Subcellular Mechanism for Ca\(^{2+}\)-Dependent Enhancement of Delayed Rectifier K\(^{+}\) Current in Isolated Membrane Patches of Guinea Pig Ventricular Myocytes

Jun-ichi Nitta, Tetsushi Furukawa, Fumiaki Marumo, Tohru Sawanobori, Masayasu Hiraoka

Intracellular Ca\(^{2+}\) augments delayed rectifier K\(^{+}\) current (I\(_{K}\)) in cardiac myocytes, which may play a major modulatory role in repolarization of action potentials. We investigated subcellular mechanisms for Ca\(^{2+}\)-induced enhancement of I\(_{K}\) in large-pipette inside-out membrane patches excised from isolated guinea pig ventricular myocytes. When [Ca\(^{2+}\)]\(_i\), was raised from 10\(^{-8}\) to 10\(^{-6}\) mol/L, the amplitude of I\(_{K}\) measured at +80 mV was increased from 12.0±2.2 to 19.5±3.3 pA (P<.01). The enhancement of I\(_{K}\) by Ca\(^{2+}\) was dose dependent, with an EC\(_{50}\) of 3.8×10\(^{-8}\) mol/L. A calmodulin antagonist, W7 (50 μmol/L), calmidazolium (100 μmol/L), or HT-74 (20 μmol/L), added to the intracellular solution abolished enhancement of I\(_{K}\) by Ca\(^{2+}\), whereas the inactive form of the W7 analogue, W5, had no effect on I\(_{K}\). In the presence of a protein kinase inhibitor with a relatively high specificity for protein kinase C (H7), for protein kinase A (H8 or peptide-type inhibitor PKI), or for calmodulin kinase II (KN-62) or a nonspecific inhibitor of serine/threonine protein kinases (staurosporine), increases in [Ca\(^{2+}\)]\(_i\) still enhanced I\(_{K}\). Ca\(^{2+}\)-induced enhancement of I\(_{K}\) was also observed when Mg\(^{2+}\) and ATP were omitted from the intracellular solution to delete exogenous phosphate donors and when adenyllylimidophosphatase was added to preclude trapped cytoplasmic substrates. Thus, cardiac I\(_{K}\) was enhanced by increases in [Ca\(^{2+}\)]\(_i\), at a physiological range via a calmodulin-dependent pathway, which did not involve a phosphorylation process. (Circ Res. 1994;74:96-104.)

Key Words • delayed rectifier K\(^{+}\) current • calmodulin • Ca\(^{2+}\)

The calcium ions function as a ubiquitous intracellular signal mediator to influence ionic channel activity, various enzymatic processes, and the regulation of muscle contraction. In cardiac cells, cytosolic [Ca\(^{2+}\)] ([Ca\(^{2+}\)]\(_i\)) rises after depolarization of membrane potential via Ca\(^{2+}\) influx across the plasma membrane and subsequent release from the intracellular stores.\(^1\) Delayed rectifier K\(^{+}\) current (I\(_{K}\)), one of the major outward currents conducting during the plateau of action potentials in cardiac myocytes, is shown to be the target of regulation by Ca\(^{2+}\).\(^2\)\(^-\)\(^4\) Enhancement of I\(_{K}\) by increased [Ca\(^{2+}\)]\(_i\) may limit depolarization duration and thus an amount of Ca\(^{2+}\) entry. This may act as a negative-feedback mechanism protecting cardiac myocytes from excessive entry of Ca\(^{2+}\). The rise in [Ca\(^{2+}\)]\(_i\) also occurs under various pathological conditions including ischemia/reperfusion injury.\(^5\) In these pathological situations, the regulation of I\(_{K}\) by Ca\(^{2+}\) may also act as a self-protection mechanism, since the shortening in action potential duration limits the amount of Ca\(^{2+}\) entry, diminishes contractile activity, and thus prevents further loss of high-energy substrates.

Despite its possible pathophysiological importance, the subcellular mechanism underlying regulation of I\(_{K}\) by Ca\(^{2+}\) remains unclarified. The major difficulty comes from the lack of methods for obtaining stable single-channel recordings of I\(_{K}\) in excised patches. The regulation of I\(_{K}\) by Ca\(^{2+}\) has been studied exclusively in the whole-cell clamp mode using dialyzed cells.\(^2\)\(^-\)\(^4\) However, in the dialyzed whole-cell clamp configuration, [Ca\(^{2+}\)]\(_i\) cannot be controlled precisely, and it is nearly impossible to test numbers of different intracellular solutions because of the limited efficacy of intracellular dialysis. Recently, the major component of I\(_{K}\) was reported to be due to the activity of high-density extremely-low-conductance K\(^{+}\) channels, and this current could be recorded in excised membrane patches.\(^6\)\(^-\)\(^8\) By this method, I\(_{K}\) can be recorded under the conditions in which various intracellular solutions can be changed rapidly and easily. In the present study, we used the excised membrane patch to explore the mechanism of subcellular regulation of I\(_{K}\) by Ca\(^{2+}\).

