Inward current generated by Na–Ca exchange during the action potential in single atrial cells of the rabbit

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To investigate the underlying ionic mechanism of the late plateau phase of the action potential in rabbit atrium the whole-cell patch-clamp technique with intracellular perfusion was used. We recorded the inward current during repolarizations following a brief 2 ms depolarizing pulse to +40 mV from a holding potential of between −70 and −80 mV. The development of this current coincides with the onset of the late plateau phase of the action potential. Peak activation of the current occurs about 10 ms from the beginning of the depolarizing pulse, and it decays spontaneously with a slow timecourse. Its voltage dependency from −40 mV to +40 mV shows very steep activation (−40 to −20 mV) and shows almost the same maximum magnitude between −10 mV and +40 mV. This behaviour is quite different from that of the calcium current. The inward current and the late plateau phase of the action potential were both abolished by the application of 5 mM EGTA, 1 μM ryanodine and by reducing the Na⁺ gradient. The fully activated current–voltage relation of the inward current was plotted as the difference current before and after treatment with Ryanodine, Diltiazem, 20 mM Na⁺ inside or 30% Na⁺ outside and shows an exponential voltage dependence with the largest magnitude of the current occurring at negative potentials. The current–voltage (I–V) curve was well fitted by the Na–Ca exchange equation, \(i = A \exp \left(-\frac{(1-r)EF}{RT}\right)\). The results suggest that the inward current contributes to the generation of the late plateau phase of the rabbit atrial action potential, and is activated by intracellular calcium released from the sarcoplasmic reticulum. Sarcoplasmic reticulum calcium release appears to be triggered both by the membrane voltage and by the calcium current. It is concluded that the inward current is generated by Na–Ca exchange.

Introduction

It has long been thought that the calcium current plays a crucial role in the maintenance of the plateau of the action potential (Beeler & Reuter 1970). However, in rat ventricular cells and rabbit atrial cells, it appears that the calcium current plays a role chiefly in the early stage of the action potential; it has been suggested that the late component of the plateau at negative potentials is associated with an additional inward current activated by internal calcium, and that

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this current is secondarily dependent upon the calcium current that releases further calcium from the intracellular stores (Mitchell et al. 1984b; Schouten & Ter Keurs 1985; Hilgemann 1986; Hilgemann & Noble 1987). This inward current, together with the late plateau phase of the action potential, was abolished by substitution of lithium for extracellular sodium, by Ryanodine, or by intracellular EGTA (Mitchell et al. 1984b; Mitchell et al. 1987; Y. E. Earm and W. K. Ho, unpublished data) and these results are consistent with the view that it is activated by intracellular calcium and that sodium is a charge carrier. The existence of this calcium-activated inward current was first shown directly by using caffeine to induce calcium release from the sarcoplasmic reticulum (sr) and then observing the development of inward Na–Ca-exchange current (Mechmann & Pott 1986). A Na–Ca-exchange current was also characterized in guinea-pig ventricular myocytes by a whole-cell-clamp technique by using internal dialysis (Kimura et al. 1986; Earm & Irisawa 1986; Kimura et al. 1987). However, it is not yet clear whether this inward exchange current is activated during normal electrical activity, and if it is, how much net electrical charge it carries. Given that such a current flows, it is also important to determine its timecourse and voltage dependence.

We report here experiments first demonstrating that in rabbit atrial cells there is a calcium-dependent inward current flowing during the normal action potential and that it is a Na–Ca exchange current activated by intracellular calcium, which is released from the sarcoplasmic reticulum. We further investigated this inward current with regard to its voltage dependence, sodium and calcium dependence, and its sensitivity to calcium-channel blockers. Part of this work has been published in abstract form (Earm et al. 1989a).

**Methods**

Single atrial cells of rabbit were isolated by a method similar to that described by Kimura et al. (1987). Briefly the heart was perfused with low Ca2+-Tyrode solution (30–50 µM Ca2+) containing collagenase (20 mg per 50 ml, Sigma, type I or Worthington, type II) for 10–15 min by using a Langendorff perfusion system. Atrial tissue was dissected out and mechanically agitated to disperse the cells and then stored in low-CP, high-K+ medium in the refrigerator.

The solution used to superfuse atrial cells contained (in millimoles per litre): NaCl, 140; KCl, 5.4; CaCl2, 1.8; MgCl2, 1; NaH2PO4, 0.33; glucose, 5; HEPES, 5; adjusted to pH = 7.4 with NaOH. In experiments in which sodium was reduced, NaCl was replaced by equimolar LiCl. The internal solution of the patch electrode normally contained (in millimoles per litre): K-aspartate, 110; Mg-ATP, 5; di-Tris-creatine phosphate, 5; MgCl2, 1; KCl, 20; HEPES, 5, EGTA, 0.1; adjusted to pH = 7.4 with KOH. For a Cs+-rich internal solution to block potassium currents, K-aspartate was replaced by equimolar Cs-aspartate and KCl by TEA-C1, and the pH was adjusted to 7.4 by using CsOH. To make an internal solution with calcium concentration [Ca2+] equal to 63 nM (pCa = 7.2), we simply added 0.05 mM Ca2+ to the normal solution that contained 0.1 mM EGTA. In some experiments (as in figure 3) we added 10 µM cAMP to the internal solution to obtain greater activation of the slow component of inward current (Earm et al. 1989b).
During experiments, cells were superfused (1 ml min⁻¹) at 35–37 °C.

