

Properties of "Creep Currents" in Single Frog Atrial Cells

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ABSTRACT Changes in membrane current in response to an elevation of $[Na]_i$ were studied in enzymatically dispersed frog atrial cells. Na loading by either intracellular dialysis or exposure to the Na ionophore monensin produces changes in membrane current that resemble the "creep currents" originally observed in cardiac Purkinje fibers during exposure to low-K solutions. Na loading induces a transient outward current during depolarizing voltage-clamp pulses, followed by an inward current in response to repolarization back to the holding potential. In contrast to cardiac Purkinje fibers, Na loading of frog atrial cells induces creep currents without accompanying transient inward currents. Creep currents induced by Na loading are insensitive to K channel antagonists like Cs and 4-aminopyridine; they are not influenced by doses of Ca channel antagonists that abolish i_{Ca} , but are sensitive to changes in $[Ca]_o$ or $[Na]_o$. A comparison of the time course of development of inward creep currents with the time course of decay of i_{Ca} also indicates that inward creep currents are not tail currents associated with i_{Ca} . Inward creep currents can also be induced by experimental interventions that increase the i_{Ca} amplitude. Exposure to isoproterenol enhances the i_{Ca} amplitude and induces inward creep currents; both can be attenuated by Ca channel antagonists. Both inward and outward creep currents are blocked by low doses of La, independently of La's ability to block i_{Ca} . It is concluded that (a) creep currents are not mediated by voltage-gated Na, Ca, or K channels or by an electrogenic Na,K pump; (b) inward creep currents induced either by Na loading or in response to an increase in the amplitude of i_{Ca} are triggered by an elevation of $[Ca]_i$; and (c) creep currents may be generated by either an electrogenic Na/Ca exchange mechanism or by a nonselective cation channel activated by $[Ca]_i$.

INTRODUCTION

A variety of agents and experimental manipulations that produce an increase in $[Na]_i$ are known to increase the strength of contraction of the heart. Some of these include an increase in heart rate and exposure to agents that increase the

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Na permeability of the sarcolemma (grayanotoxin, batrachotoxin, monensin) and agents or interventions that block the Na,K pump (strophanthidin, low-K⁺ solutions). In each of these cases, the link between intracellular accumulation of Na and augmentation of Ca influx is believed to involve a sarcolemmal Na/Ca exchange mechanism (see Langer, 1982, and Chapman, 1983, for recent reviews).

In some cardiac preparations, interventions that lead to an increase in [Na]_i produce aftercontractions accompanied by transient inward currents (Lederer and Tsien, 1976). These are believed to be due to a nonselective channel activated by oscillations in [Ca]_i (Kass et al., 1978*a, b*); however, the involvement of an electrogenic Na/Ca exchanger was also suggested as an alternative explanation. The ionic basis for the transient inward current is presently controversial, since subsequent studies have not been able to measure genuine reversal potentials for the transient inward current (Karagueuzian and Katzung, 1982; Van Ginneken, 1983; H. F. Brown et al., 1984).

In this article, we examine the influence of intracellular Na loading on membrane currents in enzymatically dispersed single frog atrial myocytes. Our results show that increases in [Na]_i produce changes in membrane current that resemble the "creep currents" originally observed in cardiac Purkinje fibers during exposure to low-K⁺ solutions (Eisner and Lederer, 1979*a*). Increases in [Na]_i in frog atrial cells, in contrast to those in mammalian preparations, produce dramatic increases in mechanical activity but do not induce transient inward currents or aftercontractions. Inward creep currents are also induced by concentrations of isoproterenol that enhance i_{Ca} . It is concluded that inward creep currents induced either by Na loading or by exposure to isoproterenol occur in response to an elevation of [Ca]_i. The possibility that creep currents may be generated by an electrogenic Na/Ca exchange mechanism is examined in the following article (Hume and Uehara, 1986).

A preliminary report of some of these results has been made (Uehara and Hume, 1985*a*).

METHODS

The procedure for enzymatic dispersion of single frog atrial myocytes has been described previously (Hume and Giles, 1981) and the voltage-clamp method used, a single suction micropipette technique, is similar to that described earlier (Hume and Giles, 1983). The data were displayed on a storage oscilloscope (model 5103N, Tektronix, Inc., Beaverton, OR) and simultaneously recorded on an FM instrumentation tape recorder (model 4DS, Racal Recorders, Inc., Corina, CA) at a bandwidth of DC to 1 or 3 kHz. Both current and voltage records were later digitized offline with a 12-bit analog-to-digital converter (DT2782, Data Translation, Marlboro, MA) at sampling intervals ranging from 0.2 to 1 ms and stored in a PDP 11-23 (Digital Equipment Corp., Maynard, MA) computer for analysis.

Two techniques were used to load isolated cells with Na. Most cells were exposed to the Na ionophore monensin for 10–15 min (at concentrations ranging from 5×10^{-6} to 3×10^{-5} M). In some experiments, micropipettes with a larger tip diameter (1.0–2.0 μ m) were used for both internal dialysis and whole-cell voltage clamp (Hamill et al., 1981). For Na loading of cells, pipettes were filled with a solution containing 150 mM K⁺

gluconate, 5 mM HEPES (pH 7.2), and 20 mM NaCl. For Cs loading of cells, pipettes were filled with 150 mM Cs aspartate and 5 mM HEPES (pH 7.2). It was possible to establish gigaseals using pipettes with both small tip diameters (Hume and Giles, 1983) and larger tip diameters (Hamill et al., 1981). In both cases, the rupture of the sarcolemma was indicated by an abrupt voltage deflection that indicated the cell's resting membrane potential. Pipette tip resistances ranged between 3 and 8 M Ω and, in many experiments, conventional series resistance compensation was used to reduce the uncompensated series resistance to \sim 2 M Ω .

The Ringer's solution contained (mM): 110 NaCl, 2.5 KCl, 2.5 CaCl₂, 5.0 MgCl₂, 5 HEPES, 10 glucose, 15 sucrose, and was saturated with 100% O₂. The pH was titrated to 7.4 by addition of NaOH. All solutions contained tetrodotoxin (TTX; 3×10^{-6} M; Sigma Chemical Co., St. Louis, MO). In experiments in which NaCl was reduced, sucrose was added to the Ringer's solution in order to keep the osmolarity constant at 282 mosmol. 0-Ca Ringer's solution was identical to normal Ringer's except that CaCl₂ was replaced by equimolar substitution of MgCl₂. Monensin was purchased from Calbiochem-Behring Corp. (La Jolla, CA); CdCl₂, LaCl₃, and *l*-isoproterenol-*d*-bitartrate were purchased from Sigma Chemical Co. Diltiazem was kindly provided by Marion Laboratories (Kansas City, MO) and nisoldipine by Miles Institute (New Haven, CT). Monensin was first dissolved in polyethylene glycol in order to make a 10-mM stock solution. All other drugs were directly dissolved in Ringer's solution. All experiments were carried out at room temperature (22–23°C).

RESULTS

Under control conditions, after establishment of a gigaseal and rupture of the sarcolemma, resting membrane potentials ranged from -90 to -83 mV (see also Table I in Hume and Giles, 1983) at 2.5 mM [K]_o. After the stability of the seal was verified, the voltage-clamp circuit was activated and the holding potential was set near the resting membrane potential (the level of current required to maintain the holding potential therefore represents the zero-current level). 200–300-ms voltage-clamp depolarizing pulses were then applied at a frequency of 0.33 Hz in 10-mV increments to potentials between -70 and $+60$ mV in order to generate a control current-voltage relationship (in the presence of 3×10^{-6} M TTX). After this, cells were exposed to concentrations of monensin ranging from 5×10^{-6} to 3×10^{-5} M. After \sim 10 min, changes in the membrane current stabilized and the current-voltage relationship was measured again. An example of the changes in membrane current produced by monensin is illustrated in Fig. 1A.

