, the protocol was repeated in the same cell, and lithium was applied 15 ms after the upstroke of the last stimulated beat for a period of 1.5 s. The results in this cell were typical of eight other cells in which the number of triggered beats was reduced after inhibiting $I_{NaCa}$, but DAD remained even at the end of the lithium pulse, and contractions continued to be associated with DAD and triggered beats. Lithium had no effect on resting potential, unless applied for longer than 3 s, at which point cells begin to depolarize. Because complete exchange of NMG for sodium occurred within 310 ms (Fig. 1), we considered it sufficient to apply lithium for no longer than 2.8 s in this study.

We repeated these experiments using the perforated-patch technique to ensure that dialysis of some intracellular component did not influence results obtained by standard patch-clamp technique. Action potentials were recorded in standard external and internal solutions, with $E_{Cl}$ equal to −50 mV. Triggered activity and DAD were evoked after a train of 15 stimulated beats at a 500-ms BCL in the presence of 1 µM isoproterenol and 5 µM ouabain. This protocol was repeated, and lithium was applied 15 ms after the upstroke of the last stimulated beat for a period of 1.5 s. As was the case with the standard patch-clamp technique, although the number of triggered beats after a train was reduced, triggered activity and DAD continued to be observed after substitution of external sodium with lithium in seven midmyocardial cells.

Any effect of lithium to reduce the number of triggered beats did not arise directly from a lithium-induced shift of the activation threshold for sodium channels to more positive potentials. Sodium currents were measured under conditions that permitted adequate voltage control. Standard patch-clamp technique was used to record currents at 22°C from a holding potential of −110 mV. Solutions were potassium free, pipettes contained 10 mM EGTA, and both internal and external sodium were reduced to 5 mM. The current-voltage relation for sodium current was measured in 5-mV increments over a voltage range between −80 and −40 mV. The protocol was repeated after replacing external sodium with 5 mM lithium. In nine cells, lithium had no effect on the −60 mV threshold of the sodium current.

Hille (16) reports the ionic permeability ratios of lithium over sodium to range between 0.93 and 1.1, depending on cell type and species. Experiments were performed to investigate whether exchanging lithium for sodium might reduce sodium channel conductance, and thus decrease the ability of these channels to trigger an action potential. Standard patch-clamp technique was used to record action potentials in normal external solution. Pipettes contained normal ions and 10 mM EGTA to reduce calcium-activated conduc-
tances. A train of action potentials was elicited at a BCL of 500 ms, and the change was made from external sodium to lithium. In eight cells, action potentials could be elicited for an average of 25.3 ± 0.8 s after switching to lithium. During investigations to determine the mechanisms underlying DAD, our substitutions never exceeded 2.8 s. We conclude that a lithium-induced reduction in sodium-channel conductance should not directly contribute to a decrease in triggered activity.

We have previously reported that oscillating inward currents recorded in sodium-free solutions in calcium-overloaded cells were blocked by SITS (38). Figure 9 shows the effects of external sodium replacement on the $I_{\text{TI}}$ that underlie DAD. Currents and cell shortening were obtained in standard external and internal solutions containing 1 µM isoproterenol and 5 µM ouabain, with $E_{\text{Cl}}$ equal to −50 mV. Calcium-activated currents were recorded at −80 mV after a 500-ms pulse to 0 mV. In Fig. 9A, $I_{\text{TI}}$ are shown accompanied by contractions. In Fig. 9B, lithium was applied 250 ms after the beginning of the step to 0 mV for a period of 2.8 s. Lithium abolished inward $I_{\text{NaCa}}$, but $I_{\text{TI}}$ due to $I_{\text{Cl(Ca)}}$ persisted and continued to be associated with contractions.