Materials and Methods

Preparation

Enzymatic dissociation of single ventricular myocytes from guinea pig hearts was done using essentially the same method as previously reported from our laboratory.\(^9\)

Solutions

The internal (bath) solution contained (mmol/L) potassium aspartate, 140; MgCl\(_2\), 1; CaCl\(_2\), 1; EGTA, 11; ATP, 5; and HEPES, 5; the pH was adjusted to 7.3 with KOH. Concentrations of free Ca\(^{2+}\) (pCa 9 to 5) and free Mg\(^{2+}\) (0.8 mmol/L) in the internal (bath) solution were estimated on the basis of

Received December 24, 1992; accepted September 28, 1993.

From the Second Department of Internal Medicine (J.N., F.M.), Faculty of Medicine, and the Department of Cardiovascular Disease (T.F., T.S., M.H.), Medical Research Institute, Tokyo Medical and Dental University, Yushima, Bunkyo-ku, Tokyo, Japan.

Correspondence to Masayasu Hiraoka, MD, PhD, Department of Cardiovascular Disease, Medical Research Institute, Tokyo Medical and Dental University, 1-5-45, Yushima, Bunkyo-ku, Tokyo 113, Japan.
apparent stability constants for Ca\(^{2+}\)-EGTA, K\(_7\)-ATP, and MgCl\(_2\), according to the calculation proposed by Tsien and Rink.\(^{10}\) 1-(5-Isoquinolinesulfonyl)-2-methylpiperezine dihydrochloride (H7), N-[2-(methylamino)ethyl]-5-isouquinolinesulfonamide dihydrochloride (H5), N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W7), and N-(6-aminohexyl)-1-naphthalenesulfonamide hydrochloride (W5) were purchased from Seikagaku Co, Tokyo, Japan; a peptide-type inhibitor of protein kinase A (PKA) was purchased from Sigma Chemical Co, St Louis, Mo. They were added to the bathing solution on each experimental day at final concentrations as described in the text from stock solutions in distilled water. 1-[N,O-Bis(1,5-isouquinolinesulfonf)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62, Sigma), staurosporine (Sigma), and 3-(2-benzothiazolyl)-4,5-dimethoxy-N-[3-(4-phenylpiperidinyl)propyl]benzenesulfonamide (HT-74, Takeda Chemical Industries LTD, Osaka, Japan) were added to the bathing solution at final concentrations described in the text from stock solutions in dimethyl sulfoxide (DMSO). 5'-Adenyllylimidodiphosphate (AMP-PNP) was purchased from Sigma. Glibenclamide, a gift of Hoechst Japan, Tokyo, was dissolved as a 0.2 mmol/L stock solution in 2% DMSO and diluted into the test solution to obtain the final concentration indicated in the text. The final concentration of DMSO contained in each test solution was less than 0.1%. The external (pipette) solution contained (mmol/L) N-methylglycine, 132; KCl, 4.8; MgCl\(_2\), 2; CaCl\(_2\), 1; glucose, 5; and HEPES, 10; the pH was adjusted to 7.4 with HCl.

**Recording Methods**

Membrane currents were recorded in an inside-out patch-clamp configuration described by Hamill et al\(^{11}\) using glass pipettes with a diameter of 3 to 4 μm. The resistance of the pipette was 1 to 4 MΩ when filled with the internal solution. The electrode was connected to the input stage of a patch-clamp amplifier (Axopatch-1C, Axon Instruments, Inc, Foster City, Calif) with a feedback resistance of 100 MΩ. Electrical connection to the pipette and to the bath was made through a Ag/AgCl half-electrode. The junction potential of each electrode was adjusted to zero potential between the pipette solution and the bath solution immediately before each cell was approached and was checked again at the end of each experiment. When the difference in junction potential between two measurements was more than 2 mV, the value of the membrane potential was corrected accordingly. The depolarizing test pulses were applied every 15 seconds. The current signals were digitized on-line by a 12-bit resolution Labmaster A/D converter (TeMcar Scientific Solutions, Burlingame, Calif) under the control of an IBM-PS/2 personal computer and were stored on a hard disk. Data were analyzed using a software program (pClamp, version 5.5.1, Axon Instruments). Leak subtraction was made using the P/4 protocol on pClamp. All experiments were performed at a temperature of 33° to 35°C.

**Data Analysis**

Data are expressed as mean±SEM, and their statistical significance was evaluated by Student’s paired t test, where appropriate. A value of P<.05 was considered significant.