Chemicals and drugs used in this study included; Ryanodine (Penick), Diltiazem (Tanabe); all other chemicals were obtained from Sigma.

The cells were voltage clamped or current clamped by using a whole-cell patch-clamp apparatus (List, EPC-7) according to the original technique developed by Hamill et al. (1981) with an intracellular perfusion technique (Soejima & Noma 1984; Earm & Irisawa 1986). Glass electrodes with resistances of 2–3 MΩ were used.

The data were recorded on a pulse code modulator (pcm) data recorder (NF, 880) for future analysis. Data were also displayed on a digital oscilloscope (Hitachi, 6041, or Nicolet, 9024) and pen recorder (Harvard oscillograph) and could then be directly reproduced onto an X-Y recorder (Graphtec, WX 2400).

**Results**

We used small rabbit atrial cells having sizes in the range of 8–10 μm in diameter, 80–100 μm in length and 6.4 pl (n = 55) in average volume. This volume estimate is based on assuming the cell to be cylindrical. The cells are between spindle and rod-shaped, which may mean that we may have slightly overestimated the cell volume. The series resistance under whole-cell-clamp conditions varied between 5 and 8 MΩ. The total capacitance of atrial cells, which was either measured from the integral of the capacitive transient in response to small voltage steps or which was read from the List patch-clamp amplifier by nulling the capacitive current usually was 30–40 pF. The input resistance was around 1 GΩ. The time constant of capacitive current was in the range of 150–350 μs. The characteristics of our atrial cells are thus smaller than those reported for other rabbit atrial cells elsewhere (Clark et al. 1988; Giles & Imaizumi 1988). We applied pulses of 2 ms or, in some cases, 5 ms duration to +40 mV and the capacitive current settled down very quickly. We also tested the possibility of inadequate voltage control by inserting a second microelectrode to monitor membrane potential during depolarizing pulses. The results showed that the voltage deviation from the imposed square clamp was small and was limited to the first 1 ms (see figure 3). This deviation was attributable to activation of sodium current as it was eliminated by applying 30 μM TTX. It is worth noting though that activation of the slow current was not significantly changed. We therefore conclude that we could control the membrane potential rapidly enough to do the experiments described.

Atrial myocytes had resting potentials in the range −65 to −75 mV and action potentials of about 110 mV in amplitude and 100–250 ms in duration (90% repolarization). Action potentials resulting from low frequency stimulation (0.025 Hz) showed two distinctive repolarization phases; early rapid repolarization and a late, low-level plateau phase. These values and shapes are very similar to those described previously (Hilgemann 1986; Giles & Imaizumi 1988).

Figure 1 shows an action potential and the currents evoked by repolarization pulses after a brief 2 ms depolarization to +40 mV. In figure 1a, the action potential and the current at −40 mV were recorded at a fast sweep speed (the stimulation frequency is 0.025 Hz). The late plateau phase of the action potential usually developed in the voltage range −30 to −40 mV and corresponded closely with the inward current activation at −40 mV (figure 1a). The onset of the late
plateau phase of the action potential occurs just after the peak of inward current.
The inward current activated by the depolarization to +40 mV decreases in magnitude as the potential value at which it is recorded becomes more positive but it is difficult to measure the amplitude of peak inward current at more depolarized potentials because of contamination with transient outward current (figure 1b, upper record). During the pre-pulse, sodium current and transient outward current were activated but are largely offscale, and have been omitted for clarity. Because activation of transient outward current at depolarizations positive to about —40 mV hampered the analysis of the inward current in which we were interested, we attempted to block the transient outward current as well as other potassium currents by using a Cs⁺-rich internal solution.

In the lower record of figure 1b, current activation in Cs⁺ internal solution by a double-pulse protocol is shown, and it can be seen that the outward current component that appears in the upper trace was almost completely abolished and that over the whole range of potentials applied, only inward currents remained. Another line of evidence suggesting that there is inward current activation is shown in figure 1c. In the presence of 1 mM 4-aminopyridine (4-AP), which is known to be a relatively specific blocker of the transient outward current (Hille 1984), inward current activation shows little change, but the initial outward decaying component seen at membrane potentials positive to —30 mV was blocked and net inward current can then be recorded at these voltages.