Before exposure to monensin (C), 300-ms voltage-clamp steps to $+20$ mV elicited a TTX-insensitive inward current that has previously been identified under identical conditions as i_{Ca} (Hume and Giles, 1983). There is very little activation of the plateau K current by these relatively short pulses because of its delayed onset. Observation of the cell revealed that the cell contracted during each voltage-clamp pulse, and relaxed after the termination of the pulse. After exposure to monensin (M), there was a shift of membrane current in the outward direction during the depolarizing voltage-clamp step, followed by the appearance of an inward current upon repolarization of the membrane potential back to the holding potential. Observation of the cell during this protocol revealed that an

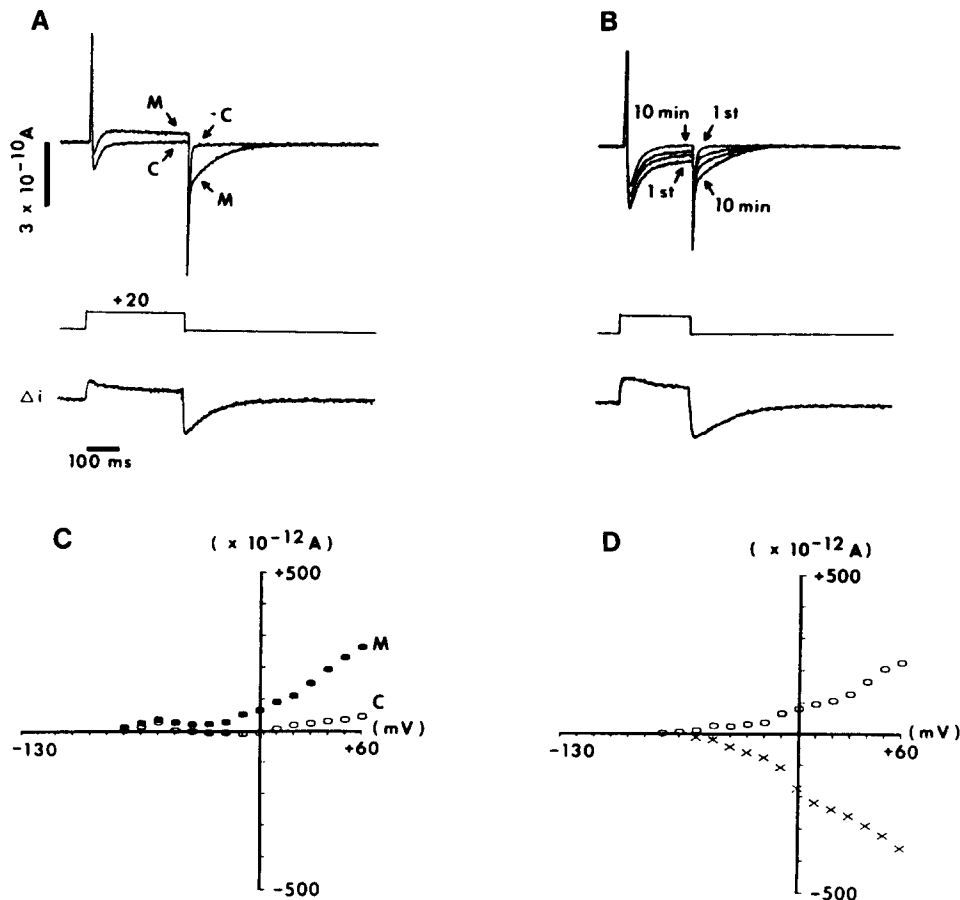


FIGURE 1. Changes in membrane current induced by Na loading of single atrial cells. (A) Superimposed membrane currents elicited by the application of 300-ms voltage-clamp pulses (0.33 Hz) from a holding potential of -80 to $+20$ mV before (C) and after 10 min exposure to 3×10^{-5} M monensin (M). Middle trace: membrane voltage; bottom trace: digitally subtracted difference current (Δi). Exposure to monensin produced an increase in outward current during the depolarizing pulse, and an inward current when the potential was changed back to the holding potential. (B) Superimposed membrane currents elicited by a series of repetitive voltage-clamp pulses to $+20$ mV in a different cell. In this cell, a pipette with a larger tip diameter was used for both voltage clamp and intracellular dialysis with a solution containing 20 mM NaCl (see Methods). Currents were recorded immediately after gigaseal formation and membrane rupture (labeled "1st") and then after additional intervals of 1, 3, and 10 min. In response to intracellular dialysis with NaCl, there was an outward shift in current during depolarizing voltage-clamp pulses and the progressive appearance of an inward current when the potential was returned to the holding potential. The bottom trace is the digitally subtracted difference between current elicited immediately after membrane rupture and current elicited 10 min later. (C) Current-voltage relationship (measured at the end of 300-ms pulses) for the experiment shown in A. Open circles: control; solid circles: obtained after 10 min exposure to 3×10^{-6} M monensin. The difference current-voltage relationship induced by Na loading (monensin - control) is plotted in D (open circles). The peak amplitude of the inward current induced by Na loading is also plotted (X's) as a function of the potential of the preceding voltage-clamp pulse (always repolarized to -80 mV).

increase in contractile activity accompanied the monensin-induced outward shift in current during the voltage-clamp pulses, compared with that observed under control conditions. This is consistent with earlier studies, which have shown that monensin produces a positive inotropic effect in myocardial preparations (Sutko et al., 1977). Coincident with the decay of the inward current immediately after the depolarizing voltage pulse, a relaxation of contractile activity can be observed. Changes in membrane current produced by Na loading of atrial cells are shown as the digitally subtracted difference current (Δi) in Fig. 1A. Na loading induces an outward current during the voltage-clamp step that partially decays, followed by an inward current that decays after repolarization back to the holding potential. Similar current changes have been observed in well over 70 different atrial cells exposed to various concentrations of monensin. At higher doses, or if pulses were applied at faster frequencies, there appeared to be a progressive retardation of the mechanical relaxation that followed each voltage-clamp step, and the cell eventually became rounded up, which is probably indicative of a state of Ca overload.

Although it has been demonstrated that monensin has a higher specificity for transporting Na over other monovalent cations (Pressman and Heeb, 1972; Pressman et al., 1975), it is important to verify that the observed changes in membrane current produced by this compound can be attributed to an increase in $[Na]_i$. Therefore, in several experiments a pipette with a large tip diameter (containing an intracellular dialysis solution that included 20 mM NaCl) was gently placed on the sarcolemma of the cell and negative pressure was applied to the pipette interior. In some trials, this resulted in the formation of a gigaseal and a subsequent rupture of the membrane (see Fig. 1B). Repetitive 200-ms voltage-clamp pulses were then applied at 0.33 Hz from a holding potential of -80 to $+20$ mV. The initial currents (labeled "1st") recorded were similar to those observed in the control in Fig. 1A and consisted primarily of i_{Ca} . During subsequent voltage-clamp pulses, a gradual shift in current in the outward direction was observed, followed by a gradually increasing inward current upon repolarization to the holding potential. The time required for the observed changes in membrane current may reflect the time necessary for internal dialysis to occur. The bottom trace in Fig. 1B shows the digitally subtracted difference between the current elicited immediately after membrane rupture and the current elicited by an identical voltage-clamp step 10 min later. Overall, these current changes closely resemble those produced by exposure to monensin (A) and provide evidence that a major action of monensin is to increase $[Na]_i$.

The complete 300-ms current-voltage relationship before (open circles) and after Na loading (solid circles) with monensin is plotted in Fig. 1C. The difference current-voltage relationship induced by Na loading is plotted in Fig. 1D (open circles). It is clear that Na loading, measured at 300 ms, produces an increase in outward current at nearly all potentials positive to the holding potential and that the increase is progressively larger at more positive potentials. Fig. 1D also shows the peak inward current (as a function of the potential of the voltage-clamp step) induced by Na loading that occurs during the return to the holding potential at -80 mV (\times 's).