**Results**

**Characteristics of I\(_K\) in Excised Patches**

To increase the likelihood of activating I\(_K\) channels, long depolarizing voltage steps of 2.5-second duration were applied to the positive potential. Fig 1A shows superimposed currents that were elicited by a depolarizing test pulse to +80 mV, followed by repolarizing pulses to various potentials between −100 and +50 mV in 10-mV steps. Depolarizing pulses elicited a slowly activating and noninactivating outward current. This current was deactivated after repolarization with time, displaying a slow decay of tail currents. Fig 1B illustrates current-voltage relations of tail currents constructed from seven patches. The reversal potential was −73.0±2.7 mV. This value is close to the equilibrium potential for K\(^{+}\) in the condition in which [K\(^{+}\)]\(_o\) was 4.8 mmol/L and [K\(^{+}\)]\(_i\) was 140 mmol/L, estimated by the Nernst equation. The measurements of reversal potential were repeated at three different [K\(^{+}\)]\(_o\) values, 4.8, 24, and 48 mmol/L. In Fig 1C, the average reversal potential is plotted against [K\(^{+}\)]\(_o\). The data were fitted by a linear regression, resulting in a slope of approximately 50 mV per 10-fold change in [K\(^{+}\)]\(_o\). These data suggest K\(^{+}\) as a main charge carrier for this current. When the time course of activation of this current was examined the time course of activation of this current was examined the time course of activation of this current. In this and following analyses, we used data obtained from slowly activating outward currents during repolarizing pulses rather than from tail currents because the amplitude of I\(_K\) tails was usually too small to be analyzed in detail. After the leak subtraction was made using the P/4 protocol, the activation of I\(_K\) was fitted by a single exponential function, and an example at a membrane potential of +80 mV is shown in Fig 2A. The activation kinetics of I\(_K\) in the excised patch could be more complicated, as have been reported for the I\(_K\) in the whole-cell clamp configuration,\(^{12-14}\) which was also suggested by our own data in the slight deviation of I\(_K\) activation from a monoexponential fit at very early
times (see Fig 2). Yet, we used a monoexponential equation simply to characterize the major component of the time course of \( I_\text{K} \) activation. Thus, the activation of \( I_\text{K} \) may be expressed as

\[
I_\text{K}(t) = I_\text{K}(\infty) - A \cdot \exp(-t/\tau)
\]

where \( A \) represents the extrapolated amplitude at the start of test pulse (\( t=0 \)), \( \tau \) is the time constant, \( I_\text{K}(t) \) is the amplitude of \( I_\text{K} \) at \( t \), and \( I_\text{K}(\infty) \) is the steady-state level of \( I_\text{K} \). To further confirm that the time course of \( I_\text{K} \) activation could be fitted by a single exponential function, we plotted a logarithm of \( [I_\text{K}(\infty) - I_\text{K}(t)] \) against the time after the start of the depolarizing pulse (Fig 2B). The data clearly show that the time course of \( I_\text{K} \) could be expressed as a single exponential function. The mean time constant \( (\tau) \) obtained from 13 patches was 341 ± 29 milliseconds, which was in good agreement with the data by Walsh et al.\(^6\) This current was blocked by internally applied 5 mmol/L Ba\(^{2+}\) (Fig 3A) or 10 mmol/L tetraethylammonium (data not shown). When these findings are taken together, it is reasonable to assume that this current represents \( I_\text{K} \), probably its slow component \( (I_{\text{Ks}}) \).\(^{12,13}\)

**Effects of Intracellular Ca\(^{2+}\) on \( I_\text{K} \)**

Fig 3B shows the effects of changing [Ca\(^{2+}\)]\(_i\) on \( I_\text{K} \) elicited by a 2.5-second depolarizing pulse to +80 mV from a holding potential of −30 mV. When [Ca\(^{2+}\)]\(_i\) was increased from \( 10^{-8} \) to \( 10^{-6} \) mol/L, the amplitude of slowly activating \( I_\text{K} \) as well as the steady-state current level at a holding potential of −30 mV was increased. At a holding potential of −30 mV, the inward rectifier K\(^+\) current shows almost complete inward rectification, and \( I_\text{K} \) does not reach the threshold for its activation. Thus, the change in steady-state current level may be due to the change in other unspecified current(s), or the surface-charge screening effect by Ca\(^{2+}\) may also be involved. To minimize the influence of changes in steady-state current level, the amplitude of \( I_\text{K} \) was measured from the current level at the beginning of a 2.5-second depolarizing pulse to a steady-state current level. As described in the whole-cell clamp mode,\(^{15}\) \( I_\text{K} \) in guinea pig ventricular myocytes developed slowly during a
depolarizing pulse and did not reach a steady-state level at the end of a 2.5-second depolarizing pulse. Thus, a monotonically extrapolated level at 2.5 seconds was taken as a steady-state level of $I_k$ [$I_k(\infty)$ in Equation 1]. The increase in $[\text{Ca}^{2+}]_i$, from $10^{-6}$ to $10^{-4}$ mol/L enhanced the amplitude of $I_k$ from 12.0±2.2 to 19.5±3.3 pA (n=7, P<.01).

It is widely observed that $I_k$ in the whole-cell clamp configuration shows rundown after starting intracellular dialysis, which might interfere with the data analysis for relatively long experimental protocols. To evaluate the magnitude of rundown for macroscopic $I_k$ in the excised-patch configuration, we monitored the amplitude of $I_k$ every 15 seconds for 1 or 2 hours at pCa 8 and pCa 6. Fig 3C shows a typical example of such experiments. Unlike $I_k$ in the whole-cell clamp configuration, macroscopic $I_k$ recorded in the excised-patch configuration showed only a minimum degree of rundown. We performed a total of six experiments for this protocol, and the degree of rundown was less than 10% in each experiment.