Peak activation of the inward current at —70 mV occurs 5–10 ms from the beginning of the stimulus (prepulse to +40 mV), and the magnitude of the inward current was in the range —200 to —600 pA (315 ± 52 pA, mean ± s.d., n = 55) at —70 mV and —100 to —200 pA (157 ± 23 pA, n = 43) at —40 mV. The inactivation time constant at —40 mV is in the range 10–20 ms. In some cases the timecourse of the inactivation showed two components: an initial fast one, followed by a later slow one (see Earm et al. (1989)). If we postulate that this inward current is activated by internal calcium and reflects Ca²⁺ release from the sarcoplasmic reticulum, the timecourse of peak activation and inactivation of the current is much faster than that of the Fura-2 Ca²⁺-signal measured in rat ventricular myocytes (Cannell et al. 1987). However, the timecourse of peak activation is in a similar range to the inward-current tail recorded in guinea-pig ventricular myocytes (Egan et al. 1986; 1989). Reasons for this difference will be discussed later (see Discussion).

We tested the effect of pre-pulse duration on the current activation. Figure 2a shows the current activation after voltage-clamp depolarizations to +40 mV for durations ranging between 1 and 10 ms. It can be seen that following the shorter depolarizations (from 1 to 3 ms), a biphasic inward current is recorded, which decays with the same timecourse and reaches a maximum negative value following the 3 ms clamp pulse. As the prepulse duration is further prolonged, the biphasic nature of the response is no longer apparent and a fast inward current is recorded that is difficult to separate from the capacitive current. In figure 2c the effect of holding potential on the current activation is illustrated. When the cell was clamped at —40 mV for some time before giving the test depolarization to +40 mV for 2 ms, the inward current recorded at —70 mV was monophasic and
Figure 1. The inward current and action potential in atrial cells of the rabbit. In (a) the inward current recorded at $-40$ mV after depolarizing the membrane from $-80$ to $+40$ mV for 2 ms (upper trace) is shown superimposed upon the action potential (lower trace). The voltage protocol is given in the inset. The timecourse of the inward current coincides with the development of the late plateau. In (b) the currents in control (K$^+$ inside: top trace) and in Cs$^+$ solution (middle trace) were recorded at various levels of repolarization (inset). Top and middle traces were obtained in the same cell. (c) shows superimposed current records obtained in control (○) and in 4-aminopyridine (4-AP) (●) solution at various potentials. It can be seen that the transient outward current that obscures the beginning of the inward current records between about $-30$ and $+10$ mV was abolished by Cs$^+$ and 4-AP. Stimulation frequency was 0.025 Hz.
However, if the membrane is held at $-70 \text{ mV}$ before giving the test pulse, the more slowly decaying, biphasic inward current is activated. When voltage-clamp pulses of $-40 \text{ mV}$ from $-70 \text{ mV}$ for different durations preceded the pulse of $+40 \text{ mV}$ for 2 ms and the membrane was returned to $-70 \text{ mV}$, inward current was recorded (figure 2b). It can be seen that following the shortest prepulse ($1 \text{ ms}$), a biphasic inward current was activated, but that following a 6 ms prepulse, only the monophasic, slowly decaying inward-current tail was seen whereas after the 11 ms prepulse, little inward current of any kind was observed. These results suggest that calcium release from the sarcoplasmic reticulum can be dependent upon the membrane potential (Hilgemann & Noble 1986) (but see Earm & Noble (1990) for a discussion of whether this dependence is necessarily direct), i.e. that the membrane potential can modify Ca$^{2+}$ release so that the release at two different potentials can be very different even if the calcium currents are similar. The consequent increase in $[\text{Ca}^{2+}]_{i}$ could then trigger the sodium-calcium exchange mechanism thus activating inward current (see Discussion).

To compare the inward current ($i_{\text{in}}$), with the calcium current ($i_{\text{Ca}}$), the voltage dependence of the inward current activation and Ca$^{2+}$ current is illustrated in figure 3. To rule out the possibility of serious voltage escape caused by activation of Na$^{+}$ current during the depolarizing pulses, we placed a second patch electrode ($V_{2}$) 30–40 $\mu$m away from the voltage-clamping electrode ($V_{1}$) and monitored membrane voltage during the experiment (figure 3a, b (inset)). When we applied depolarizing pulses of 5 ms duration there was a small loss of control during the first 1 ms during the activation of the sodium current. Thereafter, the voltage was well controlled. The initial voltage deviation was abolished when the Na$^{+}$ current was blocked with 30 $\mu$m TTX. The activation of the inward current, $i_{\text{in}}$ was compared in the control case (with the small voltage deviation) and after adding TTX. No significant difference was found (figure 3b). In both cases the voltage dependence of $i_{\text{in}}$ was quite different from that of the calcium current, which usually activates from about $-30 \text{ mV}$ and reaches a peak at about $+10 \text{ mV}$ with the magnitude of peak current in the range $-200$ to $-800 \text{ pA}$ ($350 \pm 85 \text{ pA}, n = 22$); further depolarization results in a decrease in the amplitude of $i_{\text{Ca}}$ (the dotted line in figure 3c). Peak activation of $i_{\text{in}}$, however, has a different voltage dependence both in the control and in the presence of TTX (figure 3a). The current $i_{\text{in}}$ was activated by 5 ms depolarizing pulses of different amplitude and recorded at $-70 \text{ mV}$. As can be seen in figure 3a, b, this inward current was activated by depolarizations positive to and including $-40 \text{ mV}$. It rapidly increases towards a peak at about $-10 \text{ mV}$. Usually there was very little difference between the amounts of current activated following depolarizations over the range from $-10 \text{ mV}$ to $+40 \text{ mV}$. The apparent reversal potential of the calcium current was near $+60 \text{ mV}$, but the inward current did not show a reversal even at potentials of $+80$ to $+100 \text{ mV}$.