The similarity between the changes in membrane current produced by exposure to monensin and those produced by direct internal dialysis with Na^+ via the

pipette implies that these alterations in membrane current are the direct result of an elevation of $[Na]_i$ and are not attributable to some particular property of the ionophore. Monensin is a carboxylic ionophore that may exist as a zwitterion in solution and therefore would be electrically neutral (Pressman, 1976). However, since the net change at physiological pH is uncertain, it is not possible to rule out a voltage-dependent action of the ionophore. Although the voltage dependence of the currents induced by monensin exposure might be attributed to some voltage dependence of the permeation properties of the ionophore, very similar voltage-dependent changes in current occur after intracellular dialysis with Na. The similarity between the currents induced by both methods of Na-loading cells also makes it unlikely that the ionophore produces an elevation of $[Na]_i$ that is voltage dependent. In addition, no measurable changes in membrane holding current were observed during monensin exposure in our experiments. In most subsequent experiments, monensin was used to Na-load atrial cells, since we have found empirically that it is much easier to titrate both the electrical as well as the mechanical responses with monensin compared with intracellular dialysis with Na: too large an elevation of $[Na]_i$ results in immediate, irreversible contracture and cell death. In addition, with large-diameter pipettes, rapid intracellular dialysis frequently occurs, which makes control measurements difficult to obtain in the same cell.

Several different hypotheses might explain the changes in membrane currents that are observed after atrial cells are Na loaded. It is well known that in the heart the Na,K pump is electrogenic and can be stimulated by internal Na (Glitsch, 1972). Are the changes in membrane current induced by Na loading of atrial cells mediated by an electrogenic Na,K pump? The outward current observed in Na-loaded cells during depolarizing voltage-clamp pulses might be mediated by K channels similar to those recently reported to be activated by $[Na]_i$ in isolated guinea pig ventricular myocytes (Kameyama et al., 1984). The time course of the outward current induced by Na loading is also very similar to the transient outward K current (I_{to} or I_{to}), which has previously been described in some cardiac preparations (Kenyon and Gibbons, 1979; Siegelbaum and Tsien, 1980; Josephson et al., 1984). Is the inward current observed at the end of depolarizing pulses in Na-loaded cells a tail current associated with i_{Ca} ? Although recent studies in a variety of preparations (Hagiwara and Ohmori, 1982; Eckert and Ewald, 1983; A. M. Brown et al., 1983) indicate that tail currents associated with Ca channels are an order of magnitude faster than the currents observed here, much slower tail currents associated with a noninactivating component of i_{Ca} have been reported in intact frog ventricle (Morad and Tung, 1982).

Finally, the changes in current induced by Na loading of single frog atrial cells resemble the "creep currents" observed in cardiac Purkinje fibers during exposure to either low- $[K]_o$ solutions (Eisner and Lederer, 1979a) or strophanthidin (Lederer and Tsien, 1976), two conditions that also are expected to produce an increase in $[Na]_i$. In these studies, creep currents were postulated to result from the same underlying mechanisms believed to be responsible for the accompanying transient inward currents (TI's): either (a) a Ca-activated channel that was selective for both Na and K (Kass et al., 1978b), which was subsequently described

by Colquhoun et al. (1981), or (b) an electrogenic Na/Ca exchange mechanism. Both mechanisms, which have been postulated to be responsible for the development of TI's, must also be considered potential mechanisms responsible for the creep currents produced by Na loading of single frog atrial cells.

Dependence of Creep Currents on K

The dependence of creep currents on $[K]_o$ was examined in order to determine whether creep currents are mediated either by an electrogenic Na,K pump or by voltage-gated membrane K channels. Exposure to K-free solutions has been shown to inhibit Na,K pump activity in cardiac Purkinje fibers (Eisner and Lederer, 1979b; Gadsby and Cranefield, 1979). We examined whether transient exposure to 0 $[K]_o$ would prevent the development of creep currents in Na-loaded isolated atrial cells. Although previous studies (Eisner and Lederer, 1979a) have shown that 0- $[K]_o$ solutions block the Na,K pump and lead to the development of creep currents as well as TI's, it was important to verify that the creep currents induced by Na loading using the ionophore monensin can also be observed in 0- $[K]_o$ solutions.

Fig. 2 shows the results from an experiment in which $[K]_o$ was changed from 2.5 to 0 mM. Panel A shows superimposed membrane currents elicited by identical voltage-clamp depolarizations to 0 mV before and 10 min after switching to 0-K Ringer's solution. A transient inward i_{Ca} is elicited in both solutions; the primary effect of the 0-K Ringer's solution was a small inward shift in holding current required to maintain the holding potential at -90 mV. The current-voltage relationships (measured at the end of 200-ms voltage-clamp pulses) in 2.5 and 0 mM $[K]_o$ are plotted in panel C. In 0 $[K]_o$, there is a positive shift in the zero-current potential, a reduction of inward rectification, and a decrease in the slope conductance at negative membrane potentials. These effects are similar to the changes in the steady state current-voltage relationship observed in Purkinje fibers in response to brief exposure to 0- $[K]_o$ solutions (Eisner and Lederer, 1979a, 1980). Exposure to monensin (1×10^{-5} M) in the 0- $[K]_o$ solution resulted in the development of creep currents (B). The voltage dependence of the outward creep current as well as the inward creep currents in 0- $[K]_o$ solution is very similar to that observed in normal Ringer's solution (Fig. 1D). Exposure to 0-K Ringer's solution produced changes in the current-voltage relationship of isolated atrial cells that are consistent with inhibition of an electrogenic Na,K pump. The ability of Na loading to produce creep currents in 0-K solutions makes it unlikely that an electrogenic current caused by the Na,K pump contributes substantially to the generation of creep currents. Longer exposure to 0-K solutions often results in the appearance of creep currents in single atrial cells in the absence of the Na ionophore (not shown), a result consistent with earlier experiments in Purkinje fibers (Eisner and Lederer, 1979a).

To examine the possible involvement of a transient outward K current or a K current activated by $[Na]_i$ in the generation of the outward creep current induced by Na loading, creep currents were examined at two different $[K]_o$ concentrations. If the outward creep current is mediated by a K channel, then an elevation

of $[K]_o$ would be expected to reduce the driving force for K and reduce the amplitude of the outward creep current. Fig. 3 shows the results of such an experiment. At 2.5 mM $[K]_o$, 200-ms voltage-clamp pulses were applied to a cell at a frequency of 0.33 Hz (to +10 mV from a holding potential of -90 mV)