Fig 4 displays dose-response relations for Ca$^{2+}$-induced enhancement of $I_k$, in which the amplitude of $I_k$ at a fixed membrane potential of +80 mV was measured at each [Ca$^{2+}$], and was normalized to the value at pCa 9. Data were fitted by a modified Hill equation in the following form:

$$I = 100 \times \left[1 + \frac{I_{\text{max}} \times [\text{Ca}^{2+}]^h}{K_h + [\text{Ca}^{2+}]^h}\right]$$

where I represents the amplitude of $I_k$ expressed as a percentile of the amplitude of $I_k$ at pCa 9, $I_{\text{max}}$ is the maximal $I_k$, h is the Hill coefficient, and $K_h$ is the pCa level causing half-maximal enhancement. The intracellular Ca$^{2+}$ increased the amplitude of $I_k$ in a dose-dependent manner, and the increase in $I_k$ amplitude reached a plateau at pCa 6. The Hill coefficient was 1.4, and the Ca$^{2+}$ concentration that causes half-maximal enhancement was 3.8±10$^{-4}$ mol/L.

Effects of intracellular Ca$^{2+}$ on steady-state activation of $I_k$ was studied. Membrane potential was depolarized from a holding potential of -30 mV to various test potentials between -100 and +100 mV in 10-mV steps for 2.5 seconds. Fig 5 illustrates steady-state activation of $I_k$ as a function of the test potential. The threshold for the activation of this current was approximately -30 to -20 mV at pCa 6, and this current increased in amplitude with increasing membrane potential. At neither pCa 6 nor pCa 8 did the activation of $I_k$ reach the maximum plateau after a 2.5-second depolarization to +100 mV.

**Intracellular Mechanisms Involved in Enhancement of $I_k$ by Increased [Ca$^{2+}]$**

It has been reported that $I_k$ was modulated by protein kinases A and C,4,7,15-17 To examine whether or not phosphorylation by protein kinase A or C was involved in Ca$^{2+}$-induced enhancement of $I_k$, the effects of H7, a general protein kinase inhibitor having a high specificity especially for protein kinases A and C, or H8, a relatively specific protein kinase A inhibitor, were studied. Fig 6 shows a representative experiment studying the effect of change in [Ca$^{2+}]$, on $I_k$ in the presence of 20 μmol/L H7. This concentration of H7 (20 μmol/L) is more than six times higher than the $K_c$ (3 μmol/L) for protein kinase A and more than three times higher than the $K_h$ (6 μmol/L) for protein kinase C.18 In the presence of H7, the elevation of [Ca$^{2+}]$, from $10^{-8}$ to $10^{-4}$ mol/L also caused an increase in $I_k$. In the presence of H7 (20 μmol/L), the averaged amplitude of $I_k$ at pCa 8 was 13.2±1.0 pA and that at pCa 6 was 22.2±1.5 pA (n=5, P<.01). This magnitude of enhancement of $I_k$ (68±11%) was similar to that in the absence of H7 (63±28%) (P=NS).

Similar results were obtained in the presence of 10 μmol/L H8, a concentration more than eight times higher than the $K_c$ (1.2 μmol/L) for protein kinase A.18 In the presence of H8, the average amplitude of $I_k$ at pCa 8 was 11.9±1.6 pA and that at pCa 6 was 19.4±1.5 pA (P<.01). The magnitude of enhancement of $I_k$ (63±13%) was also similar to that in the absence of H8 (63±28%) (P=NS). We also tested the effects of other types of protein kinase inhibitors. In the presence of 5 μmol/L PKI, a peptide-type inhibitor of protein kinase A, and 5 μmol/L staurosporine, a non-specific inhibitor of serine/threonine protein kinases, the magnitude of enhancement of $I_k$ was not significantly different from that occurring in the absence of any protein kinase inhibitors (68±24% [n=3] and 61±19% [n=3], respectively). PKI is known to inhibit protein kinase A competitively, with a $K_i$ of 2.3 nmol/L.19 Staurosporine is...
known to inhibit protein kinases A\textsuperscript{20} and C,\textsuperscript{21} Ca\textsuperscript{2+} / calmodulin-dependent protein kinase II (CaM kinase II),\textsuperscript{22} and other serine/threonine protein kinases with IC\textsubscript{50} values of less than 10 nmol/L. These data suggest that phosphorylation by neither protein kinase A nor C may be involved in the enhancement of IK by intracellular Ca\textsuperscript{2+}.