Depolarization beyond about $+40 \text{ mV}$ results in a decrease in the current activation. The voltage dependence of this current is very similar to that of intracellular calcium transients measured with Fura-2 in rat ventricular myocytes (Berlin et al. 1987).

The observation that the voltage dependence of the inward-current activation is quite different from that of the calcium current suggests that the activation of
Figure 2. Effects of pulse duration and the level of the conditioning pre-pulse on the activation of the inward current. When the duration of the pulse to +40 mV increased from 1 ms to 10 ms the activation of the inward current clearly shows an increasing envelope (a). However, following pre-pulses longer than 5 ms the biphasic appearance of current activation disappears and only the current decay is seen, which becomes so rapid that it merges with the capacitive transient. When pre-pulses to −40 mV of various duration preceded the pulse to +40 mV for 2 ms, the current activation was maximum following 1 ms at −40 mV and then decreased gradually with increasing durations of the pre-pulse (b). In (c) the effect of holding potential (usually about −70 mV) was tested. When the membrane holding potential was decreased to −40 mV, the inward current activated by depolarization to +40 mV was very substantially reduced (right trace).

The inward current is not simply a reflection of the calcium current but rather occurs in response to intracellular calcium transients (Berlin et al. 1987). The above results support the hypothesis that when the calcium current does trigger the release of calcium from the SR, then only a small fraction of calcium current is required to initiate a virtually maximal calcium release provided the SR is fully
Figure 3. Comparison of the voltage dependence of the inward ($i_{in}$) current and the calcium current ($i_{Ca}$). To exclude possible contamination by $i_{Na}$ or inadequate voltage control, a second patch electrode ($V_2$) was placed 30–40 μm away from the voltage-clamping electrode ($V_1$) so that membrane potential could be measured independently during depolarizing pulses. The results are compared in the control and in the presence of 30 μM TTX (see insets of (a) and (b)). The voltage records at $V_2$ show that there was a small deviation from the clamp voltage during the initial 1 ms in the control records, but that this deviation is removed when the sodium current is blocked by TTX. After 1 ms the voltage is reasonably well-controlled in both cases. Step depolarizing pulses to a range of positive potentials were applied for 5 ms and the activation of $i_{in}$ in control (a) and in 30 μM TTX (b) is shown. At −50 mV there was no inward current but from −40 to −20 mV there is very steep voltage dependence, then from −10 mV to +40 mV virtually the full magnitude of $i_{in}$ was activated. Above +40 mV the activation of this inward current diminished. The current–voltage relations of $i_{in}$ in control (•), in 30 μM TTX (■) and that of $i_{Ca}$ (------) are shown in (c), where the ordinate represents the normalized value of current magnitude and the actual current traces for $i_{Ca}$ are not shown here. The I–V curve for the calcium current shows the typical bell-shaped appearance with maximum activation at +10 mV, whereas that of $i_{in}$ shows a steeper initial activation pattern with peak activation over the wide range −10 to +40 mV.
loaded. Again, as suggested above, increased [Ca$^{2+}$]$_i$ could activate the Na–Ca exchange system in the inward direction (Noble 1984; Hilgemann & Noble 1986; Cannell et al. 1987).

**Effect of intracellular Ca$^{2+}$ concentration**

We attempted to change the intracellular Ca$^{2+}$ concentration in three ways; by application of Ryanodine, by increasing the intracellular EGTA and by internal dialysis of Ca$^{2+}$ through the perfusion pipette. The late plateau phase of the action potential in rabbit atrial cells is suppressed by increasing the intracellular EGTA from 0.1 mM to 0.5 mM (figure 4a, upper trace). In voltage-clamp records (figure 4a, lower trace) the inward current decreased when the internal EGTA was changed to 0.5 mM (solid symbols). This decrease corresponded well with the reduction in size of the late plateau of the action potential. When the concentration of intracellular EGTA was increased to 5 mM, the inward current was completely abolished (data not shown here). We also tested the effect of intracellular EGTA by using a different protocol. When the micropipette contained 0.1 mM EGTA, a voltage-clamp pulse of +40 mV for 2 ms from −70 mV activated the inward current as described earlier. Just after rupture of the cell membrane, the first