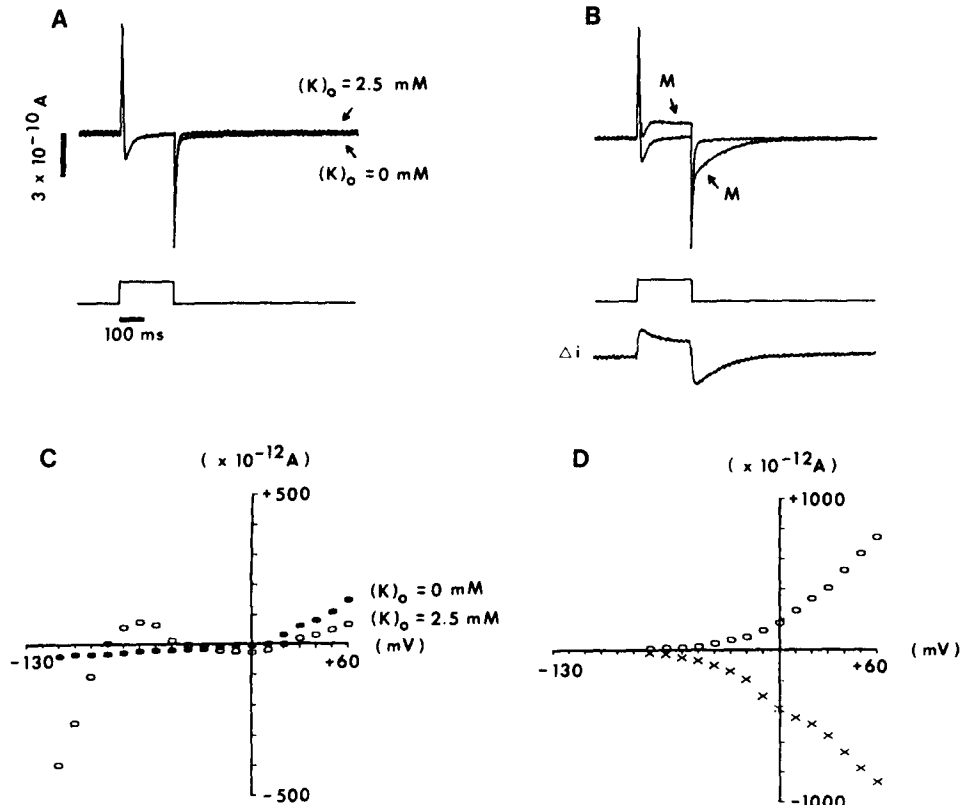


FIGURE 2. Effects of 0 $[K]_o$ on the current-voltage relationship and creep currents in atrial cells. (A) Superimposed membrane currents elicited by a 200-ms voltage-clamp pulse from -80 to +10 mV at 2.5 and 0 mM $[K]_o$. (B) Superimposed membrane currents elicited by 200-ms voltage-clamp pulses from -80 to +10 mV before and after 10 min exposure to 3×10^{-5} M monensin at 0 mM $[K]_o$; the middle trace is membrane voltage and the bottom trace is the digitally subtracted difference current induced by Na loading. (C) Current measured at the end of a 200-ms voltage-clamp pulse as a function of voltage. (D) Voltage dependence of the outward creep current (circles) and inward creep current (x's) induced by Na loading. The measurement of the difference current-voltage relationship was the same as in Fig. 1.

under control conditions and then $[K]_o$ was changed to 25 mM and the protocol was repeated (top traces). At 2.5 mM $[K]_o$, the voltage-clamp pulse elicited a transient inward i_{Ca} similar to that observed in Fig. 1. After changing $[K]_o$ to 25 mM, the current required to maintain the holding potential at -90 mV shifted

inward, which is consistent with an expected depolarization of the cell's resting membrane potential (to approximately -48 mV in this particular cell). The voltage-clamp depolarization to $+10$ mV elicited a transient inward i_{Ca} of the same magnitude as that observed at 2.5 mM $[K]_o$. At this point, the cell was exposed to monensin (10^{-5} M) for 10 min at 25 mM $[K]_o$. Repetitive voltage-clamp pulses to $+10$ mV were then reapplied. Na loading produced the typical changes in membrane currents observed previously: an outward shift in current during the pulse, followed by an inward current upon the return to the holding potential (right trace, middle row). After this, the cell was superfused with Ringer's solution containing 2.5 mM K and 10^{-5} M monensin and the clamp protocol was repeated.

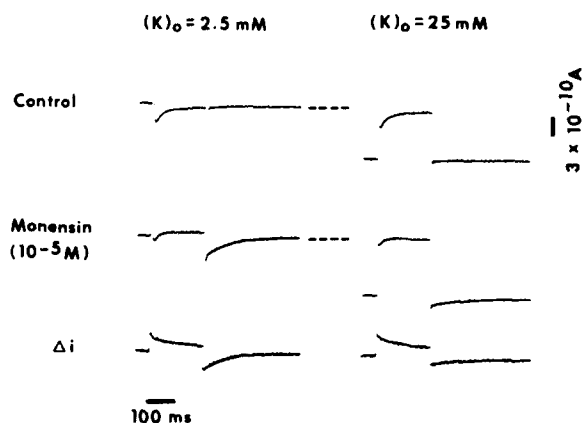


FIGURE 3. Influence of changes of $[K]_o$ on creep currents induced by Na loading of atrial cells. Repetitive 200-ms depolarizing voltage-clamp pulses to $+10$ mV were applied under control conditions with 2.5 mM $[K]_o$ and after changing to 25.0 mM $[K]_o$. The top traces are membrane currents elicited under these conditions. The cell was exposed to 1×10^{-5} M monensin in Ringer's solution containing 25.0 mM K for 10 min and then exposed to 1×10^{-5} M monensin in Ringer's solution containing 2.5 mM K. The middle traces are membrane currents elicited under these conditions, and the bottom traces are the difference currents (Δi) induced by Na loading compared at 2.5 (left) and 25.0 (right) mM $[K]_o$.

The outward and inward creep currents induced by Na loading at 25 and 2.5 mM $[K]_o$ are shown as the subtracted difference currents (Δi ; bottom row of traces in Fig. 3). Despite the considerable difference in driving force at the two $[K]_o$ concentrations, the outward creep currents induced by Na loading are nearly identical. This observation suggests that the outward creep current is unlikely to be mediated by a conventional K channel. In this particular experiment, there was some decrease in the amplitude of the inward creep current at 25 compared with 2.5 mM $[K]_o$. In a total of six identical experiments at 2.5 and 25 mM $[K]_o$, there was no difference in the amplitude of the outward creep induced by Na loading; in three experiments, the amplitude of the inward creep current was reduced at 25 mM $[K]_o$, and in three additional experiments, there was no change in the amplitude of the inward creep current at 25 mM $[K]_o$.

In order to test further the possible involvement of voltage-gated K channels in the generation of creep currents, the effects of two K channel antagonists, Cs and 4-aminopyridine (4-AP), were examined. Some cells were internally loaded with Cs by using pipettes with larger tip diameters (1–2 μm) filled with 150 mM

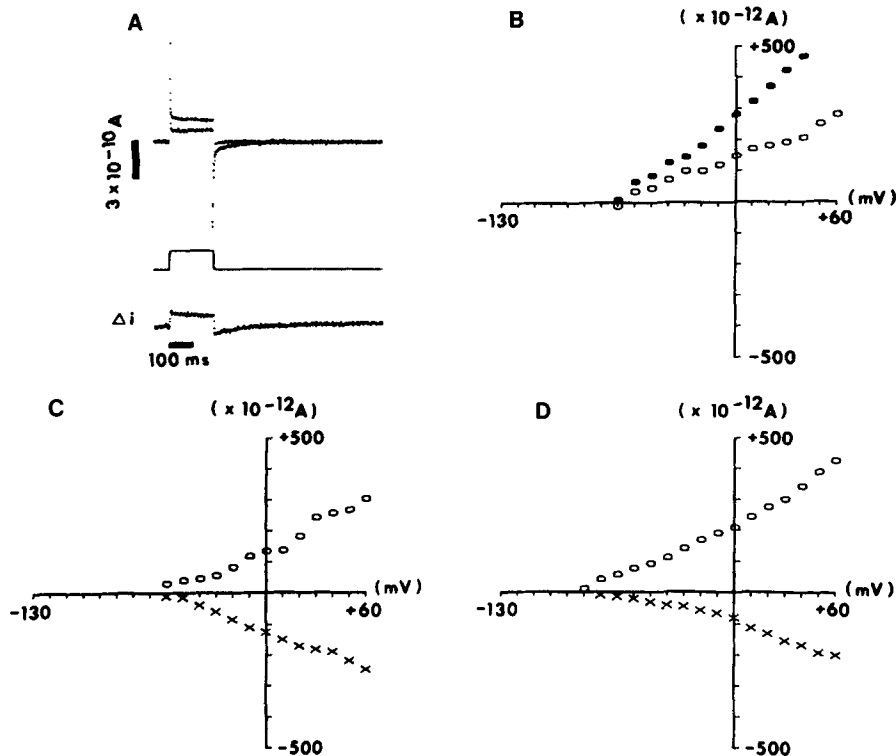


FIGURE 4. Effect of K channel antagonists on creep currents in Na-loaded atrial cells. (A) Superimposed membrane currents elicited by 200-ms voltage-clamp pulses applied from -70 to 0 mV before and after 10 min exposure to 3×10^{-5} M monensin, recorded from a cell that was continuously dialyzed with Cs aspartate (see Methods). The middle trace is membrane voltage and the bottom trace is the digitally subtracted difference current. (B) 200-ms current-voltage relationship from the same cell as in A, before (open symbols) and after (solid symbols) Na loading. (C) The difference current-voltage relationship from the data in B is plotted (circles). The voltage dependence of the inward creep current is also shown (\times 's). (D) Voltage dependence of outward and inward creep currents induced by exposure to 3×10^{-5} M monensin from another cell, which was not dialyzed but exposed continuously to Ringer's solution containing 5 mM 4-AP.