We next tested a possible involvement of calmodulin in the Ca\textsuperscript{2+}-induced enhancement of IK. Fig 7 shows a representative experiment studying the elevation of [Ca\textsuperscript{2+}]\textsubscript{i} on IK in the presence of calmodulin antagonist W7 (50 μmol/L) or W5 (50 μmol/L), which has less specificity for inhibiting the calmodulin effect. This concentration of W7 is approximately twice as high as the IC\textsubscript{50} value (approximately 30 μmol/L) for calmodulin, and the concentration of W5 is approximately a quarter of the IC\textsubscript{50} value (approximately 210 μmol/L).\textsuperscript{23,24} The enhancement of IK by the elevation of [Ca\textsuperscript{2+}]\textsubscript{i} was almost completely abolished in the presence of W7, whereas it was still clearly observed in the presence of W5. Fig 8 shows summarized data obtained from six experiments. In the presence of W7 (50 μmol/L), the amplitude of IK was not enhanced by the elevation of [Ca\textsuperscript{2+}]\textsubscript{i} from pCa 8 (10.1±2.0 pA) to pCa 6 (11.2±2.1 pA) (P=NS), whereas it was increased from 17.1±2.8 pA at pCa 8 to 25.0±4.7 pA at pCa 6 (P<.01) in the presence of W5 (50 μmol/L). Furthermore, we also tested the effects of different classes of calmodulin antagonists, calmidazolium (100 nmol/L, n=5)\textsuperscript{22} and HT-74 (20 μmol/L, n=4).\textsuperscript{26} In the presence of calmidazolium (100 nmol/L) or HT-74 (20 μmol/L), the Ca\textsuperscript{2+}-induced enhancement of IK was abolished. In the presence of calmidazolium (100 nmol/L), the amplitude of IK was 10.9±2.9 pA at pCa 8 and 11.3±2.6 pA at pCa 6 (P=NS); the corresponding values in the presence of HT-74 (20 μmol/L) were 13.0±1.9 and 13.4±2.3 pA, respectively (P=NS).

We took a different approach to test the hypothesis that the Ca\textsuperscript{2+}-induced enhancement of IK was mediated by a Ca\textsuperscript{2+}/calmodulin-dependent pathway; we perfused the intracellular aspects of membrane patches for 15 minutes with the solution containing no Ca\textsuperscript{2+} and a high concentration of the Ca\textsuperscript{2+}-chelating agent EGTA (11 mmol/L). In this way, endogenous calmodulin attached to the intracellular aspect of a patch membrane could be detached and washed away.\textsuperscript{27} Fig 9 shows the effect of intracellular Ca\textsuperscript{2+} in this condition. The amplitude of IK in the solution at pCa 6 after exposure to Ca\textsuperscript{2+}-free solution was less than that in the solution at pCa 8, although slightly higher than that in the Ca\textsuperscript{2+}-free solution. Similar findings were observed in all six patches tested. We also examined whether exogenously applied calmodulin had any effects on IK; however, enhancement of IK by exogenously applied calmodulin (1 to 3 μmol/L, purchased from Sigma) was not appreciable at pCa values from 8 to 6 (n=6, data not shown).

Ca\textsuperscript{2+}/calmodulin complexes are known to mediate diverse biologic reactions through a pathway involving phosphorylation by CaM kinase II. Therefore, we next tested the effect of a selective inhibitor of CaM kinase II, KN-62 (3 μmol/L).\textsuperscript{28} As shown in Fig 10, in the presence of KN-62 (3 μmol/L), the elevation of [Ca\textsuperscript{2+}]\textsubscript{i} clearly enhanced the amplitude of IK. From four patches, in the presence of KN-62 (3 μmol/L) the mean amplitude of IK was increased from 9.0±1.6 pA at pCa 8 to 20.8±5.2 pA at pCa 6 (P<.01). The finding that
staurosporine, a nonspecific protein kinase inhibitor with a specificity for CaM kinase II, could not attenuate Ca$^{2+}$-induced I$_K$ enhancement further argued against the role of CaM kinase II in I$_K$ enhancement by Ca$^{2+}$.

To further support the idea that a Ca$^{2+}$/calmodulin complex can enhance I$_K$ via a phosphorylation-independent pathway, we examined the effect of intracellular Ca$^{2+}$ on I$_K$ in the solution containing no ATP or Mg$^{2+}$. Even in this condition in which no exogenously applied substrate for protein kinases was present, Ca$^{2+}$ could enhance the amplitude of I$_K$, and calmodulin antagonist W7 (50 μmol/L) attenuated the Ca$^{2+}$-induced enhancement of I$_K$ (Fig 11). Similar findings were obtained in all five patches tested. Even in the absence of ATP or Mg$^{2+}$ in the intracellular solution, the possible involvement of endogenous ATP trapped to the intracellular side of the excised membrane patch may be possible. To exclude this possibility, we examined the effect of changing in [Ca$^{2+}$] in the presence of AMP-PNP (3 mmol/L). Ca$^{2+}$ could enhance the amplitude of I$_K$ even in the presence of AMP-PNP (3 mmol/L) (Fig 12). Thus, the enhancement of I$_K$ by Ca$^{2+}$ was mediated by calmodulin, but a phosphorylation process might not be involved.