![Figure 4](http://rspb.royalsocietypublishing.org/)
Figure 5. Effect of Ryanodine on the action potential and the inward current. (a) Illustrates the superimposed action potential records before (○) and after (●) treatment with 1 μM ryanodine. Ryanodine suppressed the activation of the late plateau. B, shows that Ryanodine abolished most of the inward current over a wide potential range (arrow indicates the current activation in 1 μM Ryanodine and voltage protocols are shown in the inset). The duration of the pulse to +40 mV from −70 mV was 2 ms. The fact that the current becomes net outward at potentials positive to −30 mV is because transient outward current is also being activated (see figure 1 and related text).

Record of the inward current usually showed a large activation and a very slow decay (figure 4b, open circles in upper and lower traces), but in the steady state the overall inward current (solid circles) became smaller, the time to peak faster and the decay of current much faster. This result suggests that intracellular calcium enhanced by the brief depolarization was gradually buffered by the 0.1 mM EGTA as it slowly diffused into the cell and that the decrease of the inward current then reflects this reduced calcium concentration. The effect of EGTA is most marked on the slowest component of current, so that the more rapid component remains and shows a faster time to peak (Earm et al. 1989b).

Ryanodine is thought to interfere with calcium release from the SR and has been reported to abolish a slow inward-current tail in rat ventricular myocytes (Mitchell et al. 1987). Figure 5 shows the effect of 1 μM Ryanodine on the action potential
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(figure 5a, solid circle) and the inward current (figure 5b, arrow). Ryanodine suppressed the late plateau phase of the action potential and completely abolished the inward current over a large range of membrane potentials. The above findings are consistent with the view that this inward current is activated by intracellular calcium released from the sr.

As the inward Na–Ca-exchange current is activated by internal calcium, it was of interest to examine the effect of an increase in intracellular calcium concentration on the inward current following a step pulse and on the accompanying atrial-cell action potential. Figure 6a illustrates the records of action potentials in a Ca$^{2+}$ free (solid circle) and in [Ca$^{2+}$] = 63 nM (pCa = 7.2) (open circle) internal solution. When the internal [Ca$^{2+}$] was changed to 63 nM, the late plateau phase of the action potential was greatly reduced. Under voltage-clamp conditions the

![Figure 6](http://rspb.royalsocietypublishing.org/)

**Figure 6.** Effect of intracellular [Ca$^{2+}$] = 63 nM (pCa = 7.2) on the action potential and the inward current. When the internal solution of the pipette was changed from Ca-free (●) to 63 nM (○), the late plateau of the action potential was abolished and initial repolarization was slowed (a). In (b), the effect of 63 nM [Ca$^{2+}$] on the inward current activation is shown. The magnitude of the inward current at 63 nM (○) was almost identical to that in a Ca$^{2+}$-free internal solution (●) but in [Ca$^{2+}$] = 63 nM its inactivation timecourse was accelerated. The current level was shifted in the outward direction earlier than under control conditions, possibly implying increased activation of calcium-dependent potassium currents. The voltage protocol for the activation of the inward current is shown in the inset.
peak inward current was neither increased nor decreased substantially (figure 6b, open circles). However, the inactivation timecourse of the inward current became faster and the total current became outward at more negative potentials ([Ca\(^{2+}\)] = 63 mM) than it did in Ca\(^{2+}\)-free internal solution. The interpretation of this experiment is unclear. We had expected that an increase in internal [Ca\(^{2+}\)] would increase both the plateau phase of the action potential and augment inward-current activation.

Suppression of the late plateau of the action potential and acceleration of the inactivation of the inward current might perhaps be a secondary consequence of either change in calcium release from the SR or the increase of the calcium-activated potassium current or both.

**Effect of sodium gradient**

Lowering the sodium concentration to 30% by substituting LiCl for NaCl caused a reduction in action potential duration with a decrease in the late plateau phase.