Cs aspartate for both internal dialysis and whole-cell voltage clamp. Fig. 4 shows results from a cell that was internally dialyzed with Cs aspartate. Panel A shows superimposed membrane currents elicited by 200-ms depolarizing voltage-clamp steps from -80 to 0 mV before and after exposure to monensin (3×10^{-5} M).

Na loading produces typical changes in current: an outward creep current during the depolarization and an inward creep current in response to repolarization back to the holding potential. Fig. 4, *B* and *C*, shows that in a cell internally dialyzed with Cs, monensin produces changes in the current-voltage relationship (200 ms) nearly identical to those previously observed (Fig. 1). In this particular cell, before exposure to monensin, a characteristic i_{Ca} was absent, which might be related to the "rundown" of i_{Ca} previously observed in internally dialyzed, isolated myocytes (Irisawa and Kokubun, 1983). The data in panel *D* are from another cell, which was not internally dialyzed. This cell was exposed first to Ringer's solution containing 5 mM 4-AP and then to monensin (3×10^{-5} M) and 4-AP. Despite the presence of 4-AP, Na loading produced typical outward and inward creep currents. Overall, the data from Figs. 3 and 4 strongly argue against the involvement of a voltage-gated K conductance in mediating the creep currents observed in Na-loaded atrial cells.

Dependence of Creep Currents on i_{Ca} and $[Ca]_o$

The next series of experiments was designed to investigate a possible relationship between i_{Ca} and the creep currents induced by Na loading. Fig. 5 shows results from an experiment that tested whether a dose of a Ca channel antagonist that blocked i_{Ca} would influence the development of the creep currents. This cell was not internally dialyzed and was continuously exposed to both TTX ($3 \mu\text{M}$) and Cd (1×10^{-5} M) in order to eliminate both i_{Na} and i_{Ca} , before and after exposure to monensin (3×10^{-5} M). Panel *A* shows superimposed membrane currents elicited by identical voltage-clamp depolarizations before and after Na loading. Despite the absence of i_{Ca} , both outward and inward creep currents were induced (Δi trace), which showed typical voltage dependence (*B*). This experiment is important in that it shows that the digitally subtracted difference currents, which were previously derived from records that contained i_{Ca} and attributed to the creep currents, were not contaminated by possible changes in i_{Ca} . In the absence of i_{Ca} , Na loading produces an outward creep current that slowly decays during the voltage-clamp pulse and an inward creep current after the voltage-clamp pulse. The data in Fig. 5 indicate that the inward creep current is not likely to be a "tail current" associated with i_{Ca} since it persists in the absence of i_{Ca} . Similar results have also been obtained after block of i_{Ca} by other Ca channel antagonists, such as diltiazem and the dihydropyridine derivative nisoldipine.

The possible relationship of the inward creep current to i_{Ca} was further examined in another experiment by comparing the time course of inactivation or decay of i_{Ca} and the time course of the development of the inward creep current. The objective of this experiment was also to determine whether the inward creep current is related to or is distinct from i_{Ca} tail currents. Fig. 5*C* is an example of an envelope-of-tails experiment, which clearly demonstrates that the magnitude of the inward creep current induced by Na loading becomes larger the longer the potential is held at a depolarized level, and that this occurs well after the transient i_{Ca} has subsided. This result excludes the possibility that the inward creep current is a tail current associated with the transient i_{Ca} . The

possibility that the inward creep current is a tail current associated with a noninactivating component of i_{Ca} (e.g., Morad and Tung, 1982) can also be excluded; such a tail current would be expected to reach a maximum and remain constant and also to exhibit sensitivity to Ca channel antagonists.

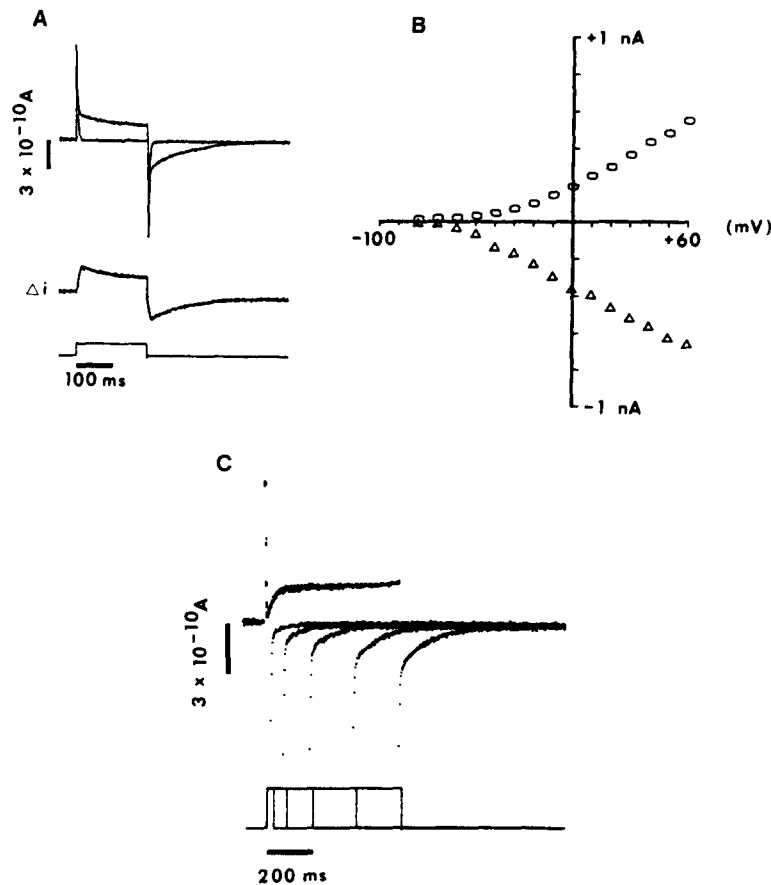


FIGURE 5. Relationship between creep currents and i_{Ca} . (A) Superimposed membrane currents elicited by 200-ms voltage-clamp depolarizations from -90 to -10 mV before and after 10 min exposure to 3×10^{-5} M monensin. All solutions contained 3×10^{-6} M TTX and 5×10^{-5} M Cd to eliminate i_{Na} and i_{Ca} . (B) Voltage dependence of outward creep currents and inward creep currents induced by Na loading (same method of measurement as in Fig. 1). (C) Superimposed membrane currents from another Na-loaded atrial cell (in the absence of Ca channel antagonist) elicited by voltage-clamp depolarizations to $+20$ mV of 25, 90, 200, 400, and 600 ms duration.

The time course of the development of the inward creep current in Fig. 5C does not appear to correspond to the time course of any current activated during the clamp depolarization. Because the control trace has not been subtracted from the trace obtained after Na loading, the outward creep current is not readily apparent. However, as shown in Fig. 5A, as well as in Figs. 1–4, the outward

creep current amplitude declined during the time course of depolarizing clamp steps. The time course of the development of the inward creep current corresponds to the decline of the outward creep current activated during the depolarization (see also Fig. 4 of the following article).

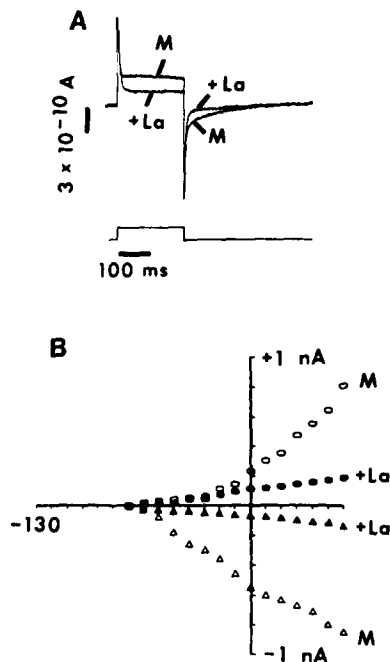


FIGURE 6. Effect of La on creep currents in single atrial cells. In this experiment, the cell was continuously exposed to both 3×10^{-6} M TTX and 5×10^{-5} M CdCl_2 in order to eliminate both i_{Na} and i_{Ca} . 200-ms voltage-clamp pulses were applied to potentials ranging from -80 to $+60$ mV in 10-mV increments before and after exposure to 1×10^{-5} M monensin (M). The cell was then exposed to 1×10^{-5} M monensin and 1×10^{-5} M LaCl_3 (+ La) for 10 min and the voltage-clamp protocol was repeated. (A) Superimposed membrane currents elicited by a clamp step to $+10$ mV after Na loading (in the continuous presence of TTX, CdCl_2 , and monensin) and after exposure to La (in the continuous presence of TTX, CdCl_2 , and monensin). (B) Current-voltage relationship of the difference current (measured at 200 ms) induced by Na loading (monensin - control). Also shown is the peak amplitude of the inward creep current induced by Na loading as a function of the potential of the preceding voltage-clamp pulse (same as in Fig. 1; open triangles). Both the outward and inward creep currents are significantly attenuated after exposure to LaCl_3 .

