Mechanism of Increased Amplitude and Duration of the Plateau With Sudden Shortening of Diastolic Intervals in Rabbit Ventricular Cells

Masayasu Hiraoka and Seiko Kawano

Action potentials and membrane currents were recorded from isolated single ventricular cells from rabbit hearts using the suction pipette whole-cell clamp method. Action potentials elicited after short diastolic intervals of < 2 seconds showed an increase and prolongation of the plateau compared to those elicited after a 10-second rest period. The recovery of the tetrodotoxin-insensitive secondary inward current revealed a transient increase at short diastolic intervals above the level of full recovery (after 10 seconds). The increased secondary inward current recovery, however, was voltage-dependent, and the period of its increase did not cover the entire diastolic intervals of the action potential overshoots, suggesting the contribution of another ionic current to the changes in potential. During depolarizing voltage steps, from + to −20 mV, a rapid activating and then inactivating outward current was elicited, which overlapped the calcium current. This outward current exhibited time- and voltage-dependent properties similar to those of the transient outward current in Purkinje and other cardiac preparations. The recovery of the transient outward current was slow, achieving only 75% of its full level at 2 seconds, whereas the same level of calcium current recovery was achieved at 200 milliseconds. The application of 4-aminopyridine suppressed most of the transient outward current, and the rest of the current was abolished by caffeine or Co²⁺. The 4-aminopyridine sensitive transient outward current exhibited slow recovery kinetics compared to those of the other or calcium current, and its inhibition caused elimination of the augmented plateau during electrical restitution. The application of verapamil or Co²⁺ for inhibition of secondary inward current also abolished the action potential overshoot. These results indicate that an increase and prolongation of the plateau at short diastolic intervals are produced by the slower recovery from inactivation in the 4-aminopyridine-sensitive transient outward current than that in the calcium current. (Circulation Research 1987;60:14-26)

Previously, we reported that APD in premature excitations of canine ventricular muscles exhibited a transient prolongation at short diastolic intervals and that the prolonged APD was associated with an increased slow inward current during its recovery period. However, the mechanism of increased slow inward current was not clarified from that study. On the other hand, Kukushkin and coworkers suggested a possible contribution of the transient outward current to the rate-dependent change in APD, but they did not study its precise role in the determination of APD in premature excitations.

Recently, methods for isolating a single cardiac cell and for recording membrane potentials and currents using this preparation have been introduced. This technique facilitates accurate measurement of action potentials and ionic currents without interactions from adjoining cells. Furthermore, single-cell studies have disclosed that the calcium current has a much larger amplitude and faster kinetics than predicted from the slow inward current measured using multicell preparations. Single-cell studies have also revealed the presence and the role of the transient outward current in different cardiac preparations other than Purkinje fibers. Therefore, the present study was done to elucidate the mechanism of APD prolongation upon sudden shortening of diastolic intervals using this refined technique applied to single ventricular cells from rabbit hearts, since this tissue has been shown to devel-
op similar changes in APD to those we observed in dog ventricles.16,17

Materials and Methods
Single ventricular cells from rabbit hearts were obtained by an enzymatic dissociation procedure. Rabbits weighing 2–3 kg were anesthetized with pentobarbital sodium (40–50 mg/kg) while the blood was heparinized (300 IU/kg, sodium salt). Using a Langendorf apparatus, the excised hearts were perfused with 0.04% collagenase (Sigma, type 1) dissolved in low-Ca²⁺ Tyrode solution containing 50 μM Ca²⁺. The heart was then stored in Krebs-bicarbonate (KB) solution at 4°C for 60 minutes. Small pieces of the ventricular wall were gently shaken in a beaker containing the KB solution to dissociate the tissue into single cells, and these were kept at room temperature before use. Isolated cells were then introduced into the recording chamber, where prewarmed Tyrode solution was superfused at a rate of 2–3 ml/min. Temperature of superfusates in the recording chamber was kept at 33–35°C. The recording chamber was set on the stage of an inverted phase-contrast microscope (Diaphot, Nikon Co., Tokyo). Isolated cells were either round or rod shaped, and usually 20–40% of cells were rod shaped in Tyrode solution containing 1.8 mM Ca²⁺.

Single rod-shaped cells having smooth surfaces with clear striations were selected for the electrical measurements. In some experiments, single cells from guinea pig ventricles were isolated according to the method described, and they were used for the electrophysiological experiments as indicated in the text.