When solutions contained normal potassium, replacement of external sodium did not result in an outward shift in currents, as had been previously observed in potassium-free solutions. This lack of outward shift in potassium-containing solutions is consistent with the failure of lithium to modify resting potential in calcium-overloaded cells (Figs. 8 and 10). We examined whether the outward shift in potassium-free solutions due to reverse-mode $I_{\text{NaCa}}$ (Fig. 3) might be offset by a lithium-induced decrease in net outward current contributed by $I_{\text{K1}}$. $I_{\text{K1}}$ was recorded in sodium-free internal and external solutions in the presence of 300 µM CdCl$\text{2}$ to block all calcium transients. While holding at −85 mV, near the typical resting potential of these cells, lithium was applied, and an inward current continued to develop over the course of application. We did not further investigate the degree that this inward current offsets reverse-mode $I_{\text{NaCa}}$, although we did observe that cells depolarized when lithium was applied for longer than 3 s in complete Tyrode solution.

We next examined if increasing the driving force for inward $I_{\text{Cl(Ca)}}$ at resting potentials might permit sustained triggered beats in the presence of lithium. Although $E_{\text{Cl}}$ is often portrayed as being −50 mV, evidence also exists for reversal at a more depolarized potential (34, 36, 42). Figure 10 shows the effects of inhibiting $I_{\text{NaCa}}$ on DAD and triggered activity when intracellular chloride was elevated. Standard patch-clamp technique was used to record action potentials and cell shortening. Internal solution contained 147 mM chloride, with $E_{\text{Cl}}$ equal to −1 mV. In A, last stimulated beat is shown at left, followed by 2 triggered beats. Each beat was associated with a contraction (bottom trace). In B, protocol was repeated in same cell, and lithium was applied 15 ms after upstroke of last stimulated beat for a period of 2.8 s. Last stimulated beat was followed by a triggered beat and 2 DAD, each associated with a contraction.

Fig. 9. Effects of external sodium replacement on transient inward currents that underlie DAD when solutions contained normal internal and external chloride, with $E_{\text{Cl}}$ equal to −50 mV. In A, transient inward currents are shown accompanied by contractions. In B, protocol was repeated, but lithium was applied 250 ms after beginning of step to 0 mV for a period of 2.8 s.

Fig. 10. Effects of inhibiting $I_{\text{NaCa}}$ on DAD and triggered activity when intracellular chloride was elevated. Standard patch-clamp technique was used to record action potentials and cell shortening. Internal solution contained 147 mM chloride, with $E_{\text{Cl}}$ equal to −1 mV. In A, last stimulated beat is shown at left, followed by 2 triggered beats. Each beat was associated with a contraction (bottom trace). In B, protocol was repeated in same cell, and lithium was applied 15 ms after upstroke of last stimulated beat for a period of 2.8 s. Last stimulated beat was followed by a triggered beat and 2 DAD, each associated with a contraction.
beats. The second triggered beat was elicited from a slowly rising DAD, and each beat was associated with a contraction (Fig. 10A, bottom trace). In Fig. 10B, the protocol was repeated in the same cell, and lithium was applied 15 ms after the upstroke of the last stimulated beat for a period of 2.8 s. The last stimulated beat was followed by a triggered beat and two DAD, each associated with a contraction. Increasing the driving force for inward $I_{Cl(Ca)}$ did not noticeably increase triggered activity in the presence of lithium. In seven cells, multiple triggered beats were recorded in normal sodium, but replacement of sodium converted this activity to a single triggered beat followed by DAD.

Figure 11 shows the effects of external sodium replacement on transient inward currents when solutions contained elevated external chloride, with $E_{Cl(Ca)}$ equal to $-1 \text{ mV}$. In Fig. 11A, $I_{Ti}$ are shown accompanied by contractions. In Fig. 11B, lithium was applied 250 ms after the beginning of the step to $0 \text{ mV}$ for a period of 2.8 s. Lithium abolished inward $I_{NaCa}$, but $I_{Ti}$ due to $I_{Cl(Ca)}$ persisted and continued to be associated with contractions. Similar results were obtained in six cells.