Are the creep currents induced by Na loading insensitive to all Ca channel antagonists? Fig. 6 shows an experiment that tested the sensitivity of the creep currents to the inorganic Ca channel antagonist lanthanum. This cell was preincubated in Ringer's solution that contained 5×10^{-5} M CdCl_3 and then was Na-loaded by exposure to 10^{-5} M monensin in the continued presence of CdCl_3 . This dose of Cd was effective in suppressing i_{Ca} and after a 10-min exposure to

monensin, typical creep currents were observed (M in panel A). A plot of the outward creep current (measured at 200 ms) induced by monensin as a function of potential, as well as the peak magnitude of the inward creep current as a function of the prepulse potential, is shown in panel B (open circles and open triangles, respectively). The voltage dependence of the creep currents induced by Na loading in the absence of i_{Ca} (blocked by Cd) again is very similar to that observed in the presence of i_{Ca} (see Fig. 1). However, in the continued presence of monensin and CdCl₃, a low dose of LaCl₃ (10^{-5} M) significantly attenuated

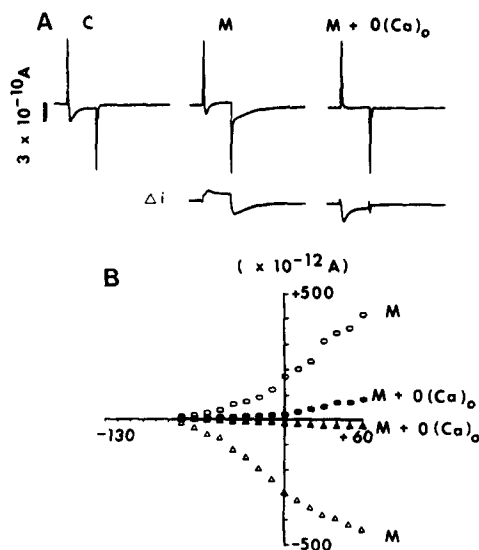


FIGURE 7. Dependence of creep currents on $[Ca]_o$. (A) Membrane currents elicited by 200-ms voltage-clamp pulses to 0 mV are shown under control conditions (C), after 10 min exposure to 5×10^{-6} M monensin (M), and then after 10 min exposure to 5×10^{-6} M monensin in 0-Ca Ringer's solution (M + 0 $[Ca]_o$). The subtracted difference currents (Δi) are also shown (either as monensin - control or as control - monensin in 0-Ca Ringer's). In this cell, Na loading induced typical changes in membrane current: an outward creep current during depolarizing pulses, followed by inward creep currents upon the return to the holding potential (-90 mV). Both outward and inward creep currents (as well as i_{Ca}) were eliminated after exposure to monensin in 0-Ca Ringer's solution. (B) Influence of $[Ca]_o$ on the creep currents induced by Na loading over a wide range of potentials. The difference currents were obtained as in Fig. 1.

both the outward and inward creep currents induced by Na loading. In other experiments, in the absence of CdCl₃, this dose of La blocks both i_{Ca} and the creep currents induced by Na loading. The ability of La to block the creep currents may therefore be an additional effect independent of its ability to antagonize i_{Ca} .

The data in Figs. 5 and 6 indicate that blockade of i_{Ca} by Ca channel antagonists does not attenuate the creep currents induced by Na loading. The dependence of the creep currents on Ca was further examined in the experiment summarized in Fig. 7. Panel A shows membrane currents elicited by 200-ms depolarizing

voltage pulses under control conditions (left), after 15 min exposure to monensin (5×10^{-6} M, middle), and then 15 min later in the continued presence of monensin after changing to Ringer's solution in which Ca was replaced by Mg (0-Ca Ringer's solution; right). The difference current induced by monensin (Δi ; monensin - control) and the difference current obtained after switching to monensin in 0-Ca Ringer's solution (Δi ; control - monensin in 0-Ca Ringer's) are also shown. In normal Ringer's solution, exposure to monensin produced typical changes in membrane current: an outward creep current during the pulse followed by an inward creep current after termination of the pulse. However, after switching to 0-Ca Ringer's solution in the continued presence of monensin, both the outward and inward creep currents (as well as i_{Ca}) were eliminated. A plot of the outward creep current (measured at 200 ms; open circles) as a function of potential and the peak amplitude of the inward creep current as a function of the prepulse potential (open triangles) induced by monensin in normal Ringer's solution and after changing to 0-Ca Ringer's solution (solid circles and triangles) is shown. Both the outward and inward creep currents induced by Na loading of atrial cells depend upon the presence of $[Ca]_o$.

Sensitivity of Creep Currents to $[Na]_o$

We have examined the sensitivity of creep currents to $[Na]_o$ by comparing Na-loaded cells in normal Ringer's solution (110 mM Na) with cells bathed in Ringer's solution in which 50% Na was replaced by sucrose. Fig. 8 illustrates typical results obtained in six cells. This experiment was carried out in the continuous presence of TTX (3×10^{-6} M) and $CdCl_2$ (5×10^{-5} M) in order to eliminate both i_{Na} and i_{Ca} . Fig. 8A shows superimposed currents that were elicited by two identical voltage-clamp depolarizing steps (200 ms duration to +10 mV from $V_h = -90$ mV) obtained in the presence of monensin (10^{-5} M) in normal Ringer's solution (M) and after exposure to 50% Na Ringer's for 10 min (M + $\frac{1}{2}$ $[Na]_o$). 50% Na Ringer's solution produced an increase in the magnitude of the outward creep current during the depolarization and reduced the magnitude of the transient inward creep current. The difference current-voltage relationship produced by Na loading in normal Ringer's solution (M) and after changing to 50% Na Ringer's (M + $\frac{1}{2}$ $[Na]_o$, containing the same dose of monensin) is plotted in Fig. 8B. A reduction of $[Na]_o$ increased the magnitude of the outward creep current (circles) at all potentials positive to the holding potential (-90 mV) but decreased the magnitude of the inward creep current (triangles) recorded on repolarization to -90 mV after all depolarizing voltage-clamp steps positive to the holding potential.

The data of Fig. 8 provide evidence that both the outward and inward creep currents induced by Na loading are sensitive to changes in $[Na]_o$. From these experiments, however, it is not obvious why the outward and inward creep currents change in different directions in response to a reduction in $[Na]_o$. A reduction of both outward and inward creep currents would be expected if the major effect of low $[Na]_o$ were merely to reduce the ability of monensin to elevate $[Na]_i$. (The differential response of the creep currents to a reduction of $[Na]_o$ may, however, be consistent with a shift in the apparent reversal potential

of the mechanism responsible for generating the creep currents; see the following article.)