The composition of the Tyrode solution was (in mM): NaCl, 144.0; NaH₂PO₄, 0.33; KCl, 4.0; CaCl₂, 1.8; MgCl₂, 0.53; glucose, 5.5; and 5-HEPES-KOH buffer (pH 7.4). The KB solution contained glutamic acid, 70.0; taurine, 15.0; KCl, 30.0; KH₂PO₄, 10.0; MgCl₂, 0.5; glucose, 11.0; EGTA, 0.5; and HEPES-KOH buffer (pH 7.4). The pipette solution consisted of KCl, 150.0; ATP, 5.0 (as dipotassium salt, Sigma Chemical Co., St. Louis, Mo.); and 5-HEPES-NaOH buffer (pH 7.2). Tetrodotoxin (TTX; Sankyo Co.), 4-aminopyridine (4-AP; Sigma), caffeine HCl (Wako Pure Chemical Co.), and verapamil HCl (Eisai Co.) were prepared on the day of each experiment from stock solutions to the final concentration in the Tyrode solution as indicated in the text. The Co²⁺ solution was made by adding 2 mM CoCl₂, to the Tyrode solution. All the solutions were aerated with 100% O₂.

The single-pipette, whole-cell clamp method was applied to single cells to record membrane potential and currents using an EPC-7 amplifier (List Medical Electronic, Darmstadt, West Germany). Glass pipettes were drawn out to a tip diameter of 2–4 μm. The electrode resistance was 1–3 MΩ after heat polishing of the electrode tip and filling with the pipette solution. After the pipette tip was pressed against the cell surface, a gentle negative pressure of 30–60 cm H₂O was applied to the interior of the electrode. When a seal resistance of 5–100 GΩ was established, a resting potential of −80 to −90 mV was usually recorded. In the whole-cell clamp mode, the electrode resistance in series with the cell membrane was compensated to minimize the duration of the capacitative surge on the current trace. The action potential was recorded from a single cell in the current-clamp mode and by passing depolarizing current of suprathreshold intensity (less than 5 milliseconds in duration) through the pipette. The membrane current was measured in the voltage-clamp mode and by applying various protocols of pulses as indicated in the text.

Before starting the electrical measurements, the configurations of either action potentials or membrane currents during depolarizing pulses increased to 0 mV from the holding potential of −40 mV for 5–10 minutes with the pulse application at 0.1 Hz. If the action potential duration at 0 mV or the amplitude of the peak inward current, mostly representing the calcium current, did not differ by more than 5% of their values in successive measurements, the preparations were judged stable and further experiments were allowed to proceed. This was based on our observations that the repolarization phase of action potentials and the calcium current seemed to be the most sensitive indicators of cellular conditions, since changes in these parameters always preceded cellular damage and death. Once the stable condition was achieved, it usually lasted for 30–40 minutes in our preparations and sometimes > 60 minutes in the presence of Co²⁺. Therefore, our measurements were mainly completed within 30 minutes. The exception to this rule was when Co²⁺ was applied, at which time the experiments occasionally extended to 60 minutes. After the end of each experiment the junction potential was measured. All the values of junction potential in these experiments were within 2 mV, and therefore no correction was made for it in the data analysis.

The parameters measured and their definitions in the present experiments were AP-1, the first action potential elicited after a 10-second rest period; AP-2, the action potential after AP-1 with paired stimulation; APD₉₀, action potential duration at the 0 mV level; APD₉₀₀, action potential duration at 90% repolarization; plateau height, the maximal level of the plateau (Phase 2) after the initial peak (Phase 0) of the action potential; diastolic interval (DI), the interval between the end of full repolarization of AP-1 and the beginning of AP-2 depolarization; and Iₘ, a TTX-insensitive secondary inward current, mostly representing the calcium current (IₐCa) but contaminated by the outward current, depending on the pulse protocols as discussed in the text. Iₘ was measured under conditions in which the fast Na⁺ current (IₗNa) was inactivated by applying TTX and/or by depolarizing the holding potential at −40 mV, or less negative. In the present experiments we used 6.26 or 12.5 μM TTX to inactivate IₗNa. To exclude contamination of Iₘ, the holding potential was set at −60 mV, since our preliminary experiments (3 cells) had shown that 6.26 μM TTX abolished Iₘ on depolarization from −60 mV but failed to suppress it from a −70 mV or more negative holding potential. Sometimes, even 20 μM TTX was not enough to block
$I_{\text{Na}}$ at a holding potential of $-80 \text{ mV}$ or more negative. Secondly, the amplitude of $I_\text{Na}$ increased with increasing membrane depolarizations during the pulses and attained a maximum value between 0 and $+10 \text{ mV}$. The $I_\text{Na}$ with this kind of voltage dependency was reversibly abolished in the Ca$^{2+}$-free solution (4 cells), suggesting that the current represented $I_{\text{Na}}$. Therefore, the cells exhibiting $I_\text{Na}$ of the characteristics described above were chosen for the current measurement.) Additional definitions are peak $I_\text{Na}$, the value of peak $I_\text{Na}$ relative to a 0 current level ($I_{\text{Na,0}}$) was also measured as the difference between the peak inward current and the holding current, and the results of this analysis were similar to those of the peak $I_\text{Na}$ analysis; and $I_{\text{Na,0}}$, transient outward current. ($I_{\text{Na,0}}$ amplitude was measured by the difference between the maximal point of $I_{\text{Na}}$ and the minimal point of the current trace during the depolarizing voltage steps. To avoid contamination of the inward currents in the $I_{\text{Na}}$ measurements, Co$^{2+}$ was applied to block $I_{\text{Na}}$ and TTX to inhibit $I_{\text{Na}}$. Furthermore, the test voltages of $+$60 or $+70 \text{ mV}$, which were close to their expected reversal potentials and would have small amplitudes, were selected except in cases when the voltage dependence of $I_{\text{Na}}$ was examined or as otherwise stated in the text.)