**Discussion**

Previous studies using the whole-cell clamp technique have shown that I$_K$ in guinea pig ventricular myocytes is enhanced by an increase in [Ca$^{2+}$], but the intracellular mechanism by which Ca$^{2+}$ regulated I$_K$ has not been fully understood. In the present study, we confirmed by using excised cell-free patches that I$_K$ in guinea pig ventricular myocytes was indeed enhanced by increased intracellular Ca$^{2+}$ in the range of physiological levels. The novel finding in the present study is that Ca$^{2+}$-induced enhancement of I$_K$ occurs via a calmodulin-dependent pathway, but the action seems not to be mediated via a phosphorylation reaction.

**Macroscopic I$_K$ Recorded in Excised Membrane Patches**

Recent reports suggested that the current analysis using excised membrane patches was an invaluable tool in the study of regulatory mechanisms of I$_K$ independent of Ca$^{2+}$.

**Fig 8.** Bar graphs showing the effects of calmodulin antagonists W7 (A) and W5 (B) on the Ca$^{2+}$-induced enhancement of delayed rectifier K$^+$ current (I$_K$). The amplitude of I$_K$ was measured from six experiments in the presence or absence of 50 μmol/L W7 or 50 μmol/L W5 at pCa 8 or pCa 6. Results are expressed as mean±SEM. Please note that, when pCa was changed from 8 to 6, the amplitude of I$_K$ was not increased in the presence of W7, whereas it was increased in the presence of W5 with a magnitude similar to that in the absence of calmodulin antagonists. *P<.05.

**Fig 9.** Plot showing the effects of increases in [Ca$^{2+}$], on delayed rectifier K$^+$ current (I$_K$) amplitude after the intracellular aspect of the membrane was perfused with the solution containing no Ca$^{2+}$ and a high concentration of Ca$^{2+}$-chelating agent EGTA (11 mmol/L) for approximately 20 minutes. The amplitude of I$_K$ was decreased in the Ca$^{2+}$-free solution, and I$_K$ was not enhanced by increasing [Ca$^{2+}$], to pCa 6.

**Fig 10.** Plot showing the effect of KN-62, a Ca$^{2+}$/calmodulin-dependent protein kinase II antagonist, on the Ca$^{2+}$-induced enhancement of delayed rectifier K$^+$ current (I$_K$). The increase in [Ca$^{2+}$], from pCa 8 to pCa 6 enhanced macroscopic I$_K$ even in the presence of 3 μmol/L KN-62.
of cytoplasmic components. Before discussing the modulation of \( I_K \) by intracellular Ca\(^{2+} \), in this method, we have to consider whether the slowly activating outward current observed in excised membrane patches indeed represents \( I_K \) recorded in the whole-cell clamp experiments, and if so, which component(s) of \( I_K \) this current represents. This current had a threshold for activation at approximately −30 to −20 mV, displaying slowly activating outward currents during depolarizing pulses without inactivation. The current increased in amplitude with membrane depolarization but did not reach full activation after a 2.5-second depolarization even up to +100 mV. On repolarization, the current decayed with time, thus exhibiting slowly deactivating tail currents. The reversal potential of tail currents at \([K^+]_o\) of 4.8 mmol/L was −73 mV on average, and the value at different levels of \([K^+]_o\) revealed a slope of 50 mV per a 10-fold change in \([K^+]_o\), indicating that the main carrier of this current was \( K^+ \). These results are in good agreement with the properties of \( I_K \) recorded from guinea pig ventricular myocytes with the whole-cell clamp configuration from various laboratories.\(^2\)\(^{-}\)\(^4\)\(^{12}\)\(^{13}\)\(^{15}\)\(^{-}\)\(^{17}\)\(^{29}\)\(^{30}\) It has been suggested that \( I_K \) in guinea pig ventricular myocytes is the composite of at least two components, rapidly activating \( I_K \) (\( I_{Ks} \)) and slowly activating \( I_K \) (\( I_{Kp} \)).\(^{12}\)\(^{13}\) Recently, it was reported that another component, \( I_{Kp} \), which activated very rapidly and was conductive at plateau potential, might exist in guinea pig ventricular myocytes.\(^{14}\) \( I_K \) may not be the main constituent of the slowly activating outward current in excised membrane patches, because this slowly activating outward current did not show significant inward rectification and the time course of current activation was much faster for \( I_{Ks} \) than for the slowly activating outward current. This current is also unlike \( I_{Ks} \) because its activation kinetics were much slower than those of \( I_{Ks} \) (less than 10 milliseconds). Furthermore, it was reported that \( I_{Ks} \) did not require intracellular Ca\(^{2+} \) for its activation. Since the time course and voltage dependence of its activation are similar to those of \( I_{Ks} \) in guinea pig ventricular myocytes, it is most likely that the slowly activating outward current recorded in excised membrane patches mainly represents \( I_{Ks} \) in whole-cell clamp experiments, as suggested by Walsh et al,\(^6\) although contamination by \( I_{Kp} \), \( I_{Kp} \), or other unspecified components could not be completely excluded. It was also reported that modulations of the slowly activating outward current in excised membrane patches by protein kinases A and C and GTP-binding protein were similar to those in \( I_{Ks} \) in guinea pig ventricular myocytes.\(^7\)\(^{28}\)