Inward Creep Currents Are Induced by Ca Loading

Our results thus far have shown that both outward and inward creep currents are consistently observed under conditions in which $[Na]_i$ is elevated. Furthermore, these changes in membrane current are unlikely to be directly mediated

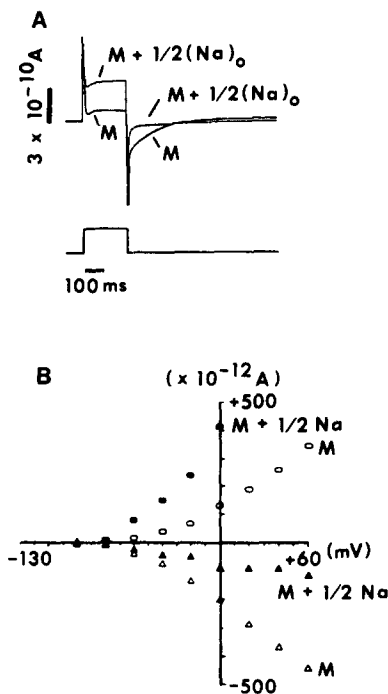


FIGURE 8. Dependence of creep currents on $[Na]_o$. In this experiment, the cell was continuously exposed to both 3×10^{-6} M TTX and 5×10^{-5} M $CdCl_2$ in order to eliminate both i_{Na} and i_{Ca} . (A) Membrane currents elicited by 200-ms voltage-clamp pulses to +10 mV were obtained from an Na-loaded atrial cell (10 min exposure to 1×10^{-5} M monensin [M]) and then after exposure to 50% Na Ringer's containing monensin ($M + 1/2 [Na]_o$). (B) Current-voltage relationship of the outward and inward creep currents induced by Na loading in normal Ringer's solution (open symbols) and in 50% Na Ringer's (solid symbols). The difference curves were obtained using the same procedure described in Fig. 1.

by a K channel or by i_{Ca} but are sensitive to changes in $[Ca]_o$ and $[Na]_o$. If the creep currents are produced by either a Ca-activated nonselective channel (Kass et al., 1978b) or by an electrogenic Na/Ca exchanger, then it might be expected that they would also show some sensitivity to changes in $[Ca]_i$.

Fig. 9 illustrates results from an experiment that tested whether an increase in $[Ca]_i$ (in the absence of any attempt to elevate $[Na]_i$) could produce changes in membrane current similar to the creep currents induced by Na loading of cells.

Panel A shows superimposed currents elicited by repetitive 200-ms voltage-clamp depolarizations to +10 mV under control conditions and during superfusion with isoproterenol (5×10^{-7} M). β -Adrenergic stimulation produced the expected result: a progressive increase in the amplitude of i_{Ca} . Note, however, that coincident with the enhancement of the i_{Ca} amplitude during the voltage-clamp pulse, there was also a progressive enhancement of the amplitude of what appears to be an inward "tail current" upon the return to the holding potential. Since the amplitude of i_{Ca} is increased nearly threefold by this dose of isoproterenol, it is reasonable to also expect an increase in i_{Ca} tail currents. However, an envelope-of-tails test (B) revealed that the amplitude of the apparent inward tails got larger the longer the potential was held at a depolarized level, and continued to increase well after the transient i_{Ca} had subsided. This apparent "tail current" produced by a relatively high dose of isoproterenol bears a striking similarity to the inward creep current induced by Na loading (see Fig. 5).

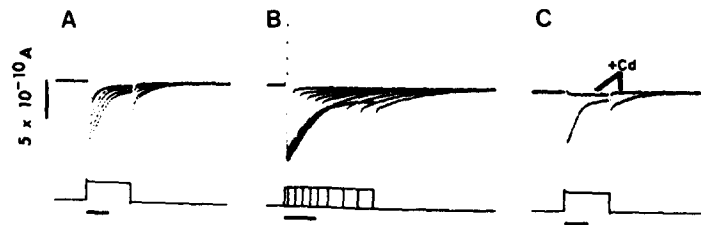


FIGURE 9. Inward creep currents induced in response to increases in i_{Ca} amplitude. (A) Repetitive voltage-clamp pulses were applied (0.33 Hz) to +10 mV under control conditions and during superfusion with 5×10^{-7} M isoproterenol. (B) Superimposed currents elicited by depolarizing clamp pulses to +10 mV for durations of 10, 30, 60, 80, 100, 130, 180, 200, and 280 ms (all in 5×10^{-7} M isoproterenol). (C) Superimposed membrane current elicited by a voltage-clamp pulse to +10 mV in the presence of 5×10^{-7} M isoproterenol and then after changing to a solution containing 5×10^{-7} M isoproterenol and 1×10^{-3} M $CdCl_2$. Horizontal calibrations are 100 ms.

These results suggest that similar inward creep currents are produced by conditions that elevate either $[Na]_i$ or $[Ca]_i$. However, as shown in Fig. 9C, in contrast to the insensitivity of inward creep currents induced by Na loading to Ca channel antagonists, the inward creep currents induced by an elevation of $[Ca]_i$ via enhancement of i_{Ca} were attenuated by Ca channel antagonists like Cd (1×10^{-3} M).

DISCUSSION

The changes in membrane current that are observed after single frog atrial cells are loaded with Na are remarkably similar to the "creep currents" that were originally observed in papillary muscles and Purkinje fibers after exposure to strophanthidin (Lederer and Tsien, 1976) or to low-K solutions (Eisner and Lederer, 1979a). Transient inward currents (TI's) and aftercontractions accompanied the creep currents under both of these conditions, which would be

expected to produce an increase in $[Na]_i$. In contrast, in frog atrial cells, increases in $[Na]_i$ result in the development of both outward and inward creep currents without accompanying TI's or aftercontractions. The underlying mechanisms responsible for the development of TI's and aftercontractions have received considerable attention in recent years, but the relationship between TI's and creep currents or the mechanism responsible for creep currents has not been studied in any detail. Na-loaded frog atrial cells offer the opportunity to study the properties of creep currents in the absence of accompanying TI's.

Na loading of single frog atrial cells, either by exposure to the ionophore monensin or by intracellular dialysis with Na, produces similar changes in membrane current: an increase in outward current during depolarizing voltage-clamp pulses (with an apparent time-dependent sag or creep in current) followed by a transient inward ("tail" or creep) current upon the return of the membrane potential to the holding potential. The possible mediation of these changes in current by either K channels or i_{Ca} was examined. Despite the similarity between the time course of the outward creep current and the transient outward current, which has been described in some cardiac preparations (Kenyon and Gibbons, 1979; Siegelbaum and Tsien, 1980; Josephson et al., 1984), and the recent report of a K channel activated by $[Na]_i$ (Kameyama et al., 1984), the outward creep current observed in Na-loaded frog atrial cells is not sensitive to changes in $[K]_o$ or to the K channel antagonists Cs and 4-AP. Therefore, the activation of a K channel is probably not an important factor in the generation of the outward creep current. Na channels are also unlikely to be involved in the generation of the creep currents, since all solutions contained TTX.

In some experiments, the inward creep current appeared to be reduced in amplitude at higher $[K]_o$. It is not clear how consistent this result is or whether it indicates a genuine dependence of the inward creep current on the transmembrane K gradient. It should be pointed out that in these experiments no attempt was made to maintain constant osmolarity when $[K]_o$ was elevated, since decreases in $[Na]_o$ alone influence the creep currents (cf. Fig. 8). Changes in the inward creep current in response to elevations of $[K]_o$ may therefore be complicated by osmotic effects or, alternatively, the ionophore's selectivity for Na^+ over K^+ may be reduced when $[K]_o$ is elevated.

The inward creep current observed on repolarization of the membrane potential back to the holding potential could be interpreted as a tail current associated with i_{Ca} in Na-loaded cells. However, our experiments show that concentrations of either organic or inorganic Ca channel antagonists that block i_{Ca} had no effect on the inward creep current, and an envelope-of-tails experiment revealed that the amplitude of the inward creep currents continued to increase well after i_{Ca} had decayed (Fig. 4). These experiments indicate that it is unlikely that the inward creep currents induced by Na loading are directly mediated by i_{Ca} ; however, some relationship between creep currents and Ca is suggested by the observation that removal of $[Ca]_o$ attenuates both inward and outward creep currents induced by Na loading.

A relationship between inward creep currents and Ca is also suggested by the observation that doses of isoproterenol that enhance i_{Ca} induce an inward creep

current that resembles the inward creep currents induced by Na loading. An envelope-of-tails experiment on the isoproterenol-induced inward creep current also suggested that the creep current is not directly caused by i_{Ca} , but the inorganic antagonist Cd blocks both i_{Ca} and the inward creep current in the continued presence of isoproterenol. This result can be explained if the inward creep current observed in the presence of isoproterenol is produced indirectly by an elevation of $[Ca]_i$ via enhancement of i_{Ca} , and is therefore reversed when i_{Ca} is blocked. This explanation is consistent with the observation that the larger i_{Ca} observed at higher $[Ca]_o$ is frequently accompanied by inward creep currents that also are blocked by low doses of organic Ca channel antagonists (see Fig. 2 of Uehara and Hume, 1985b). A scheme that explains the actions of Ca channel antagonists on the inward creep currents induced by either an elevation of $[Na]_i$ or by enhancement of i_{Ca} is shown in Fig. 10.

