Role of Nitric Oxide in Ca\(^{2+}\) Sensitivity of the Slowly Activating Delayed Rectifier K\(^{+}\) Current in Cardiac Myocytes

Chang-Xi Bai, Iyuki Namekata, Junko Kurokawa, Hikaru Tanaka, Koki Shigenobu, Tetsushi Furukawa

Abstract—Sarcoplasmic Ca\(^{2+}\) entry is a vital step for contraction of cardiomyocytes, but Ca\(^{2+}\) overload is harmful and may trigger arrhythmias and/or apoptosis. To maintain the amount of Ca\(^{2+}\) entry within an appropriate range, cardiomyocytes have feedback systems that tightly regulate ion channel activities in response to the changes in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)). The elevation of [Ca\(^{2+}\)]\(_i\), directly regulates Ca\(^{2+}\) entry pathway, the L-type Ca\(^{2+}\) channel.\(^{1-3}\) It also alters the duration of action potential plateau and regulates the duration where the L-type Ca\(^{2+}\) channel can open. The slope of action potential plateau is mainly dependent on the balance between L-type Ca\(^{2+}\) channel currents (\(I_{calc}\)) and delayed rectifier K\(^{+}\) currents. Delayed rectifier K\(^{+}\) currents consist of two components: the rapidly activating component (\(I_{Ks}\)) that is sensitive to a class III antiarrhythmic drug E-4031 and the slowly activating component (\(I_{Kd}\)) that is E-4031-resistant.\(^4\) Elevations in [Ca\(^{2+}\)]\(_i\), selectively enhances the \(I_{Ks}\) component.\(^4,5\) The range of [Ca\(^{2+}\)]\(_i\), that regulates \(I_{Kd}\) (between 10\(^{-8}\) and 10\(^{-6}\) mol/L) is in the range that alters during the cycle of contraction and relaxation of cardiac myocytes.\(^6\) Thus, the Ca\(^{2+}\) sensitivity of \(I_{Kd}\) may potentially act as a physiological feedback system to regulate [Ca\(^{2+}\)]\(_i\).

To maintain the amount of Ca\(^{2+}\) entry within a narrow range, cardiomyocytes develop feedback systems that tightly regulate ion channel activities and thereby Ca\(^{2+}\) entry in response to the changes in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)). The elevation of [Ca\(^{2+}\)]\(_i\), directly regulates Ca\(^{2+}\) entry pathway, the L-type Ca\(^{2+}\) channel.\(^{1-3}\) It also alters the duration of action potential plateau and regulates the duration where the L-type Ca\(^{2+}\) channel can open. The slope of action potential plateau is mainly dependent on the balance between L-type Ca\(^{2+}\) channel currents (\(I_{calc}\)) and delayed rectifier K\(^{+}\) currents. Delayed rectifier K\(^{+}\) currents consist of two components: the rapidly activating component (\(I_{Ks}\)) that is sensitive to a class III antiarrhythmic drug E-4031 and the slowly activating component (\(I_{Kd}\)) that is E-4031-resistant.\(^4\) Elevations in [Ca\(^{2+}\)]\(_i\), selectively enhances the \(I_{Ks}\) component.\(^4,5\) The range of [Ca\(^{2+}\)]\(_i\), that regulates \(I_{Kd}\) (between 10\(^{-8}\) and 10\(^{-6}\) mol/L) is in the range that alters during the cycle of contraction and relaxation of cardiac myocytes.\(^6\) Thus, the Ca\(^{2+}\) sensitivity of \(I_{Kd}\) may potentially act as a physiological feedback system to regulate [Ca\(^{2+}\)]\(_i\).

Key Words: ion channels ■ nitric oxide synthases ■ calmodulin ■ protein-protein interaction ■ caveolin

In a previous article, we showed that \(I_{Ks}\) enhancement by elevation of [Ca\(^{2+}\)]\(_i\), was inhibited by a calmodulin (CaM) inhibitor, but not by an inhibitor of CaM-dependent kinase II.\(^6\) Although this finding suggests that the allosteric regulation of CaM is crucial for Ca\(^{2+}\)-sensitive \(I_{Ks}\) alterations, the detailed underlying mechanism remains unknown. Recently, we found that \(I_{Kd}\) was enhanced by nitric oxide (NO) via a cGMP-independent mechanism.\(^7\) Because allosteric interaction of Ca\(^{2+}\)/CaM complex with NO synthase (NOS) is a major mechanism of NOS activation and NO release,\(^8,9\) we tested in the present study if NO plays an important role in the Ca\(^{2+}\)-sensitive modulation of \(I_{Kd}\) in cardiac myocytes.