All the values were expressed as a mean ± standard error (SE). Statistical analysis was done using the paired or nonpaired t test, and $p < 0.05$ was considered significant.

**Results**

**Changes in the Action Potential Plateau and $I_\text{Na}$ on Sudden Shortening of Diastolic Intervals in Rabbit Ventricular Cells**

When ventricular cells were stimulated by regular stimuli applied after a rest period of 10 seconds, the evoked action potential showed a small but distinct Phase 1 after its initial peak, followed by a smooth repolarizing Phase 2. Occasionally, a secondary depolarization after an initial peak with a notch between them was noted, depending on the preparations. When the second stimulus was given with various coupling intervals after the first action potential (AP-1), the second action potential (AP-2) had a decreased or absent Phase 1 and developed a prominent plateau attaining a level higher than that of AP-1. The APD was longer in AP-2 than in AP-1, and this was especially pronounced at APD$_2$. These characteristic changes were noted in all AP-2 elicited at coupling intervals <2 seconds. One series of experimental records demonstrating these changes in AP-1 and AP-2 is shown in Figure 1.

In our previous studies,$^{4,5}$ we observed that the APD prolongation was associated with an increased slow inward current when measured by a single sucrose-gap voltage-clamp method during premature excitations in dog ventricular muscle. Therefore, changes in $I_\text{Na}$ were measured using single ventricular cells with a stimulation protocol similar to that shown in Figure 1. In the voltage-clamp mode, two successive pulses of 200 milliseconds duration to 0 mV from the holding potential of $-40 \text{ mV}$ were applied after a rest period of 10 seconds with various diastolic intervals between the two. This method gave a measure of the recovery of $I_\text{Na}$. The experimental records of this measurement from a single preparation are shown in Figure 2. Although $I_\text{Na}$ at a diastolic interval of 50 milliseconds was smaller after a 10-second rest period, those at 250 and 500 milliseconds were larger than the first. At a diastolic interval of 2,000 milliseconds, the amplitudes of $I_\text{Na}$ at the first and second pulses were almost the same.

In the recording of the current-clamp mode, this preparation also showed action potential changes similar to those shown in Figure 1. Analyses of both parameters in action potentials and in $I_\text{Na}$ vs. diastolic intervals examined with a single preparation are shown in Figure 3. Action potentials elicited at short diastolic intervals (<2 seconds) showed longer APD$_2$ and attained a larger amplitude of the plateau height than those at 10 seconds. $I_{\text{Na}}$ was also shown to increase in the second depolarizing pulses elicited at short diastolic intervals compared to those at 10-second intervals. In 7 preparations, both action potential parameters and $I_{\text{Na}}$ were examined using an experimental protocol similar to those shown in Figures 1–3. All the results revealed a prolongation of APD$_2$ and an increase in the plateau height and $I_{\text{Na}}$ at short diastolic intervals of <2 seconds compared with those at 10-second intervals, although the diastolic intervals demonstrating the maximum values of these parameters varied from cell to cell. In action potential measurements of 7 cells, the plateau height was $32.6 ± 2.5 \text{ mV}$ (mean ± SE; $n = 7$) in AP-1 and the maximum plateau height of AP-2 attained during the electrical restitution was a mean of $44.3 ± 3.1 \text{ mV}$ in AP-2. The two values were signifi-
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![Diagram](image)

**Figure 2.** Experimental records demonstrating increased amplitudes of \( I_s \) during the recovery period. The experimental protocol is shown at the top. Two depolarizing pulses of 200-milliseconds duration to 0 mV were applied from the holding potential of \(-40 \text{ mV} \) after a 10-second rest period with various intervals (<2 seconds) compared to those at 10-second intervals. The maximal \( I_s \) attained during its recovery period appeared to be a voltage-dependent process.