In this experiment, and that shown in Fig. 9, lithium does not appear to inhibit oscillating inward current, as one might predict for an inhibitor of inward $I_{NaCa}$. This is even more confusing given the similar degree of cell shortening after applying lithium. We observed that this was a feature of taking a 500-ms step to a depolarized potential. Under these conditions, substitution of external sodium and activation of reverse-mode sodium/calcium exchange caused undue loading of the cell with calcium, making comparisons between currents qualitative rather than quantitative. This increased contractility was rarely reflected in the cell shortening record because not all movement was along the longitudinal axis of the cell. One aspect of this increased calcium loading that is obvious in Fig. 11B is a lithium-induced increase in frequency of oscillations.

DISCUSSION

We are the first to report the relative amplitudes of $I_{NaCa}$ and inward $I_{Cl(Ca)}$ under conditions that provoke DAD and triggered activity in canine M cells. Our decision to characterize $I_{NaCa}$ as the sodium-sensitive conductance was made based on a need to preserve $I_{Cl(Ca)}$. Whereas others have used millimolar concentrations of nickel to characterize $I_{NaCa}$, such applications could have posed problems under our experimental conditions. Because of its inhibitory effects on $I_{Ca}$, nickel might indirectly reduce $I_{Cl(Ca)}$ (37). Nickel-sensitive current would then represent components of both $I_{NaCa}$ and $I_{Cl(Ca)}$ and result in overestimation of $I_{NaCa}$. Sodium substitution effectively blocks $I_{NaCa}$, without reducing $I_{Cl(Ca)}$ (40). Unfortunately, defining $I_{NaCa}$ as the sodium-sensitive current is also not benign, since substitution-induced increases in contractility can complicate interpretation of the difference current and lead to a larger $I_{Cl(Ca)}$. These concerns were addressed, first by limiting the time spent in sodium-free solution and second by comparing the results obtained with and without sodium in the pipette. The difference between $I_{NaCa}$ and $I_{Cl(Ca)}$ was greater after reducing reverse-mode exchange, suggesting that determinations of $I_{Cl(Ca)}$ made with sodium in the pipette were exaggerated. When controlling for changes in contractility, $I_{Cl(Ca)}$ was found to be $\sim 70\%$ of the amplitude of $I_{NaCa}$. One further intricacy must be considered. Contractility is reduced when sodium is removed from the pipette. We propose that the relative amplitudes of $I_{Cl(Ca)}$ and $I_{NaCa}$ are best determined in sodium-free pipette solution, whereas the absolute amplitude of $I_{Cl(Ca)}$ might very well be closer to that found in sodium-containing pipette solution.

For our determination of $I_{Cl(Ca)}$, it is critical to know whether sodium is eliminated from the subsarcolemmal space while dialyzing a cell with sodium-free pipette solution. Although concentrations of ions in the subsarcolemmal space must certainly be different from concentrations in the bulk cytosol, this restricted space does communicate with the cytosol. The question becomes, How long can sodium that enters through sodium channels be maintained in the subsarcolemmal space in the face of an infinite sink with sodium-free...
solution? Kimura et al. (20) and Hillgemann (15) found that reverse-mode exchange was abolished by eliminating internal sodium. For cells dialyzed with sodium-free pipette solution, we found that reverse-mode \( I_{\text{NaCa}} \) was nearly abolished 1 s after the end of a train of pulses designed to increase intracellular sodium. \( I_{\text{Cl(Ca)}} \) was 124 ± 23 × 10^{-3} \ pC/pF when pipettes contained sodium and 114 ± 24 × 10^{-3} \ pC/pF in the absence of internal sodium. The substantial reduction in reverse-mode \( I_{\text{NaCa}} \) due to elimination of sodium in the pipette equates to an 8% reduction in chloride flux. As shown by Fig. 3, we were unable to detect a lithium-induced increase in cell shortening when pipettes contained no sodium. If we modify our initial assumption to say that sodium-free pipette solutions do not completely abolish reverse-mode \( I_{\text{NaCa}} \), any lithium-induced increase in intracellular calcium must be quite small, and our “overestimate” of \( I_{\text{Cl(Ca)}} \) will be much less than 8%.