Characteristics of the slowly activating outward current in excised membrane patches, however, are not totally identical to those of \( I_{Ks} \). It is widely known that \( I_{Ks} \) in the whole-cell clamp configuration shows rundown after starting intracellular dialysis, whereas the slowly activating outward current in excised membrane patches did not show significant rundown. Because of similar characteristics between these two currents discussed above, it may be more likely that this difference in the magnitude of rundown is due to different experimental conditions rather than to the fact that these two currents represent totally different components of \( I_K \). Duchatelle-Gourdon et al\(^{31}\) reported that the rundown of \( I_{Ks} \) in the whole-cell clamp configuration depended on \([Mg^{2+}]_o\). The basal free \([Mg^{2+}]_o\) was estimated to be 0.8 mmol/L, and the amplitude of \( I_{Ks} \) showed rundown when the concentration of \( Mg^{2+} \) in the pipette solution was greater than 0.8 mmol/L, and it showed rundown when \( Mg^{2+} \) in the pipette solution was less than 0.8 mmol/L. In our experiment, we used a free concentration of \( Mg^{2+} \) of 0.8 mmol/L in the intracellular solution. This may be one possible explanation for the lack of significant rundown in the slowly activating outward current in our experiment. However, we cannot exclude other possible explanations, such as the possibility that some cytosolic components related to rundown were washed away in the excised membrane patch; thus, further experiments are needed to determine the precise reason for the lack of rundown in this current.

**Effects of Intracellular Ca\(^{2+} \) on \( I_K \)**

The elevation of \([Ca^{2+}]_i\) augmented the amplitude of \( I_K \) in the excised membrane patches. These findings were also consistent with the data in the whole-cell clamp experiments.\(^8\)\(^{32}\) Kass\(^{32}\) reported that the delayed rectifier outward current (\( I_{Kr} \)) recorded using a conventional two-microelectrode voltage-clamp arrangement from the cardiac Purkinje fiber of calf or dog was not activated by intracellular Ca\(^{2+} \). His conclusion was drawn from the finding that, in the presence of a Ca\(^{2+} \) channel blocker, nisoldipine, Ca\(^{2+} \) channel current and contractile activity were abolished, whereas the amplitude of outward current tails that follow the depolarizing pulses was not suppressed. In his study, in the presence of nisoldipine, no slowly activating outward currents were observed during depolarizing pulses, leaving outward currents without clear time dependence, which was apparently different in current kinetics from the slowly activating outward current in the excised patch. Thus, in the cardiac Purkinje fiber of calf or dog, the main component(s) of outward current might differ from that in guinea pig ventricular myocytes. Another possible explanation for the lack of Ca\(^{2+} \) sensitivity in \( I_K \) was that \([Ca^{2+}]_i\) below the level for inducing muscle contraction could affect \( I_K \). It was reported that, in chemically skinned rat ventricular muscles, the threshold for skinned fiber contraction was at a pCa of approximately 6.5.\(^{33}\) At this concentration, intracellular Ca\(^{2+} \) enhanced \( I_K \) to almost a maximum degree. Thus, it might be possible that in his experiment.


[Ca\(^{2+}\)] did not decrease to the level at which the amplitude of \(I_K\) would be modulated.

### Subcellular Mechanism for Ca\(^{2+}\)-Induced \(I_K\) Enhancement

The fact that Ca\(^{2+}\)-induced enhancement of \(I_K\) occurred in excised patches may imply that intracellular Ca\(^{2+}\) acts directly on channel protein or via some components bound to the intracellular side of cell membrane. Many biologic reactions dependent on Ca\(^{2+}\) occur through a pathway involving protein kinase C or the Ca\(^{2+}\)-binding protein calmodulin. The finding that the presence of calmodulin antagonists, W7, calmidazolium, or HT-74, abolished Ca\(^{2+}\)-induced enhancement of \(I_K\) raises a possibility that a calmodulin-dependent pathway is important for the modulation of \(I_K\). It was reported in nerve tissue, however, that W7 per se inhibited a K\(^+\) current and a Ca\(^{2+}\) current independent of a calmodulin pathway.\(^{34}\) Inability of W5 to suppress Ca\(^{2+}\)-induced augmentation of \(I_K\) may support a calmodulin-dependent pathway rather than a nonspecific action of W7, because W5 lacking chlorine in its molecule has less specificity for inhibiting the effect of calmodulin. We also found that the Ca\(^{2+}\)-induced enhancement of \(I_K\) did not occur after the intracellular side of the membrane patches was perfused for 15 minutes with solution containing no Ca\(^{2+}\) and high concentration of EGTA. The application of a high concentration of Ca\(^{2+}\)-chelating agent EGTA for a relatively long duration has been used to detach the membrane-bound form of endogenous calmodulin.\(^{20}\) Thus, this finding appears to provide additional evidence that Ca\(^{2+}\)-induced \(I_K\) was mediated by endogenously present calmodulin. We also tested whether exogenously applied calmodulin could mimic the Ca\(^{2+}\)-induced enhancement of \(I_K\) but found no augmentation of Ca\(^{2+}\) action on \(I_K\) by exogenously applied calmodulin up to 3 \(\text{mol/L}\) (data not shown). Therefore, we speculate that calmodulin can mediate the Ca\(^{2+}\)-induced \(I_K\) enhancement only if calmodulin is present adjacent to the \(I_K\) channel, and exogenously applied calmodulin could not approach the channel in the biologic membrane. Alternatively, it is also possible that calmodulin has different actions in different tissues and that the calmodulin we used (harvested from bovine brain) does not work in the action that takes place in cardiac cells. It should also be noted that our data did not argue against the modulation of \(I_K\) by protein kinases A and C.\(^{24,27,37,39,40}\) We actually found that application of H7 or H8 diminished the amplitude of \(I_K\) at a given concentration of Ca\(^{2+}\) (data not shown).