In our previous studies, the APD prolongation in premature excitations was abolished in the presence of verapamil and Mn\(^{2+}\), which blocked the slow inward current. Therefore, the effects of verapamil and Co\(^{2+}\) on rabbit ventricular action potentials and \( I_s \) were studied to examine whether or not \( I_s \) could contribute to the increased plateau and its prolongation of AP-2. Figure 4 shows one such experiment. In the control, APD\(_0\) prolongation in AP-2 compared with AP-1 was observed for excitations elicited at diastolic intervals of <2 seconds. A transient increase in \( I_s \) during its recovery period was also noted at diastolic intervals of 300-1,000 milliseconds. The application of verapamil eliminated these APD\(_0\) prolongations at diastolic intervals of <1 second and produced complete inhibition of \( I_s \), washout of the drug caused incomplete recovery of \( I_s \), with reappearance of a transient overshoot during its repolarization as well as the APD\(_0\) prolongation of the short diastolic excitations, although APD\(_0\) of AP-1 continued to be shortened. Therefore, the inhibition of \( I_s \) resulted in a disappearance of the APD\(_0\) prolongation of AP-2 at short diastolic intervals. Similar results as shown in Figure 4 were confirmed in 3 other cells after verapamil application and in 2 cells after 2 mM Co\(^{2+}\).

The results shown in Figures 3 and 4 may indicate another point. While action potential parameters such as APD, and plateau height showed increased values at diastolic intervals between 50 and 2,000 milliseconds compared with those at 10-second intervals, \( I_s \) increased at diastolic intervals only between 300 and 1,000 milliseconds. This indicates the possibility that a factor other than \( I_s \) is involved in the prolongation of APD\(_0\) and that increased plateau height of AP-2 is elicited at short diastolic intervals. This point was further examined.

**Transient Outward Current and Its Contribution to Rabbit Ventricular Action Potential**

Figure 5 represents the changes in membrane current in response to depolarizing voltage steps from the holding potential of \(-60 \text{ mV} \) in a single ventricular
The current amplitude increased with increased membrane depolarizations during pulses and reached its peak above +70 mV (Figure 6B). The current resembled the $I_{\text{To}}$ originally described in sheep Purkinje fibers. Six preparations treated with TTX and Co$^{2+}$ were demonstrated to have an activation threshold of $I_{\text{To}}$ at $-30$ mV (2 cells) and $-20$ mV (4 cells). Therefore, $I_{\text{To}}$ was activated at membrane potentials above $-20$ mV to form a net membrane current due to an overlap with $I_{\text{Ca}}$.

Since $I_{\text{To}}$ of Purkinje fibers has been shown to exhibit voltage-dependent inactivation, the development of $I_{\text{To}}$ in rabbit ventricular cells was examined at different holding potentials. $I_{\text{To}}$ was inactivated at depolarized holding potentials, and its inactivation was removed with hyperpolarization (Figure 6C). This property was also confirmed in 4 cells.

If $I_{\text{To}}$ is activated on application of depolarizing voltage steps + to $-20$ mV and has recovery kinetics similar to those demonstrated in Purkinje fibers, it could contribute to the formation of the early phase of repolarization in rabbit ventricular action potential and its rate-dependent changes (Figures 1 and 3). Therefore, the recovery of $I_{\text{To}}$ was examined by the double-pulse method. Figure 7A shows one of the experimen-
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Natural text:

tal records taken from a cell treated with 6.26 μM TTX and 2 mM Co²⁺. Two depolarizing pulses to +60 mV from the holding potential of −60 mV were applied in succession with various diastolic intervals after a rest period of 10 seconds. Iₒ elicited by the second pulses were small at short diastolic intervals. With prolongation of the diastole, the amplitude of Iₒ became larger, but even at an interval of 2,000 milliseconds it was not as large as after a 10-second rest, indicating a slow recovery process. Similar results were obtained from 5 preparations treated with 6.26 μM TTX and 2 mM Co²⁺. Their degree of Iₒ recovery at 2-second diastolic intervals achieved 75 ± 2% (n = 5) of the level of its full recovery (10-second diastolic interval).

Effects of 4-Aminopyridine and Caffeine on Action Potentials and Iₒ of Rabbit Ventricular Cells

The results indicated that Iₒ and its slow recovery kinetics might have been responsible for the increased plateau height and prolonged APDₒ in action potentials elicited at short (2-second) diastolic intervals. To ascertain the contribution of Iₒ to the above changes in