\( I_{\text{NaCa}} \) was the largest contributor to calcium-activated current at the resting potential in isoproterenol-treated cells. Consistent with these voltage-clamp findings, \( I_{\text{NaCa}} \) was large enough to cause DAD and triggered activity in the absence of other calcium-activated conductances. Our results are consistent with those reported by Kass et al. (19), who determined the sodium dependence of \( I_{\text{T1}} \) in cardiac Purkinje fibers. A correlation between \( I_{\text{NaCa}} \) and \( I_{\text{T1}} \) or DAD has also been demonstrated for human atrial cells, canine ventricular and atrial cells, and the sinoatrial node of the rabbit (2, 3, 32, 33, 35). \( I_{\text{T1}} \) in guinea pig ventricular cells was abolished when external sodium was reduced to 25% of normal, although mechanical oscillations continued, suggesting that \( I_{\text{NaCa}} \) is the major component of \( I_{\text{T1}} \) in this species (9). In canine coronary sinus, transient replacement of sodium with either lithium or sucrose caused a 30% decrease in \( I_{\text{T1}} \) amplitude, leaving open the possibility of further contributions from additional calcium-activated conductances (33). Conversely, a dissociation between forward-mode sodium/calcium exchange and \( I_{\text{T1}} \) was demonstrated for rabbit ventricular cells and rabbit Purkinje fibers (10, 12–14, 28).

Han and Ferrier (13) determined that isoproterenol-induced \( I_{\text{T1}} \) was dependent on calcium entry via reverse-mode sodium/calcium exchange. They suggested that isoproterenol directly activates reverse-mode sodium/calcium exchange, although a recent study by Main et al. (23) has found no evidence for \( \beta \)-adrenergic stimulation of the exchanger. Calcium entry via reverse-mode sodium/calcium exchange was observed in the present study, but this loading of the cell with calcium only occurred after external sodium was reduced and the reversal of the exchanger was significantly altered. In effect, reverse-mode sodium/calcium exchange was an artifact of our experimental procedure. Moreover, calcium entry via the exchanger was abolished by elimination of internal sodium, without dramatically reducing the ability of isoproterenol to evoke \( I_{\text{T1}} \). \( I_{\text{Cl(Ca)}} \) represented  ~40% of the total calcium-activated current at the resting potential in isoproterenol- and ouabain-treated cells. It is probable that the relative amplitudes of \( I_{\text{Cl(Ca)}} \) and \( I_{\text{NaCa}} \) will vary based on the method used to induce calcium overload and DAD. Whereas ouabain will elevate internal sodium and reduce the contribution of \( I_{\text{NaCa}} \) to the total calcium-activated conductance, isoproterenol appears to increase the relative contribution of \( I_{\text{NaCa}} \). Other studies have used high extracellular calcium or BAY K 8644 to induce DAD, and the relative importance of the two calcium-activated conductances might be different from what we report. Our choice of isoproterenol and ouabain was made because we did not want to dramatically alter extracellular divalent ions, and isoproterenol alone did not reproducibly produce multiple triggered beats after a train of stimulated action potentials.

Positive to \( E_{\text{Cl}} \), oscillating currents consisted of both inward and outward components, indicating that more than one conductance contributed to \( I_{\text{T1}} \). We attributed the oscillating outward component to \( I_{\text{Cl(Ca)}} \), since it was reduced significantly when \( E_{\text{Cl}} \) was made equal to the step potential. In the absence of \( I_{\text{NaCa}} \), we have previously shown that calcium overload-induced oscillating inward current is blocked by SITS and absent at \( E_{\text{Cl}} \) (38). Reversal of \( I_{\text{T1}} \) has been demonstrated for rabbit, sheep, and calf Purkinje fibers and rabbit ventricular myocytes (12–14, 19, 21).