Materials and Methods

The investigation was conducted in accordance with the rules and regulations of the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University.

Quantification of [Ca\(^{2+}\)]\(_i\)

Quantification of [Ca\(^{2+}\)]\(_i\), was performed as described previously.\(^10\) (See the Expanded Materials and Methods section in the online data)
supplement available at http://circres.ahajournals.org.) Briefly, guinea pig ventricular myocytes were loaded with 10 μmol/L indo-1-AM (Dojin), and Ca\(^{2+}\) transients were elicited by field-stimulation at 1 Hz. Cells were excited at 360 nm from a Xenon lamp and the emission bands, 395 to 415 nm and 470 to 490 nm, were separated (W-VIEW system, Hamamatsu Photonics), detected by a high-speed cooled CCD camera (HISC, Hamamatsu Photonics), and ratio of the intensity of two emission bands was calculated (Aquacosmos software, Hamamatsu Photonics).

**Patch Clamp**

Single ventricular myocytes were harvested from hearts of adult guinea pigs (n=41, white Hartrey; Saitama Experimental Animal Supply Co Ltd, Saitama, Japan). Action potentials and membrane currents were recorded with an Axopatch 200B amplifier (Axon Instruments) using the perforated configuration of the patch-clamp technique with Amphotericin B (Sigma-Aldrich), in a current-clamp mode and a voltage-clamp mode. Action potentials were elicited by passing depolarizing current pulses (<2 ms in duration at a rate of 1 Hz) of suprathreshold intensity. \(I_{\text{Ks}}\) were elicited by 3.5-second depolarizing pulses to various test potentials between −30 and +50 mV in 10-mV increments at 0.1 Hz from a holding potential of −40 mV, and amplitudes of tail \(I_{\text{Ks}}\) were measured by extrapolating from exponential fits. Compositions of solutions used are described in the online data supplement. All experiments were performed at 36±1°C. The averaged series resistance was 157±1.8 MΩ, the capacitance time constant was 2.5±0.3 ms, and the membrane capacitance was 150±13 pF.

**Immunoprecipitation and Immunoblotting**

Cardiomyocytes were isolated from adult guinea pigs (n=3). Immunoprecipitation and immunoblotting were performed as described previously (see online data supplement). Briefly, cell lysates were immunoprecipitated with a monoclonal anti-NOS3 antibody (Zymed), followed by immunoblot analysis with an anti-NOS3 antibody (Zymed), an anti-caveolin-3 (Cav-3) antibody (N-18, Santa Cruz Biotechnology), or an anti-CaM antibody (FL-149, Santa Cruz Biotechnology) followed by incubation with a horseradish peroxidase-conjugated anti-mouse IgG (DAKO Japan Co Ltd) or an anti-rabbit IgG (DAKO Japan Co Ltd). Proteins were detected using the advance enhanced chemiluminescence system (Amersham Bioscience).

**Reagents**

Chromanol 293 B was supplied by Hoechst. E-4031 was purchased from Eisai Co Ltd, A23187, W7 and W5 from Wako, SH-6 from Merck, digoxin from Nacalai Tesque, and all other reagents from Sigma-Aldrich. A23187 was prepared as a 10 mmol/L stock solution in dimethylsulfoxide (W5; 10 mmol/L), and sodium nitroprusside (SNP; 1 mmol/L) were prepared in ethanol. Stock solutions of E-4031 (5 mmol/L), W7 (10 mmol/L), W5 (10 mmol/L), N-ethylmaleimide (SMTU; 10 mmol/L), N-acetyl-l-cysteine (l-NAC; 100 mmol/L), N-acetyl-l-ascorbic acid (l-Asc; 1 mmol/L), and sodium nitroprusside (SNP; 1 mmol/L) were prepared in distilled water, and nisoldipine (10 mmol/L), SH-6 (20 mmol/L), and 2-(4-morpholino)-8-phenyl-1-(4H)-benzopyran-4-one hydrochloride (LY-294,002; 60 mmol/L) stock solutions in dimethylsulfoxide. They were diluted in the bath solution to achieve the desired concentration.

**Data Analysis**

All values are presented as mean±SE. Statistical significance was determined by repeated-measure one-way analysis of variance (ANOVA) followed by Scheffé F test. Values \(P<0.05\) are considered to be significant.