**Figure 7.** Effects of reducing extracellular sodium concentration on the action potential and the inward current. In low sodium solution (●) the late plateau of the action potential (a) was abolished and the inward currents (b) were diminished at various membrane potentials (○). 30% Na\(^+\) was replaced by equimolar lithium and voltage pulses are shown in the inset at the lower left-hand corner.
It also can be seen that inward current at various potentials was indeed suppressed by the reduction of extracellular sodium (figure 7b). The effect of reducing external sodium in diminishing the late plateau phase of the action potential and in abolishing the inward current may have been caused directly by the reduction in extracellular sodium or might perhaps be a secondary consequence due to a decrease in calcium current and modification of changes in the concentration of intracellular calcium that normally accompany the calcium current. However, the strontium current in rat cells is not reduced by low extracellular sodium when strontium is substituted for calcium in the external solution (Mitchell et al. 1983; Mitchell et al. 1984a) and injection of EGTA to buffer intracellular calcium reduces the effect of low extracellular sodium in causing a decrease in calcium current (Mitchell et al. 1987). Therefore it is unlikely that the reduction of the inward current is solely caused by a decrease of calcium current, but more likely that the inward current is carried, at least in part, by sodium in these cells. As lithium will substitute well for sodium in the conductance mechanism carrying the fast sodium current responsible for the upstroke of the action potential, but cannot be carried in place of Na⁺ by the Na–Ca-exchange mechanism, it seems likely that the inward current is carried by sodium via the Na–Ca exchange and that the late plateau of the action potential is maintained by this inward current.

![Figure 8](image-url)
However, the above results suggest that changes in intracellular sodium should also affect the inward current and the action-potential configuration. We therefore did experiments to test this possibility. When we increased the sodium in the internal solution from 0 mM Na to 20 mM the late plateau of the action potential was suppressed (figure 8a) and the inward current reduced (figure 8b, arrow). This was expected because an increase in intracellular sodium concentration decreases the driving force for Na-inflow and thus reduces the magnitude of the inward mode of the Na–Ca exchange. Nevertheless, activation of the Na-pump by internal sodium and the consequent generation of outward current could also be responsible for these effects. It is known that the outward current carried by the Na-pump is larger at more depolarized potentials than it is at the resting potential (Gadsby et al. 1985). In our experiments though, the reduction of the inward current and the late plateau of the action potential were more prominent in the hyperpolarized potential range. It is therefore likely that the observed decrease of inward current is due to the reduction in the Na-gradient.

Effect of calcium-channel blocker

Calcium-channel blockers such as Diltiazem are known to be selective in their effects in reducing currents carried by calcium (Lee & Tsien 1982). In the case of the inward current activated by intracellular calcium described above, it would be expected that abolition of the current carried by calcium would lead to an abolition of the associated rise in intracellular calcium and secondarily to a suppression of calcium-activated currents. However, the relation between activation of membrane calcium current and the development of inward current activated by intracellular calcium is not straightforward, and it is possible to block a substantial fraction of peak calcium current during the depolarizations while still activating a significant amount of the inward current (Egan et al. 1986). This assumption was tested and the results are illustrated in figure 9. The calcium current was almost blocked by 1 μM Diltiazem (figure 9a). In the absence of the calcium-channel blocker, the inward current was activated as before over a wide potential range and the same experimental protocol was then repeated in the presence of 1 μM Diltiazem (figure 9b). It can be seen that under these conditions an appreciable inward current was still recorded. Following a higher dose of 10 μM Diltiazem, the inward current completely disappeared, presumably as any residual calcium current for triggering the release of calcium from the sr was suppressed.

This observation could suggest that the inward current is activated by a rise in intracellular calcium and that provided sufficient calcium current remains unblocked by Diltiazem to trigger the release of calcium from the internal stores there will be a sufficient rise in intracellular calcium to activate the inward current, but it is not clear whether the result that we obtained is caused directly by the reduction of the inward current or indirectly by the reduction of the calcium current.
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Figure 9. Inhibition of the development of calcium current and of \( i_{in} \) by Diltiazem; 1 \( \mu \)M Diltiazem abolished most of the calcium current (a), but reduced the activation of \( i_{in} \) and 10 \( \mu \)M abolished the inward current completely (b). Voltage pulses for the current activation are shown in the bottom trace.

Current–voltage relation of the inward current

To investigate the current–voltage relations of the inward current activated by intracellular calcium, the amplitudes of the inward currents recorded at various membrane potentials should be measured. However, at moderately depolarized potentials it was almost impossible to measure the magnitude of the inward current, as activation of transient outward current shifts the current level outward (when the internal solution contained K\(^+\) and no blockers of K\(^+\)-current were present) or the inward current activation itself was too small to measure with sufficient accuracy (when the internal solution contained Cs\(^+\) or 4-AP was present in the external bathing solution). So we measured the difference between the magnitude of the control currents and the magnitude of the inward current recorded in ryanodine, Diltiazem, 20 Na\(^+\) or 30\% Na\(^+\) (see figures 5, 7, 8 and 9) and plotted the current–voltage relations with the current expressed as a fraction of the current magnitude activated at the holding potential (—70 to —80 mV). The
Figure 10. Fully activated current–voltage relation of the inward current. The difference currents measured before and after treatment of the cells with ryanodine (○), Diltiazem (●), 20 mM Na\(^+\) (□) or 30% Na\(^+\) (■) were plotted against the membrane potential. The magnitude of the difference current was normalized and expressed as a fraction of the difference current magnitude at the holding potential (usually \(-70\) mV). The relation turned out to be an exponential curve and showed steeper voltage dependence at more hyperpolarized membrane potentials.