As was the case for \( I_{\text{NaCa}} \), \( I_{\text{Cl(Ca)}} \) was sufficiently large to cause DAD in the absence of other calcium-activated conductances. We were unable to record multiple triggered beats after substituting lithium for external sodium, even though a substantial oscillating inward \( I_{\text{Cl(Ca)}} \) was present. Furthermore, increasing the driving force for inward chloride current also failed to elicit multiple triggered action potentials. These results suggest that the threshold for sodium channels has been shifted in the presence of lithium. Although we examined the effects of lithium on action potentials and sodium current threshold, these experiments were always performed using pipette solutions containing EGTA to inhibit calcium-activated conductances. Although these protocols weigh the direct effects of lithium, they abolish any subsequent outcome of a lithium-induced rise of intracellular calcium. A rise in intracellular calcium will cause a shift in the voltage dependence of sodium channel gating, such that fewer channels are available at the resting potential (7, 11). Lithium might act in this indirect manner to change membrane surface charge and contribute to a shift in sodium channel threshold. We suggest that this shift contributes to a lack of repeated triggered beats under conditions in which \( I_{\text{Cl(Ca)}} \) is known to be large.

When recording action potentials, our method used to inhibit \( I_{\text{NaCa}} \) exaggerates \( I_{\text{Cl(Ca)}} \) due to an increase in contractility. However, given similar amplitudes of \( I_{\text{Cl(Ca)}} \) and \( I_{\text{NaCa}} \) when protocol-induced changes in contractility were abolished, we propose that \( I_{\text{Cl(Ca)}} \) contributes to the normal formation of DAD. Trafford et al. (31) demonstrated that in the ferret ventricle the time courses of \( I_{\text{NaCa}} \) and \( I_{\text{Cl(Ca)}} \) are different. We concur with these findings, since oscillating currents positive to \( E_{\text{Cl}} \) appear to have a biphasic waveform (see Fig. 6), and \( I_{\text{Cl(Ca)}} \) cannot simply be scaled to equal \( I_{\text{NaCa}} \). Trafford et
al. (31) suggested that in the ferret, these differences in kinetics result from subarcoldemmal compartmentalization of calcium. Similar compartmentalization might occur in the dog, although we cannot rule out differences in calcium affinity between the exchanger and chloride channel contributing to differences in time course of the two currents.

Our results regarding $I_{\text{Cl(Ca)}}$ are consistent with an earlier demonstration of a SITS- and chloride-sensitive oscillating inward current in calcium-overloaded M cells and conform to similar findings in calcium-overloaded rabbit Purkinje fibers and ventricular cells (12–14, 21, 38). Of the very early investigations of the ionic basis of $I_{\text{TI}}$, some did not examine the contributions of a calcium-activated chloride conductance (1, 3). However, Kass et al. (19) found that substitution of external chloride did not appreciably affect strophanthidin-induced $I_{\text{TI}}$ reversal and proposed that a nonselective leak conductance or electrogenic sodium/calcium exchange was the ionic basis of $I_{\text{TI}}$. In an interesting investigation of early afterdepolarizations and DAD in canine myocytes, Volders et al. (35) demonstrated that isoproterenol-induced DAD were abolished by nickel, a nonspecific blocker of $I_{\text{Na,Ca}}$. Although they hypothesized that $I_{\text{Na,Ca}}$ alone caused DAD in these cells, the effects of nickel on $I_{\text{Cl(Ca)}}$ were not examined. It is important to establish in such a study that the time course of the block of $I_{\text{Na,Ca}}$ by nickel is the same time course as the block of DAD by nickel. Such a comparison would begin to establish whether nickel secondarily blocks additional conductances.

We have considered that the ability of isoproterenol to increase outward but not inward $I_{\text{Cl(Ca)}}$ suggests that this is not a single conductance. Different ionic channels might underlie these outward and inward currents. However, we have shown that this inward current is calcium activated, blocked by removal of chloride, and sensitive to SITS. From these experiments, we conclude that all of the current remaining after inhibition of $I_{\text{Na,Ca}}$ is $I_{\text{Cl(Ca)}}$. We can only speculate on the failure of isoproterenol to increase inward $I_{\text{Cl(Ca)}}$. Our results can be understood if two populations of chloride channels with different calcium sensitivities, and perhaps different rectification characteristics, are present in the canine midmyocardium. Papp et al. (25) have demonstrated two components of $I_{\text{Cl(Ca)}}$ in rabbit cardiac Purkinje cells, suggesting that these components could arise from spatial and temporal inhomogeneities of calcium transients, or different chloride channel populations with a different calcium sensitivity. If a second population of chloride channels exists, our results indicate a higher calcium sensitivity for those channels carrying inward current, such that saturation of calcium binding sites obviates any potential isoproterenol-induced increase in current.