It is intriguing to study how calmodulin enhances the amplitude of \(I_K\), because numerous biologic processes are known to be mediated by a Ca\(^{2+}\)/calmodulin complex. The most attractive candidate, perhaps, is CaM kinase II, because this is shown to modulate several sarcocellular ion channels; recently, it was reported that the slowly activating \(I_K\)-like outward current (\(I_{Ks}\)) expressed by microinjection neonatal mouse cardiac poly(A)^+ or complementary RNA encoding this channel was enhanced by intracellular Ca\(^{2+}\) via CaM kinase II.\(^{35}\) It was also reported that the opening of a delayed K\(^+\) current in *Eucnadrina* neurons was mediated by CaM kinase II.\(^{36}\) Therefore, we studied this aspect by examining the effects of a specific inhibitor of CaM kinase II (KN-62), a nonspecific protein kinase inhibitor with a specificity for CaM kinase II (staurosporine), or, in the absence of ATP, a substrate for protein kinases on Ca\(^{2+}\)-induced \(I_K\) activation. The elevation of [Ca\(^{2+}\)], still increased the amplitude of \(I_K\) despite of these treatments, indicating that a phosphorylation via CaM kinase II was not involved. These results suggest that a Ca\(^{2+}\)/calmodulin complex directly modulates \(I_K\), probably via an allosteric interaction with the channel protein itself or channel-associated protein. Direct interactions between channels and Ca\(^{2+}\)/calmodulin complexes have been reported; a Ca\(^{2+}\)/calmodulin complex appears to directly activate the Ca\(^{2+}\)-dependent sodium channel in excised membrane patches of Paramecium\(^{37}\) and to directly inactivate the Ca\(^{2+}\)-release channel of sarcoplasmic reticulum.\(^{38}\)

### Physiological Importance of Ca\(^{2+}\)-Induced Enhancement of \(I_K\)

Could the Ca\(^{2+}\)-induced enhancement of \(I_K\) work under physiological conditions and what might be the physiological role of this regulation? Our data showed that intracellular Ca\(^{2+}\) augmented \(I_K\) at a concentration of \(10^{-8} \text{ mol/L}\) or higher and reached a steady state at approximately \(5 \times 10^{-7} \text{ mol/L}\). The [Ca\(^{2+}\)], that causes half-maximal enhancement was 3.8 \(\times 10^{-8} \text{ mol/L}\). Considering the fact that intracellular Ca\(^{2+}\) activity in diastole in myocardium is in the range between \(10^{-8}\) and \(7 \times 10^{-8} \text{ mol/L}\) and it rises to approximately \(5 \times 10^{-6} \text{ mol/L}\) when cells are activated,\(^{39,40}\) the Ca\(^{2+}\)-induced enhancement of \(I_K\) appears to take place in the range where [Ca\(^{2+}\)] \(_i\) varies during a course of cell contraction and relaxation. Thus, under physiological conditions, Ca\(^{2+}\) that enters through voltage-dependent Ca\(^{2+}\) channels enhances \(I_K\), helping to terminate action potential repolarization and to eliminate further Ca\(^{2+}\) influx. Thus, the regulation of \(I_K\) by Ca\(^{2+}\) may work protectively for the heart under various pathological conditions. For example, the augmentation of \(I_K\) by increased [Ca\(^{2+}\)] shortens the action potential duration, thus reducing the amount of Ca\(^{2+}\) entry, thereby maintaining energy consumption at a minimum by suppressing muscle contraction and eliminating Ca\(^{2+}\) overload in cells.

### Acknowledgments

We wish to thank Noriko Fujita for excellent secretarial assistance and Yoko Sugimoto for superb technical assistance.

### References

Subcellular mechanism for Ca(2+)-dependent enhancement of delayed rectifier K+ current in isolated membrane patches of guinea pig ventricular myocytes.

J Nitta, T Furukawa, F Marumo, T Sawanobori and M Hiraoka

Circ Res. 1994;74:96-104
doi: 10.1161/01.RES.74.1.96

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/74/1/96