I–V curve, which was obtained by measuring the current difference, is shown in figure 10. This current had an exponential voltage dependence. For quantification the current was fitted by the following equation (DiFrancesco & Noble 1985):

\[
i = A \exp\left(-\frac{(1-r)EF}{RT}\right),
\]

where \(A\) is a scaling factor that determines the magnitude of the current and \(r\) is a partition parameter used in rate theory to represent the position of an energy barrier in the electrical field and that indicates the steepness of the voltage dependence of the current. \(E, F, R\) and \(T\) indicate membrane potential, Faraday number, the universal gas constant and temperature. The value of \(r\) was 0.39, which as it is smaller than 0.5, suggests that the I–V curve is not symmetrical between outward and inward components, and has a steeper I–V curve in the inward current direction. This exponential I–V relation is of the form expected if this inward current is indeed an inward component of Na–Ca exchange current.

Discussion
This paper analyses the properties of an inward current that is activated in rabbit atrial-muscle cells by a rise in intracellular calcium and could support the late plateau phase of the action potential. The evidence suggests that the current
concerned could be the inward mode of a Na–Ca-exchange mechanism. This conclusion can be reached by considering the following results.

1. An inward current activated in rabbit atrial myocytes by a brief depolarizing clamp pulse was found to have a timecourse corresponding closely with the onset of the late plateau phase of the action potential.

2. The voltage dependence of this inward current is quite different from that of the membrane calcium current.

3. The inward current and the late plateau phase of the action potential are similarly inhibited by intracellular EGTA, Ryanodine, reduced extracellular sodium or increased intracellular sodium.

4. Both the calcium current and the inward tail current are blocked by Diltiazem in a dose-dependent manner.

5. The current–voltage relation for the inward current was exponential with a steep voltage dependence at hyperpolarized potentials.

These properties of the inward current are those expected of a current contributing to the late plateau of rabbit atrial cells (Hilgemann 1986; Hilgemann & Noble 1987). Similar inward-current tails were recorded in rat ventricular cells and are thought to be responsible for the late plateau phase so distinctive in the action potential of this species. It has been established that the inward current in rat ventricle is a Na–Ca-exchange current (Mitchell et al. 1984b; Schouten & Ter Keurs 1985; Mitchell et al. 1987; Hilgemann & Noble 1987).

**Timecourse of the inward current**

The inward current reaches a peak within about 10 ms and then decays to the baseline level with much faster rising and decaying timecourses than do the intracellular calcium transients from using Fura-2 signals in rat ventricular cells (Berlin et al. 1987; Cannell et al. 1987). This difference may result from the fact that reduction of the slow component of \(i_{in}\) by EGTA makes the onset faster than normal. But it may also be attributable to the kinetic properties of calcium binding to Fura-2, leading to the Fura-2 signal decaying much more slowly than free calcium and in turn slowing the timecourse of the calcium transient (Noble & Powell 1990).

The intracellular calcium transient from using Fura-2 rises as the action potential is initiated and then falls to the original low level during the plateau phase (Berlin et al. 1987; Cannell et al. 1987). This fall in intracellular calcium may be partly due to \(Ca^{2+}\) uptake by the sr and partly due to \(Ca^{2+}\) extrusion by Na–Ca exchange. If so, Na–Ca exchange may expel \(Ca^{2+}\) most vigorously during the plateau phase. The above results clearly support the view that in mammalian cardiac muscle, most of the calcium efflux does indeed occur during and not following the action potential. This conclusion is somewhat different to the situation in amphibian cardiac cells, where slow inward tail currents have been recorded, but in which the inward current was activated very slowly during the action potential and that the exchange extrusion of calcium occurs mainly after repolarization (Hume & Giles 1983; Hume & Uehara 1986; Campbell et al. 1988). This difference probably reflects the fact that intracellular sr release of calcium is...
much faster and is a considerably more important source of intracellular calcium in mammalian cells than in amphibian cells. The extrusion of calcium by the exchange is therefore activated more rapidly in mammalian cells.

The nature of the inward current

The sensitivity of the inward current to reduced extracellular sodium or increased intracellular sodium suggests that the inward current is carried at least partly by sodium ions. Two possible calcium-activated currents that could account for this are (i) an electrogenic Na–Ca current (Mullins 1979, 1981; Kimura et al. 1986; Mechmann & Pott 1986; Kimura et al. 1987; Fedida et al. 1987) and (ii) current flowing through non-selective cation channels (Colquhoun et al. 1981).

Current components with these characteristics have been separated under patch-clamp conditions in atrial-cell myoballs by Mechmann & Pott (1986), and it appears that the concentration of calcium needed to activate electrogenic Na–Ca exchange is lower than that required to activate non-selective cation channels (Colquhoun et al. 1981; Mechmann & Pott, 1986). In our experiments with reduced extracellular sodium, the fall in the inward current when lithium ions are used as a sodium-substitute might be taken as evidence in support of electrogenic Na–Ca exchange as lithium would be expected to pass through ionic sodium channels and non-selective cation channels, but cannot be carried by the Na–Ca exchange system.