**Results**

**Elevation of \([\text{Ca}^{2+}]_i\) by A23187 Shortened Action Potential Duration and Enhanced \(I_{\text{Ks}}\)**

Addition of A23187 to the bath solution increased \([\text{Ca}^{2+}]_i\), both in the basal level and at the peak of \(\text{Ca}^{2+}\) transient (Figure 1A). The basal \([\text{Ca}^{2+}]_i\), was 0.12±0.01 μmol/L (n=10) in the control state, 0.16±0.03 μmol/L (n=5) in the presence of A23187 at 0.1 μmol/L (P=NS versus control), and 0.22±0.02 μmol/L (n=5) at 2 μmol/L of A23187 (P<0.05 versus control). The peak \([\text{Ca}^{2+}]_i\), was 0.97±0.03 μmol/L (n=10) in the control state, 1.24±0.03 μmol/L (n=5) at 0.1 μmol/L of A23187 (P<0.05 versus control), and 1.52±0.09 μmol/L (n=5) at 2 μmol/L of A23187 (P<0.05 versus control).

Application of A23187 induced a dose-dependent shortening of action potential duration and enhancement of \(I_{\text{Ks}}\), which attained a maximal value at a concentration of 2 μmol/L of A23187 (where APD20 shortened by 49%, APD90 by 20%, and \(I_{\text{Ks}}\) amplitude increased by 31%) (see online data supplement). These values are comparable to magnitude of \(\text{Ca}^{2+}\)-induced enhancement of \(I_{\text{Ks}}\) recorded in the excised giant-patch recording. Thus, in the following experiments, we used A23187 at this concentration (2 μmol/L). Once electrical access in the perforated patch configuration was established, there was a slight time-dependent reduction in APD that occurred without drug application. Application of A23187 (2 μmol/L) reversibly shortened both APD20 (Δ in Figure 1B) and APD90 (△ in Figure 1B) with a significantly greater magnitude than time-dependent reduction in APD without drug application (○ and □ in Figure 1B) (n=5, \(P<0.01\)). Treatment with A23187 (2 μmol/L) for 15 minutes shortened APD20 by 49.4±3.3% (Figure 1B, n=6, \(P<0.01\) versus spontaneous APD20 shortening), and APD90 by 19.2±3.6% (Figure 1B, n=5, \(P<0.01\) versus spontaneous APD90 shortening). The tail amplitude of \(I_{\text{Ks}}\) in the control cells exhibited a time-dependent decrease (○ in Figure 1C). Application of A23187 (2 μmol/L) reversibly increased tail \(I_{\text{Ks}}\) by 31.2±2.3% (Figure 1C, n=5, \(P<0.01\) versus spontaneous time-dependent reduction in \(I_{\text{Ks}}\)). Addition of EGTA (10 mmol/L), a \(\text{Ca}^{2+}\) chelator, to the pipette solution prevented shortening of APD and enhancement of tail \(I_{\text{Ks}}\) amplitude by A23187 (see online data supplement). These results indicate that the effects of extracellularly applied A23187 on APD and \(I_{\text{Ks}}\) depend on the elevation in \([\text{Ca}^{2+}]_i\).

**Relative Contribution of \(I_{\text{Ks}}\) and \(I_{\text{Ca,L}}\) to \(\text{Ca}^{2+}\)-Induced APD Shortening**

\(I_{\text{Ca,L}}\) amplitude is diminished by elevation in \([\text{Ca}^{2+}]_i\), via the \(\text{Ca}^{2+}\)-induced \(I_{\text{Ca,L}}\) inactivation mechanism, which also induces APD shortening. To assess relative contribution of \(\text{Ca}^{2+}\)-induced \(I_{\text{Ks}}\) enhancement and \(I_{\text{Ca,L}}\) inactivation to the APD shortening induced by A23187, we used a \(I_{\text{Ks}}\) channel blocker, chromanol 293B, and/or a \(I_{\text{Ca,L}}\) channel blocker, nisoldipine, at supramaximal concentrations. Action potentials were continuously elicited at a stimulation rate of 0.1, 0.4, 1, and 4 Hz, and after shortening of APD by A23187 (2 μmol/L) had reached a pseudo-steady state, chromanol 293B (30 μmol/L), nisoldipine (3 μmol/L), or a combination of chromanol 293B (30 μmol/L) and nisoldipine (3 μmol/L) was added in the presence of A23187 (2 μmol/L). At 0.1 Hz, chromanol 293B alone or nisoldipine alone partially reversed A23187-induced APD20 and APD90 shortening with a similar magnitude (left panel in Figure 2A): APD20 shortening was reversed by 49.4±3.3% by chromanol 293B and by...
48.9±3.1% by nisoldipine, (n=5, P=NS) (Figure 2B), and APD shortening was reversed by 54.5±3.4% by chromanol 293B and by 42.7±3.0% by nisoldipine (n=5, P=NS) (Figure 2C). A combination of chromanol 293B and nisoldipine completely reversed A23187-induced APD shortening to the control levels (Figure 2B and 2C). When stimulation rate was increased to 0.4, 1, and then 4 Hz, the magnitude of APD shortening was progressively increased with progressive increases in the fraction of APD shortening reversed by chromanol 293B, leaving the fraction reversed by nisoldipine.
constant. At 4 Hz, chromanol 293B reversed APD shortening by 74.8% and APD90 shortening by 79.8%, and nisoldipine reversed APD20 shortening by 23.6% and APD90 shortening by 20.0%. Thus, at a low stimulation rate both Ca2+-induced I\textsubscript{Ks} enhancement and Ca2+-induced I\textsubscript{Ca,L} contributed to the Ca2+-induced APD shortening with a similar magnitude. However, at a high stimulation rate, contribution of I\textsubscript{Ks} enhancement was significantly greater than contribution of I\textsubscript{Ca,L} suppression.