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Figure 4. Effects of verapamil on the prolonged plateau and the Iₒ recovery during electrical restitution. A. Effect of verapamil on AP-1 and AP-2. During the control (a), AP-2 elicited after the diastolic interval (DI) of 105 milliseconds displayed prolonged APDₒ and increased plateau height compared to those of AP-1. The application of 6.8 μM verapamil abolished the augmented plateau duration and amplitude of AP-2 (b). The washout of verapamil (c) caused a reappearance of the augmented plateau of AP-2 compared to those of AP-1, while APDₒ of AP-1 continued to be shortened. DI = Diastolic interval in milliseconds. B. Plots of the restitution of APDₒ and the effect of verapamil on it. Verapamil (△-△) produced shortening of APDₒ at 10-second intervals and abolished its overshoot at short diastolic intervals. The washout of the drug (△-△) brought about a reappearance of the overshoot, although APDₒ at 10-second intervals was further shortened compared to those in the verapamil solution. C. Plots of the Iₒ recovery in the control (○-○), with verapamil (●-●) and with washout of verapamil (○-○). The experimental protocol is shown in the inset. Two pulses of 200-millisecond duration were applied at various diastolic intervals. Iₒ showed an augmented recovery at diastolic intervals between 300 and 1,000 milliseconds. Verapamil abolished Iₒ completely, and the washout of the drug produced a reappearance of the current as well as an increased amplitude at short diastolic intervals.
Figure 5. Net membrane current during depolarizing voltage steps in normal Tyrode solution. In each panel, the top trace shows the current and the bottom trace, the voltage. Numbers at the top of each panel indicate the level of membrane depolarization during the pulses in mV. While depolarizing pulses + to 40 mV clearly produced rapidly activating and then deactivating outward current, those at +20 and +30 mV elicited an outward hump during the falling phase of the inward current (arrows).
A. Development of $I_w$ in the presence of TTX and Co$^{2+}$. A. Experimental records at different membrane depolarizations during the pulses. The top 4 (left) and 3 (right) traces indicate the current, and the bottom traces show the voltage. B. Current-voltage relation of $I_w$. $I_w$ had an apparent activation threshold at $-20$ mV. With increasing depolarizing pulses, the amplitude of $I_w$ increased to attain a plateau value above $+70$ mV. The results of (A) and (B) were obtained from the same experiment. C. Dependence of the $I_w$ development on the level of the holding potential. Two-hundred-millisecond depolarizing pulses to $+60$ mV were applied from different holding potential levels as indicated at the bottom of each pulse. The high negative holding potential induced the larger $I_w$. Data were obtained from a different preparation from those shown in (A) and (B). Although the cell was treated with 6.28 μM TTX and 2 mM Co$^{2+}$, $I_w$ was not completely blocked when the holding potential was held at $-70$ mV or a more negative level.

**Figure 6.** Development of $I_w$ in the presence of TTX and Co$^{2+}$. A. Experimental records at different membrane depolarizations during the pulses. The top 4 (left) and 3 (right) traces indicate the current, and the bottom traces show the voltage. B. Current-voltage relation of $I_w$. $I_w$ had an apparent activation threshold at $-20$ mV. With increasing depolarizing pulses, the amplitude of $I_w$ increased to attain a plateau value above $+70$ mV. The results of (A) and (B) were obtained from the same experiment. C. Dependence of the $I_w$ development on the level of the holding potential. Two-hundred-millisecond depolarizing pulses to $+60$ mV were applied from different holding potential levels as indicated at the bottom of each pulse. The high negative holding potential induced the larger $I_w$. Data were obtained from a different preparation from those shown in (A) and (B). Although the cell was treated with 6.28 μM TTX and 2 mM Co$^{2+}$, $I_w$ was not completely blocked when the holding potential was held at $-70$ mV or a more negative level.

of 2 mM 4-AP. In another six preparations, the concurrent application of 2 mM 4-AP and 2 mM Co$^{2+}$ also abolished $I_w$ completely (data not shown).

To test whether or not a slow recovery of $I_s$ actually causes an oscillatory repriming of $I_s$ examined by the procedure described in Figures 2-4, whereby recovery kinetics after pharmacological dissection of each current component were examined. Figure 10 shows one such analysis obtained from a single preparation, where (A) represents an analysis of APD$\alpha$ in normal Tyrode solution. APD$\alpha$ of AP-2 at short diastolic intervals were longer than those of AP-1 elicited after a 10-second rest period. The cell was then treated with 12.5 μM TTX to block $I_{Na}$. The recovery kinetics of $I_o$, $I_{Ca}^{\alpha}$, and $I_w$ were examined with dissection of each current component in different external solutions. In the voltage-clamp mode, double pulses of 200-millisecond duration from the holding potential of $-50$ mV to $0$ mV were applied at various diastolic intervals (Figure 10B-10D). In (B), the recovery of $I_o$ was examined. $I_o$ at diastolic intervals of 200–2,000 milliseconds were larger than those at 10 seconds. Therefore, the recovery of $I_o$ showed a transient increase by as much as 125% of the level of full recovery (after a 10-second interval). When the cell was treated with 2 mM 4-AP and 5 mM caffeine in the presence of TTX to block $I_{Na}$ and $I_{Ca}^{\alpha}$ (C), a pure $I_{Ca}^{\beta}$ and its recovery could be determined. $I_{Ca}^{\beta}$ showed a smooth monotonic recovery without a transient augmentation, achieving more than 80% of its full recovery level at a diastolic interval of 200 milliseconds. It was also noted that the peak $I_{Ca}^{\beta}$ was twice as large as $I_o$. 4-AP and caffeine were then washed out, and 2 mM Co$^{2+}$ was added in the presence of TTX to examine $I_{Ca}^{\beta}$ (D). $I_{Ca}^{\beta}$ achieved rather a slow recovery, attaining only 65% of its maximum value even at a diastolic interval of 2,000 milliseconds. Therefore, the recovery kinetics of $I_{Ca}^{\beta}$ were 10 times longer than those of $I_{Ca}^{\alpha}$. Similar results were confirmed in the other two preparations.