Ca$^{2+}$ influx and efflux

The average atrial-cell volume in this experiment was about 6.4 ± 1.1 pl and the calcium concentration in the external perfusate was 1.8 mM. In these conditions the peak magnitude of the calcium current was in the range 350 ± 45 pA, whereas the maximal amplitude of the inward current at −40 mV was in the range 157 ± 13 pA. Because the stoichiometry of the Na–Ca-exchange process is assumed to be 3:1, the charge carried by the exchange process if it expels all the calcium entering via the calcium channels will be only 50% of the charge carried by the calcium channels. Also this proposed process of calcium efflux (the inward current) occurs over a period (30 ms), which is about twice as long as the period of net calcium entry (15 ms). As the net charge transfer of the exchange current is only half that of the calcium channel current an electrical balance would be achieved if the inward current had an amplitude approximately equal to 25% of the membrane $i_{Ca}$. However, the peak value of inward current flowing during the late plateau phase of the action potential (−40 mV) was generally found to be about 45% of the maximum calcium-current amplitude. This may therefore seem to be a relatively large current for a process that is postulated to play a role in transmembrane calcium balance. Clearly, in the steady state, calcium influx must be balanced by calcium efflux, but at the low-frequency stimulation used here, where the system is not in a steady state, calcium influx via the calcium channel need not necessarily match any calcium efflux via the inward mode of Na–Ca exchange. In this respect it is worth noting that at higher frequency stimulation the rabbit atrial action potential changes its shape substantially, the late, low plateau phase becoming much less marked as the stimulation time increases (see Saito (1971)).
Current-voltage relations of the inward current

The voltage dependence of the inward current was investigated by two methods; the first method involved increasing intracellular Ca\(^{2+}\) by applying a brief depolarizing pulse to +40 mV from the resting potential and then returning to various more negative potential levels to observe the subsequent development of the current at given repolarized values. The second method was to apply 5 ms depolarizations of different magnitudes before returning the membrane to the holding potential. By using this pulse protocol it can be demonstrated that activation of the inward current reflects the voltage dependence of calcium release from the sr.

The voltage dependence of the Na–Ca exchange has been observed by using radioactive-tracer measurements. External Na-dependent Ca\(^{2+}\) efflux was reduced by membrane depolarization and increased by hyperpolarization in squid axons (Blaustein et al. 1974; Mullins & Brinley 1975; Dipolo et al. 1985) and in bovine cardiac vesicles (Reeves & Hale 1984). Noble (1986) introduced a partition parameter, the \(r\) parameter of Eyring’s rate theory, to model the Na-Ca-exchange current allowing the current to be expressed as a function of \(\exp (rEF)\).

If \(r\) is 0.5, the exchange rate will be symmetrical between inward and outward directions. In our results obtained by using the first method (figure 10), the I–V curve of the inward current shows an exponential relation and the value of \(r\) is about 0.39 (\(1 - r = 0.61\)) for the inward exchange current (Ca\(^{2+}\) efflux), which means that the Na–Ca exchange is asymmetrical and that the inward component has a steeper voltage dependence. This result is therefore very similar to that obtained for the Na–Ca exchange current obtained by using dialysed patch-clamp analysis of single guinea-pig ventricular cells (Kimura et al. 1987).

The voltage dependence of the inward current obtained by the second pulse protocol shows that no inward current was measured by depolarizing to potentials below −50 mV but \(i_{\text{in}}\) was activated from a potential of −40 mV, whereas from potentials between −20 and −10 mV almost the full magnitude of inward current was activated. We also found that the I–V relation of the inward current has a very different voltage dependence from that of the calcium current. These two observations suggest that only a very small fraction of calcium current is required to activate the inward current. If we suppose that the inward current is activated by Ca\(^{2+}\) release from the sr, then calcium release may be mediated by a mechanism that requires some calcium influx but that may also be modulated by changes in voltage alone (Cannell et al. 1987).

Finally, these experiments raise questions about the calcium release mechanism from sr in cardiac cells. It seems likely that calcium release, once activated by a small amount of calcium current, may be partially or wholly regenerative and calcium may be a cofactor rather than the sole release mechanism. An equally necessary trigger for the release mechanism could be membrane depolarization, as has already been suggested for skeletal muscle (Rios & Brum 1987). Whether this voltage dependence is direct or mediated by voltage-dependent entry of calcium via reversed Na–Ca exchange will be discussed in the following paper (Earm & Noble 1990), which uses the Hilgemann–Noble (1987) equations to model some of the key results that we have obtained.
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Na–Ca exchange in rabbit atrium