Roles of NO in APD Shortening and I\textsubscript{Ks} Enhancement by A23187

Ca\textsuperscript{2+} is a well-known activator of NOS. Recently, we reported that NO increased I\textsubscript{Ks} possibly by a mechanism dependent on direct S-nitrosylation of the channel protein, which prompted us to examine potential roles of NO in the A23187-induced enhancement of I\textsubscript{Ks}. After A23187 (2 μmol/L) had shorted APD and enhanced I\textsubscript{Ks}, we applied a nonspecific NOS inhibitor, SMTU (1 μmol/L). Effects of A23187 (2 μmol/L) on APD were partially, but significantly reversed by SMTU (Figure 3A), and those on I\textsubscript{Ks} were fully reversed back to the control levels (Figure 3B). Application of a NO scavenger, l-NAC (1 mmol/L), also reversed enhancement of I\textsubscript{Ks} induced by A23187 to the control levels (Figure 3B). Effects of A23187 on the gating properties of I\textsubscript{Ks} were also similar to those by a NO donor, SNP, which provide supporting evidence that A23187 enhances I\textsubscript{Ks} by causing the production of NO (see online data supplement).

NOS3 Is Responsible for A23187-Induced NO Release

Increase in I\textsubscript{Ks} occurs rapidly after application of A23187, which argues against activation of inducible NOS (NOS2). Therefore, we determined whether A23187 activated NOS1 or NOS3 to cause enhancement of I\textsubscript{Ks}. We used 2 different NOS inhibitors, SMTC, which has a significantly lower IC\textsubscript{50} for NOS1 (0.31 μmol/L) than for NOS3 (5.4 μmol/L), and

![Figure 3](http://circres.ahajournals.org/)

**Figure 3.** NO and NOS3 play a role in the A23187-induced APD shortening and I\textsubscript{Ks} enhancement. A, NOS inhibitor, SMTU (1 μmol/L), reversed APD shortening by A23187. a, Time course of changes in APD, and b depicts averaged percent changes in APD in the presence of A23187 alone (black bars) and in the presence of A23187 and SMTU (hatched bars). *P<0.05. B, NOS inhibitor, SMTU (1 μmol/L) (a), and a NO scavenger, l-NAC (1 mmol/L) (b), reversed the I\textsubscript{Ks} enhancement by A23187. a and b, Time course of changes in I\textsubscript{Ks}, and c depicts averaged percent changes in I\textsubscript{Ks} in the presence of A23187 alone (black bars) and in the presence of A23187 and SMTU or l-NAC (hatched bars). *P<0.05. C, NOS1 inhibitor, SMTC (3 μmol/L), did not reverse I\textsubscript{Ks} enhancement by A23187 (panel a). However, a NOS3 inhibitor, l-NIO (1 μmol/L), did reverse I\textsubscript{Ks} enhancement by A23187 (b). c, Averaged percent changes in I\textsubscript{Ks} in the presence of A23187 alone (black bars) and in the presence of A23187 and SMTC or l-NIO (hatched bars). *P<0.05, #P=NS.
Similarly, application of W7 (10 μmol/L) reversed $I_{Ks}$ enhancement by A23187 (a). However, an inactive analog of W7, W5 (10 μmol/L) (b), an Akt inhibitor, SH-6 (10 μmol/L) (c), and a PI3-kinase inhibitor, LY-294,002 (30 μmol/L) (d), did not reverse $I_{Ks}$ enhancement by A23187. B, Percent changes in $I_{Ks}$ in the presence of A23187 alone (black bars) or in the presence of A23187 with W7, W5, SH-6, or LY-294,002 (hatched bars). *P<0.05, #P=NS. C, W7 (10 μmol/L) reversed APD shortening (a), but SH-6 (10 μmol/L) did not (b). D, Percent changes in APD in the presence of A23187 alone (black bars) or in the presence of A23187 with W7 or SH-6 (hatched bars). *P<0.05, #P=NS.