**Action Potential Restitution and Recovery of $I_s$ in Guinea Pig Ventricular Cells**

The contribution of $I_s$ to the increase and prolongation of the plateau was further supported by another line of evidence. In guinea pig ventricular muscles, the action potential plateau has been shown to exhibit a smooth recovery with almost no augmentation during electrical restitution, and there have been no reports describing the clear existence of $I_s$. Therefore, changes in the action potential configurations and the recovery of $I_s$ were examined using the same protocol as that shown in Figures 1-3. In 4 ventricular cells from guinea pig hearts, action potentials revealed neither a Phase 1 nor a prominent plateau like AP-2 in rabbit cells, and no APD$\alpha$ prolongation was observed during electrical restitution. The recovery of $I_s$, which was examined by applying test depolarizations to 0 and $+40$ mV from the holding potential of either $-40$ or $-60$ mV, showed a smooth recovery but no transient increase in any examined cells showed (data not shown).
Discussion

The present study demonstrates that an increased plateau height and its prolongation appeared on sudden shortening of the preceding diastolic intervals from a slow basic heart rate in action potentials of single rabbit ventricular cells. This finding confirms previous observations made using multicell preparations of several mammalian species, including rabbit ventricles.1,2,16,17 The result further implies that the augmented plateau was not brought about by the electrotonic influence of neighboring cells (such as in the region of the Purkinje-myocardial junction23) but was caused by changes in the ionic currents forming the plateau. Our results indicate that this phenomenon is produced by inactivation of I_{Ks} at short diastolic intervals, supporting the view proposed by Kukushkin and coworkers6 and further implying that the presence and faster recovery of I_{Ks} than I_{K}, is responsible for these potential changes.

After an action potential, the membrane goes through a period of recovery, or electrical restitution.24 Two types of electrical restitution occur when premature action potentials are elicited after control responses of slow pulsing rates. In a variety of preparations, a premature action potential is characterized by a reduced amplitude of the plateau, its duration, and total APD. In contrast, other groups of tissues show

![Figure 7. Recovery from inactivation of I_{Ks}. A. Current responses induced by 2 depolarizing pulses (200-millisecond duration) to +60 mV from the holding potential of −60 mV. Double pulses were applied after a 10-second rest period. Diastolic intervals between two pulses are indicated at the top of each record in milliseconds from (a) to (c) and in seconds at (d) and (e). B. Plots of the I_{Ks} recovery, in its absolute value ( ● — ● ) or in its relative value (I_{Ks}/I_{K}) ( ○ — ● ). Data in A and B were obtained from a single preparation that was treated with 6.28 μM TTX and 2 mM Ca^{2+}.](image)

![Figure 8. Effects of 4-AP on the action potential restitution. A. Effects of 2 mM 4-AP on AP-1 and AP-2 elicited at a diastolic interval of about 100 milliseconds. Note that the prolongation of the plateau in AP-1 and AP-2 after the application of 4-AP and the longer APD of AP-1 than that of AP-2 (b), while AP-2 was longer than AP-1 in the control (a). B. and C. represent the restitution of APD and plateau height as examined using the same experimental protocols as those in Figures 1 and 3. Note that the augmented plateau at short diastolic intervals seen in the control was abolished in the 4-AP solution.](image)
overshoot of these parameters above the control values during the recovery period. We previously observed the overshoot of the plateau in premature excitations of dog ventricular muscles, which was associated with an increased slow inward current during the recovery period. While the precise mechanism of the transient increase in the slow inward current during the recovery period was not clarified from that study, similar oscillatory repriming of this current has been reported by Weingart and coworkers for calf Purkinje fibers after exposure to digitalis. In the present experiments, a similar increase in the TTX-insensitive I was noted in rabbit ventricular cells together with an increase and a prolongation of the plateau. However, the increase in I was dependent on the level of the voltages used for the depolarizing pulses or the level of the holding potential. Furthermore, the period of the increased I did not cover the entire period of the augmented plateau. This implies that the measurement of I may be hindered by contamination of another current, and the contribution of the latter factor to changes in action potential is indicated.

The exact measurement of individual ionic current in the heart is subject to serious error, since more than two currents with similar time- and voltage-dependent properties flow either in the same or in opposite directions. This problem is also applicable to the present analysis using single ventricular myocytes. A TTX-insensitive I is generally believed to mostly represent I,, and I in our preparations actually disappeared in the Ca²⁺-free solution. However, it was also shown that I,, having a similar time course to I,, was activated by depolarizing voltage steps from + to -20 mV. Therefore, I measured in normal Tyrode solution represented the net inward current and, thus, its changes in amplitude time course were brought about by an alteration not only of I but also of I,. Actually, the oscillatory repriming of I observed in normal Tyrode solution was increased in amplitude and was changed to a smooth monotonic recovery after I was blocked by 4-AP and caffeine (Figure 10). The problem of the purity of I was also applicable to the analysis of this current. Even if I and I were blocked by the application of TTX and Co⁺, the currents might contain the delayed outward K⁺ current (I) and the peak current. The amplitude of the latter in our preparations, however, was small, accounting for less than 10% of I (see Figures 5 and 6), and the activation of I was much slower than those of I (<20 milliseconds) (see Figure 5). The presence of Co⁺ may affect the amplitude of I, since the I component was already blocked in this solution (see below).