DAD and triggered activity were elicited in M cells, without activation of a nonselective cation conductance. With $I_{\text{Cl(Ca)}}$ blocked, substitution of external sodium and concomitant inhibition of $I_{\text{Na,Ca}}$ abolished all calcium-activated current, even though a substantial gradient persisted for flux through nonselective channels. Our inability to detect a leak conductance was not due to channel rectification, since we looked for both inward and outward currents carried by monovalent cations. Moreover, this conductance was absent despite application of isoproterenol and ouabain to elevate intracellular calcium, and with a clear demonstration that contractility was maintained. The lack of a nonselective cation conductance has been reported in rabbit atrial and ventricular myocytes, rabbit Purkinje cells, and canine ventricular myocytes (21, 25, 30, 38–40).

Our conclusions are in disagreement with those presented for the ionic basis of $I_{\text{TI}}$ in rabbit Purkinje fibers. Han and Ferrier (13) propose that reverse-mode sodium/calcium exchange contributes to calcium overload, but that $I_{\text{Na,Ca}}$ does not act as a charge carrier for $I_{\text{TI}}$. We are puzzled by this suggestion, since a rise in intracellular calcium should immediately result in activation of forward-mode sodium/calcium exchange and an oscillating inward current. They conclude that an inwardly rectifying nonselective cation conductance plays the most prominent role in generation of inward $I_{\text{TI}}$ with some additional contribution from $I_{\text{Cl(Ca)}}$ and that outward $I_{\text{TI}}$ is primarily carried by $I_{\text{Cl(Ca)}}$. If this were the case, their substitution of external sodium with impermeant NMG or sucrose should have resulted in oscillating outward currents at ~55 mV, rather than the inward currents they ascribe to the nonselective cation conductance (14; Figs. 5 and 6).

Voltage-clamp studies have characterized the nonselective cation conductance as linear, so it is expected that this conductance should contribute to both inward and outward $I_{\text{TI}}$ if it is present in Purkinje fibers (6, 8). Consonant with our results in the dog, investigations of calcium-activated conductances in rabbit Purkinje cells have failed to find activation of nonselective cation channels, despite elicitation of a robust $I_{\text{Cl(Ca)}}$ (25, 30).

A model of triggered activity under a maintained calcium overload similar to our experimental conditions details the relationship between the nonselective cation conductance and $I_{\text{Na,Ca}}$ in the generation of DAD (22). In this model, the nonselective cation conductance is much more important than $I_{\text{Na,Ca}}$ in generating triggered activity, in large part because of elevated internal sodium and concomitant reduction of $I_{\text{Na,Ca}}$. Internal sodium was also presumably elevated in our experiments due to blockade of the sodium pump. However, $I_{\text{Na,Ca}}$ was still larger than $I_{\text{Cl(Ca)}}$, probably because of the smaller electrochemical gradient for chloride.

Evidence from animal and human studies indicates that reentry is the primary mechanism underlying arrhythmogenesis but that focal mechanisms might initiate ventricular arrhythmias under certain pathophysiological conditions. Three-dimensional cardiac mapping performed in dogs with ischemic cardiomyopathy induced by intracoronary embolizations revealed that monomorphic ventricular tachycardia was due to focal activation of subendocardial sites (26). Similarly, d-sotalol-induced polymorphic tachyarrhythmias with characteristics of torsade de pointes were initiated by spontane-
ous premature beats originating in the subendocardium (27). Such focal activation might arise as early afterdepolarizations or DAD originating in M cells or Purkinje fibers. We have determined the underlying mechanisms for DAD in M cells. An important next step to understanding the role these two conductances play during reperefusion- and drug-induced arrhythmias will be to determine if $I_{\text{Na}}$ and $I_{\text{CIC}}$ are affected differently by intracellular pH, calcium loading of the SR, and the metabolic components of ischemia.

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