Figure 4. CaM, but not Akt, is involved in A23187-induced $I_{Ks}$ enhancement and APD shortening. A, CaM inhibitor, W7 (10 μmol/L), reversed $I_{Ks}$ enhancement by A23187 (a). However, an inactive analog of W7, W5 (10 μmol/L) (b), an Akt inhibitor, SH-6 (10 μmol/L) (c), and a PI3-kinase inhibitor, LY-294,002 (30 μmol/L) (d), did not reverse $I_{Ks}$ enhancement by A23187. B, Percent changes in $I_{Ks}$ in the presence of A23187 alone (black bars) or in the presence of A23187 with W7, W5, SH-6, or LY-294,002 (hatched bars). *P<0.05, #P=NS. C, W7 (10 μmol/L) reversed APD shortening (a), but SH-6 (10 μmol/L) did not (b). D, Percent changes in APD in the presence of A23187 alone (black bars) or in the presence of A23187 with W7 or SH-6 (hatched bars). *P<0.05, #P=NS.

Effects of Ca$^{2+}$ Loading by Digoxin and Rise in Extracellular Ca$^{2+}$ Concentration on APD and $I_{Ks}$

Finally to examine if physiologically relevant Ca$^{2+}$-loading also shorten APD and enhance $I_{Ks}$, we examined effects of digoxin and rise in extracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]). Application of digoxin at 0.2 μmol/L to the bath solution did not significantly change basal [Ca$^{2+}$], (0.10±0.01 μmol/L in the control state [n=6] and 0.13±0.02 μmol/L in the presence of digoxin at 0.2 μmol/L [n=6]; P=NS), whereas peak [Ca$^{2+}$], was increased from 0.98±0.06 μmol/L (n=6) in the control state to 1.47±0.08 μmol/L (n=6) (P<0.05). Digoxin at 2 μmol/L increased both basal [Ca$^{2+}$], from 0.10±0.01 μmol/L (n=6) in the control state to 0.20±0.01 μmol/L (n=5) (P<0.05) and peak [Ca$^{2+}$], from 0.98±0.06 μmol/L (n=6) to 1.96±0.09 μmol/L (n=5) (P<0.05) (Figure 6A). Digoxin at 0.2 μmol/L shortened APD$_{50}$ by 28.0±3.9% and APD$_{90}$ by 12.6±3.1% relative to the control, and digoxin at 2 μmol/L shortened APD$_{50}$ by 58.8±3.4% and APD$_{90}$ by 24.0±3.4% relative to the control (Figure 6B and online data supplement). L-NAC (1 mmol/L) partially, but significantly, reversed APD shortening by digoxin (Figure 6B and online data supplement). Digoxin at 0.2 μmol/L increased $I_{Ks}$ amplitude by 17.4±3.1%, and digoxin at 2 μmol/L by 37.7±7.1% relative to the control.
IKs enhancement by digoxin was fully reversed by application of L-NAC (1 mmol/L) (Figure 6B and online data supplement).

Rise in [Ca\(^{2+}\)]\(_{o}\) from the control state (1.8 mmol/L) to 2.4 mmol/L shortened APD\(_{20}\) by 27.2\(\pm\)3.9% and APD\(_{90}\) by 17.4\(\pm\)3.0%, and to 3.0 mmol/L shortened APD\(_{20}\) by 45.4\(\pm\)3.8% and APD\(_{90}\) by 26.7\(\pm\)3.2%, which were partially, but significantly reversed by L-NAC (1 mmol/L) (Figure 6C and online data supplement). Rise in [Ca\(^{2+}\)]\(_{o}\) from 1.8 mmol/L to 2.4 mmol/L, and to 3.0 mmol/L enhanced IKs by 27.9\(\pm\)8.3% and 43.8\(\pm\)11.2%, respectively, which were fully reversed by L-NAC (1 mmol/L) (Figure 6C and online data supplement).

**Discussion**

NO has been shown to play important roles in the physiological regulation of Ca\(^{2+}\) cycling of cardiomyocytes.\(^{21}\) In this study, we demonstrate that Ca\(^{2+}\) regulation of IKs channel is
another critical NO-dependent Ca\(^{2+}\) cycling system based on the following findings: (1) a NO donor mimicked Ca\(^{2+}\)-induced \(I_{\text{Ks}}\) enhancement; (2) a NOS inhibitor abolished \(I_{\text{Ks}}\) enhancement by Ca\(^{2+}\); (3) a NO scavenger also abolished Ca\(^{2+}\)-induced \(I_{\text{Ks}}\) enhancement; and (4) changes in \(I_{\text{Ks}}\) kinetics caused by a NO donor were very similar to those by Ca\(^{2+}\).