I was activated on depolarizing voltage steps + to -20 mV in rabbit ventricular cells, confirming the report by Kukushkin and coworkers using multicellular preparations. This current was always observed on strong depolarizing voltage steps from the holding potential of -60 mV in all examined preparations (more than 40 cells). Because it seemed unlikely that we had picked up a single Purkinje cell during the dissociation procedure and used it for these electrical measurements, it was thought that they were actually muscle cells. This point was further supported by the findings that these cells showed an action potential configuration typical of rabbit ventricular muscles (see Figures 1, 4, and 7). The activation voltage and time course of I were similar to those of I, and, thus, two currents overlapped each other at the plateau of voltages (see

![Figure 9. Effects of 4-AP and caffeine on the recovery from inactivation of I. A. Current responses elicited by two depolarizing pulses from -60 mV to +60 mV applied with a diastolic interval of 200 milliseconds, after a 10-second rest period. In the control (a), a larger I was induced by the first pulse, but the second I was small because of a slow recovery. In the presence of 2 mM 4-AP (b), the first I was reduced in amplitude, but the second one did not change greatly compared with those in the control. The application of 2 mM 4-AP and 5 mM caffeine concurrently (c) abolished I of both pulses. B. Recovery from inactivation of I and the effects of 4-AP and caffeine. 4-AP depressed the component having slow recovery kinetics, and caffeine abolished the fast component.](http://circres.ahajournals.org/content.figures/64/5/23.full/fig9.large.jpg)
FIGURE 10. Recovery of APD₀, \( I_{Ca} \), and \( I_{Na} \). Results were obtained from a single preparation. A. Recovery of APD₀ examined in normal Tyrode solution. Prolonged APD₀ was noted at short diastolic intervals compared to the action potential at a 10-second diastolic interval. B. Recovery of \( I_{Ca} \) examined in the solution containing 12.5 \( \mu \)M TTX. The inset shows one of the actual current responses elicited by double pulses separated by a 300-millisecond diastolic interval. A larger \( I_{Ca} \) was seen at diastolic intervals of 200 milliseconds-2 seconds. By the application of 2 mM Co²⁺, 5 mM caffeine, and 12.5 \( \mu \)M TTX, the \( I_{Ca} \) recovery shows a smooth monotonic profile without any augmentation. The inset illustrates one of the actual current responses. Note that the peak amplitude of \( I_{Ca} \) was larger than that of \( I_{Na} \) shown in B. D. Recovery of \( I_{Na} \) examined after blocking \( I_{Ca} \) and \( I_{Na} \) by the application of 2 mM Co²⁺ and 12.5 \( \mu \)M TTX. The recovery of \( I_{Na} \) was slow, achieving only 65% of the full recovery at 2 seconds. For further details, see text.

Figures 5 and 6). The presence of \( I_{Na} \) was first described in detail by Dudel and coworkers in sheep Purkinje fibers. Subsequently, Fozzard and Hiraoka examined its kinetics and voltage- and time-dependent inactivation properties. Because of its kinetics and inactivation properties, \( I_{Ca} \) contributes to the Phase 1 of Purkinje action potentials and their rate-dependent changes. Kukushkin and coworkers also attributed \( I_{Ca} \) to rate-dependent changes in rabbit ventricular action potentials slower than 1.0 Hz, which display a less prominent Phase 1 than those of Purkinje fibers. They also indicated that \( I_{Ca} \) might contribute to an overshoot of the plateau during electrical restitution, but they did not give any direct evidence to prove this point. In accordance with \( I_{Ca} \) in Purkinje and other cardiac preparations, the current exhibits voltage-dependent inactivation, and its recovery from inactivation is slow, achieving only 75% of the full recovery level at diastolic intervals of 2 seconds.