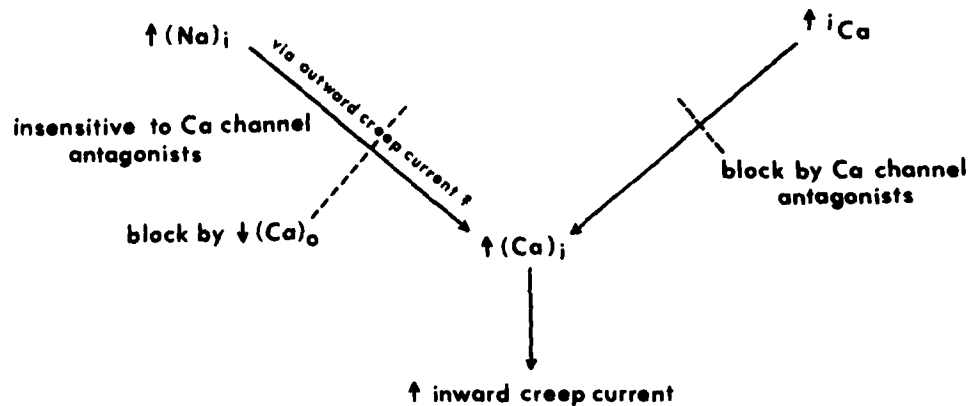


FIGURE 10. Hypothesis that accounts for the differential sensitivity of inward creep currents induced either by Na loading or by augmentation of i_{Ca} to Ca channel antagonists.

In this hypothesis, an elevation of $[Ca]_i$ is considered the direct stimulus for the inward creep current and would explain both the observed insensitivity of the inward creep currents induced by Na loading to Ca channel antagonists and the sensitivity of the inward creep currents induced by enhancement of i_{Ca} to Ca channel antagonists. This hypothesis also explains the sensitivity of the inward creep currents induced by Na loading to changes in $[Ca]_o$. It is reasonable to expect that both an increase in $[Na]_i$ and an enhancement of i_{Ca} will produce an elevation of $[Ca]_i$ in the heart, although by different mechanisms. It is important to point out that during the time course of the outward creep current in Na-loaded frog atrial cells, even in the absence of i_{Ca} (in the presence of Ca channel antagonist), the cell contracts strongly. It can be concluded, therefore, that during the time course of the outward creep current, there must be a significant increase in $[Ca]_i$.

It has previously been suggested that creep currents and TI's may be generated by similar mechanisms in papillary muscles and Purkinje fibers (Eisner and

Lederer, 1979*a*). Kass et al. (1978*b*) proposed that TI's are generated by either (*a*) a Ca-activated channel that is selective for both Na and K, as subsequently described by Colquhoun et al. (1981), or (*b*) an electrogenic Na/Ca exchange mechanism. More recent studies of the ionic basis of the TI's have provided evidence in support of the latter mechanism (Karagueuzian and Katzung, 1982; Van Ginneken, 1983; H. F. Brown et al., 1984; Arlock and Katzung, 1985). Although the exact mechanism is presently controversial (Eisner and Lederer, 1985), both potential mechanisms for the generation of TI's must be considered for the generation of creep currents in frog atrial cells.

There are important differences between the current changes induced by Na loading of single frog atrial cells and those previously described in some Na-loaded mammalian preparations, which bear upon the question of the mechanism underlying the creep currents. In Na-loaded frog atrial cells, creep currents are not accompanied by TI's or aftercontractions. This may be due to the smaller amount of releasable Ca available as a result of the smaller volume of sarcoplasmic reticulum in this species (Page and Niedergerke, 1972). Consistent with these anatomical differences, cyclic contractions caused by oscillatory release of $[Ca]_i$ from intracellular stores cannot be demonstrated in skinned frog ventricular cells (Fabiato and Fabiato, 1978), which suggests that activation of myofilaments may be more directly dependent upon transsarcolemmal Ca influx. If similar reasoning is used to explain the absence of TI's in frog atrial cells—namely that a cyclic release of internal Ca is insufficient to activate the nonselective channel responsible for generation of the TI—then it is necessary to conclude either (*a*) that a cyclic release of internal Ca is also insufficient to activate a nonselective channel underlying the creep currents, or (*b*) that activation of a nonselective channel underlying the creep currents is more dependent upon transsarcolemmal Ca influx. However, in frog atrial cells, the creep currents induced by Na loading appear to be rather insensitive to concentrations of organic or inorganic Ca channel antagonists that block Ca influx via i_{Ca} . This is in contrast to results obtained in mammalian Purkinje fibers (Kass et al., 1978*a*), which have shown that both TI's and aftercontractions are inhibited by both organic and inorganic Ca channel antagonists (see also Ferrier and Moe, 1973). The ability of Ca channel antagonists to inhibit TI's and aftercontractions in mammalian preparations may be due to their ability to eventually deplete intracellular stores of Ca (Kass et al., 1978*a*). The simplest conclusion to be drawn with regard to frog atrial cells is that TI's are not observed in response to an elevation of $[Na]_i$, because Ca-activated nonselective channels may not be present in this preparation and may not be important factors in the generation of creep currents. Our experiments, however, cannot eliminate the possibility that creep currents may be generated by a nonselective cation channel that is dependent upon Ca influx via an electrically neutral Na/Ca exchange mechanism.

An Na/H exchange mechanism is known to exist in most vertebrate cells, which is important in the regulation of intracellular pH (Aickin and Thomas, 1977; Kinsella and Aronson, 1980; Deitmer and Ellis, 1980; Piwnica-Worms et al., 1985). Therefore, any technique for elevating $[Na]_i$ in the heart would be expected to result in subsequent intracellular acidification via an Na/H exchange system. The changes in membrane current induced by Na loading of atrial cells

are not likely to be directly mediated by an Na/H exchange mechanism, since most investigations on the stoichiometry of Na/H exchange agree that it is electrically neutral and not voltage dependent (see Mahnensmith and Aronson, 1985, for review). However, intracellular acidification per se as a result of the activity of an Na/H exchanger could produce alterations in the properties of membrane channels. However, our experiments indicate that mediation of creep currents by voltage-gated Na, Ca, or K channels is unlikely. Therefore, although Na loading of atrial cells must result in some intracellular acidification, pH-induced alterations in ion channel properties are not a likely explanation for the induction of creep currents.

The creep currents observed in Na-loaded atrial cells are consistent with predictions of a thermodynamic model of electrogenic Na/Ca exchange proposed by Mullins (1979; see also Fig. 7.3 in Mullins, 1981). In this model, Na/Ca exchange would be expected to produce an outward current at potentials positive to the reversal potential of the exchanger (E_{ex}), which would be associated with Ca influx into the cell. At potentials negative to E_{ex} , a transient inward current associated with Ca efflux is predicted. Furthermore, it is believed that E_{ex} is labile and will shift as a function of the activity of the exchanger. Therefore, in the Mullins' model, any time dependence for the exchanger current would be due to transient changes in ion concentration gradients and subsequent shifts in E_{ex} of the exchanger, or due to re-establishment of ion concentration gradients by the exchanger. Careful reversal potential measurements of creep currents are needed to determine whether the time dependence of the creep currents is consistent with transient changes in ion concentration gradients.

Our experiments have also shown that low doses of La (independently of effects on i_{Ca}) block both the outward and inward creep currents in Na-loaded frog atrial cells. La has been reported to be a putative inhibitor of Na/Ca exchange in isolated sarcolemmal vesicles (Reeves and Sutko, 1979). The hypothesis that creep currents are generated by an electrogenic Na/Ca exchange mechanism is examined in the following article (Hume and Uehara, 1986).

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