To examine the mechanism underlying Ca\(^{2+}\)-sensitivity of \(I_{\text{Ks}}\), we increased [Ca\(^{2+}\)], by using a Ca\(^{2+}\)-ionophore, A23187, and recorded whole-cell \(I_{\text{Ks}}\) in the perforated patch configuration. This recording configuration is preferred to study Ca\(^{2+}\) regulation of \(I_{\text{Ks}}\) because it prevents wash-out of the intracellular signal transduction machinery. The peak [Ca\(^{2+}\)], was increased from 0.97±0.03 μmol/L in the control state to 1.24±0.03 μmol/L by A23187 at 0.1 μmol/L and to 1.52±0.09 μmol/L by A23187 at 2 μmol/L, whereas APD and \(I_{\text{Ks}}\) were barely affected by A23187 at 0.1 μmol/L and maximally changed by A23187 at 2 μmol/L. Thus, the threshold of [Ca\(^{2+}\)], to modulate APD and \(I_{\text{Ks}}\) appears to be between 1.24 and 1.52 μmol/L, which is well within the range where [Ca\(^{2+}\)], physiologically varies in cardiac myocytes. The changes in [Ca\(^{2+}\)], reached the stable level at 5 minutes after application of A23187 (see online data supplement), which is similar to the time course of changes in APD and \(I_{\text{Ks}}\) by A23187, further supporting that effects of APD and \(I_{\text{Ks}}\) by A23187 were attributable to changes in [Ca\(^{2+}\)].

In addition to Ca\(^{2+}\)-induced enhancement of \(I_{\text{Ks}}\), rise in [Ca\(^{2+}\)], causes Ca\(^{2+}\)-induced inactivation of \(I_{\text{Ca,L}}\). To understand the feedback system of Ca\(^{2+}\) entry, it is important to know relative contributions of Ca\(^{2+}\)-induced \(I_{\text{Ks}}\) enhancement and \(I_{\text{Ca,L}}\) inactivation to the Ca\(^{2+}\)-induced APD shortening. In the present study, at a low stimulation rate, both \(I_{\text{Ks}}\) enhancement and \(I_{\text{Ca,L}}\) inactivation contribute to the Ca\(^{2+}\)-induced APD shortening with a similar magnitude. However, at a high stimulation rate, A23187-induced APD shortening was reversed by 75% to 80% with chromanol 293B and only by 20% to 25% with nisoldipine. Thus, at a high stimulation rate, Ca\(^{2+}\)-induced \(I_{\text{Ks}}\) enhancement plays a dominant role in APD shortening. A23187-induced APD shortening was dependent on stimulation rate: the higher the stimulation was, the greater the A23187-induced APD shortening was. This result is consistent with a simulation study showing that \(I_{\text{Ks}}\) underlies rate-dependent adaptation of APD at fast heart rates. Fast pacing results in short diastolic intervals that prevent complete deactivation of \(I_{\text{Ks}}\), resulting in build-up of instantaneous \(I_{\text{Ks}}\) repolarizing current. In our study, elevated \(I_{\text{Ks}}\) components at higher [Ca\(^{2+}\)], may cause additional accumulation of the repolarizing current, resulting in the greater APD shortening in the presence of A23187.

We have previously demonstrated that \(I_{\text{Ca,L}}\) is suppressed by NO via a cGMP-dependent pathway. However, NO-dependent modulation of \(I_{\text{Ca,L}}\) appears not to play a significant role in the Ca\(^{2+}\)-induced \(I_{\text{Ca,L}}\) inactivation in our experiments, because neither a NO scavenger, L-NAC, nor a NOS inhibitor, SMTU, reversed the A23187-induced \(I_{\text{Ca,L}}\) suppression (see online data supplement). Because \(I_{\text{Ca,L}}\) channel inactivation is underlined by direct interaction of Ca\(^{2+}\)/CaM with the \(I_{\text{Ca,L}}\) channels that is situated in a pathway upstream of Ca\(^{2+}\)/CaM-dependent activation of NOS3, effects of NO on the \(I_{\text{Ca,L}}\) channels may be masked by a direct effect of Ca\(^{2+}\)/CaM. The finding that L-NAC and SMTU reversed A23187-induced APD shortening only partially may further implicate that a fraction of APD shortening caused by \(I_{\text{Ca,L}}\) inactivation is insensitive to NO modulators.