As to the ion transfer mechanism of \( I_{Na} \), several earlier studies have indicated that the current is mainly carried by \( K^+ \) rather than \( Cl^- \). Various findings appear to indicate that the \( K^+ \) currents contained in \( I_{Na} \) have two components, one of which depends on increased intracellular \( Ca^{2+} \) concentration ([\( Ca^{2+} \)]), either through \( Ca^{2+} \) influx via the sarcolemma or through the release of stored \( Ca^{2+} \). The other is independent of [\( Ca^{2+} \)], and is blocked by 4-AP having voltage-dependent properties. The present experiments also demonstrated that \( I_{Na} \) in rabbit ventricular cells has two components, one of which is 4-AP-sensitive with voltage-dependent properties and the other \( Ca^{2+} \)-activated, since \( I_{Na} \) in normal Tyrode solution decreased to about 28% of its original level after the application of 2 mM 4-AP alone. The rest of \( I_{Na} \) was completely abolished by the combined presence of 4-AP and caffeine or 4-AP and \( Ca^{2+} \). On other occasions, the application of \( Ca^{2+} \) blocked \( I_{Na} \) and revealed the voltage-dependent activation of \( I_{Ca} \) (Figure 6). This fraction of \( I_{Na} \) was completely blocked by 4-AP. There is ample evidence that \( I_{Ca} \) in other preparations is suppressed by 4-AP, and \( Ca^{2+} \) influx via the sarcolemma or through \( Ca^{2+} \) release from the sarcoplasmic reticulum, since \( Ca^{2+} \) is thought to block the former and caffeine to
inhibit the latter.\textsuperscript{32} The value of 28\% for the fraction of the Ca\textsuperscript{2+}-activated I\textsubscript{Na} relative to the total I\textsubscript{Na} might not represent its exact amplitude since we had to measure this fraction in the presence of I\textsubscript{Ca}. Also, the modulation of I\textsubscript{Na} changed the Ca\textsuperscript{2+}-activated I\textsubscript{Na} as seen in the cases of Ca\textsuperscript{2+} application. Caffeine might also affect I\textsubscript{Na} in addition to its action on the Ca\textsuperscript{2+} release process.\textsuperscript{22} It was further shown that the 4-AP-sensitive I\textsubscript{Na} had slower recovery kinetics than those of Ca\textsuperscript{2+}- sensitive I\textsubscript{Na} and therefore contributed to the shortening and the decrease in the plateau of the excitations elicited with slow heart rates or after long diastolic intervals. Because of the slow recovery of the 4-AP-sensitive I\textsubscript{Na}, the action potentials elicited at short diastolic intervals would attain a higher level and a longer duration of the plateau than those after a 10-second rest period. Actually, the application of 4-AP abolished the overshoot of the plateau during electrical restitution despite the fact that the Ca\textsuperscript{2+}-sensitive component was unaffected (Figures 8 and 9). On the other hand, the Ca\textsuperscript{2+}-sensitive I\textsubscript{Na} has a recovery time course as rapid as those of I\textsubscript{Na}, and it contributes to the formation of the notch after an initial peak action potential. Therefore, the inactivation of I\textsubscript{Na} at short diastolic intervals unmasks the rapidly recovering I\textsubscript{Na}, which produces an increase in the net inward current and an overshoot of the plateau. This idea was also supported by the findings that the block of I\textsubscript{Na} by the application of verapamil or Ca\textsuperscript{2+} abolished the overshoot of the plateau during electrical restitution and that the depolarizing voltage steps activating both currents increased the amplitude of the inward current in the second pulse elicited at short diastolic intervals compared to the first one after the long rest period (Figure 10B). These findings support part of our previous observations\textsuperscript{43} and the changes in action potential observed during the application of verapamil and different extracellular Ca\textsuperscript{2+} concentrations.\textsuperscript{40}

The presence of I\textsubscript{Na} and its kinetics contribute to the modulation of APD as shown in the present experiments. APD at a slow heart rate is shortened, whereas it is prolonged at a fast rate. Furthermore, the current acts to maintain or prolong APD at short coupling intervals of premature excitations in muscles, while APD of Purkinje fibers become shortened.\textsuperscript{1,2} Therefore, the disparity of refractoriness in premature excitations is increased in various parts of the ventricle, which further facilitates the genesis of arrhythmias. Since I\textsubscript{Na} overlaps with I\textsubscript{Ca}, the former may influence the slow response activity dependent on the latter. The upstroke of the slow response action potential may be augmented at short coupling intervals and depressed at long intervals due to the slow recovery of I\textsubscript{Na}. This would explain the supernormal conduction of the slow response in addition to the prolongation of APD.\textsuperscript{31}

A type of outward current similar to I\textsubscript{Na} called I\textsubscript{Na} has been observed in various tissues.\textsuperscript{31} This current has recently been shown to be modulated by increased intracellular cyclic adenosine 3':5'-monophosphate (cAMP), which speeds its inactivation.\textsuperscript{32} As a result of this effect, I\textsubscript{Na} is assumed to cause repetitive excitations during the afterdischarge. Therefore, it is of interest to know how I\textsubscript{Na} in the heart is modulated, whether by a hormone or a neurotransmitter, since the cardiac Ca\textsuperscript{2+} current is enhanced by an adrenaline-sensitive adenylate cyclase.\textsuperscript{33} Therefore, important aspects of the modulation of I\textsubscript{Na} and its precise role in cardiac electrical activity remain to be clarified.

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M Hiraoka and S Kawano

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