It is also critical to know whether Ca\(^{2+}\)-induced \(I_{\text{Ks}}\) enhancement and resultant APD shortening act in physiological and/or pathological conditions of the hearts. To address this question, we used physiologically relevant Ca\(^{2+}\) loading such as digoxin and rise in [Ca\(^{2+}\)]. Digoxin, a selective blocker of Na\(^+/\)K\(^{+}\)-ATPase is known to increase [Ca\(^{2+}\)], by indirectly enhancing activity of Na\(^{+}\)/Ca\(^{2+}\) exchanger, and in fact in our study, digoxin increased both basal and peak [Ca\(^{2+}\)]. Both application of digoxin and rise in [Ca\(^{2+}\)], induced shortening.
of APD and enhancement of \( I_{Ks} \). APD shortening was partially reversed by a NO scavenger, \( t \)-NAC, and \( I_{Ks} \) enhancement was fully reversed by \( t \)-NAC. Thus, \( Ca^{2+} \)-induced \( I_{Ks} \) enhancement and resultant APD shortening appear to be one of the physiological and pathological regulators of \( I_{Ks} \), among other well-known regulators, such as \( \beta \)-adrenergic regulation.23,24 Because \( Ca^{2+} \) enters in cells during the action potential plateau, changes in APD may affect the amount of \( Ca^{2+} \) entry, and thereby \( [Ca^{2+}]_i \). Indeed, Padmala and Demir reported that the 40% reduction in APD results in 60% reduction in \( Ca^{2+} \) entry through \( I_{Ca,L} \). 23% reduction in peak \( [Ca^{2+}]_i \), and 18% reduction in diastolic \( [Ca^{2+}]_i \). Thus, \( Ca^{2+} \)-induced APD shortening potentially acts as a negative feedback system to maintain \( [Ca^{2+}]_i \), within an appropriate range.

Cardiomyocytes express both NOS1 and NOS3.19,21 The finding that \( t \)-NIO, a relatively specific NOS3 inhibitor, completely abolished \( I_{Ks} \) enhancement by A23187, whereas SMTc, a relatively specific NOS1 inhibitor, barely affected it, strongly suggests that the activation of NOS3, rather than NOS1, is involved in the \( I_{Ks} \) enhancement. NOS3 is activated via at least two distinct pathways, a CaM-dependent pathway and an Akt-dependent pathway.17,18 In the present study, \( Ca^{2+} \)-dependent \( I_{Ks} \) enhancement was blocked by a CaM inhibitor, W7, but was barely affected by an Akt inhibitor, SH-6, indicating that a CaM-dependent activation of NOS3 participates in this phenomenon. We have previously reported that \( I_{Ks} \) is enhanced by intracellular \( Ca^{2+} \), which may be consequences of allosteric interaction of CaM rather than phosphorylation by CaM kinase II, because a CaM inhibitor, W7, but not a CaM kinase II inhibitor, KN62, inhibited the \( Ca^{2+} \)-induced \( I_{Ks} \) enhancement.5 This hypothesis is supported by our immunoprecipitation-immunoblotting data that the elevation of \( [Ca^{2+}]_i \) by A23187 switched the protein-protein interaction from NOS3-Cav-3 to NOS3-CaM. In cardiomyocytes, NOS1 and NOS3 have opposing actions in \( Ca^{2+} \) cycling: NO released from NOS3 decreases \( [Ca^{2+}]_i \) by suppressing the \( I_{Ca,L} \) channel activities, whereas NO released from NOS1 increases \( [Ca^{2+}]_i \), by activating ryanodine receptor type 2.19,21 The opposing actions of NOS1 and NOS3 in \( Ca^{2+} \) cycling are critically dependent on their spatial confinement with effector molecules: the ryanodine receptor type 2 and NOS 1 on endoplasmic reticulum and the \( I_{Ca,L} \) channel and NOS3 on sarcoclemma.19,21 The \( Ca^{2+} \)-induced enhancement of \( I_{Ks} \) can be observed in cell-free excised patch configuration,6 implicating that the spatial confinement of NOS3 with the \( I_{Ks} \) channel also takes place. Interestingly, the \( \alpha \) subunit of the \( I_{Ks} \) channel, KCNQ1, has two potential binding sites for \( Ca^{2+} \)-free CaM (apo-CaM), an IQ motif and a \( \Phi \) motif, in its intracellular C-terminal region.27 Yeast two-hybrid assay also suggests that KCNQ1 associates with apo-CaM.27 Thus, the changes in protein-protein interactions may occur in the membrane-delimited microdomains that involve Cav-3, NOS3, CaM, and KCNQ1. Because of participation of Cav-3, microdomains are likely to be in the caveola. Although the \( I_{Ks} \) channel has been suggested to colocalize with the \( \beta_3 \)-adrenergic receptor that accumulates in caveola,28 the direct demonstration of localization of components of the \( I_{Ks} \) channel, KCNQ1 and KCNE1, with the caveola remains to be shown.

Acknowledgments

This work was supported in part by Research Grant from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and a research grant from the Takeda Science Foundation. We thank Dr. Brian Delisle (University of Wisconsin) for checking English, K. Yoshida for the technical assistance, and A. Sugai for the secretarial services.

References

19. Barouch LA, Harrison RW, Skaf MW, Rosas GO, Cappola TP, Kobeissi ZA, Hobiak JA, Lemmon CA, Burnett AL, O’Rourke B, Rodoriguez ER, Huang PL, Lima JA, Berkowitz DE, Hare JM. Nitric oxide regulates the...


Role of Nitric Oxide in Ca^{2+} Sensitivity of the Slowly Activating Delayed Rectifier K^{+} Current in Cardiac Myocytes
Chang-Xi Bai, Iyuki Namekata, Junko Kurokawa, Hikaru Tanaka, Koki Shigenobu and Tetsushi Furukawa

Circ Res. 2005;96:64-72; originally published online November 29, 2004; doi: 10.1161/01.RES.0000151846.19788.E0
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

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/96/1/64

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2004/12/28/96.1.64.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
Supplementary data

Materials and methods

Quantification of \([\text{Ca}^{2+}]_i\)

Quantification of \([\text{Ca}^{2+}]_i\) was performed with fluorescence microscopy. Ventricular myocytes isolated from adult guinea pig hearts (n=5, white Hartrey) were plated on coverslips attached to an experimental chamber and preincubated for 45 min with 10 \(\mu\text{mol/L}\) indo-1-AM (Dojin, Kumamoto, Japan). After incubation, experimental chamber was placed on an epifluorescence microscope (Olympus IX70, Tokyo, Japan), and cells were washed by perfusion with the Tyrode’s solution of the following composition: 143 mmol/L NaCl, 0.33 mmol/L NaH_2PO_4, 4 mmol/L KCl, 1.8 mmol/L CaCl_2, 1.0 mmol/L MgCl_2, 5.5 mmol/L glucose and 5.0 mmol/L HEPES, and pH was adjusted to 7.4 with NaOH. The solution was gassed with 100% O_2 and warmed at 36 °C. To evoke Ca^{2+} transients, cells were field-stimulated at 1 Hz through platinum wire pairs with rectangular current pulses of 3 msec duration generated by an electric stimulator (SEN-3303, Nihon Kohden, Tokyo, Japan). Cells were excited at 360 nm from a Xenon lamp and the emission bands, 395-415 nm and 470-490 nm, were separated (W-VIEW system, Hamamatsu Photonics, Hamamatsu, Japan), detected by a high-speed cooled CCD camera (HISCA, Hamamatsu Photonics) at a time resolution of 1.95 ms, and ratio of the intensity of two emission bands was calculated after correction of background fluorescence (Aquacosmos software, Hamamatsu Photonics). In situ calibration of indo-1 fluorescence ratio values to intracellular Ca^{2+} concentration was performed as described previously (Tanaka, H., et al. Jpn. J. Pharmacol. 1996;70:235-242).

Patch-clamp

Single ventricular myocytes were harvested from hearts of adult guinea pigs (n=41, white Hartrey). Action potentials and membrane currents were recorded using the perforated configuration of the patch-clamp technique with an Axopatch 200B amplifier (Axon Instruments, Union City, CA), in a current-clamp mode and a voltage-clamp mode. Action potentials were elicited by passing depolarizing current pulses (<2 ms in duration at a rate of 1 Hz) of suprathreshold intensity. I_{Ks} were elicited by 3.5 s-depolarizing pulses to various test potentials
between –30 mV and +50 mV in 10 mV increments at 0.1 Hz from a holding potential ($V_h$) of –40 mV, and amplitudes of tail $I_{\text{k}}$ were measured by extrapolating from exponential fits. To elicit $I_{\text{Ca,L}}$, pre-pulse (100 ms) was applied to –40 mV from a holding potential of –80 mV to inactivate the Na$^+$ channels and the T-type Ca$^{2+}$ channels, following a 200-ms test pulse to 0 mV at 1 Hz. All experiments were performed at 36±1 °C.

Standard pipette solution contained 110 mmol/L aspartic acid, 30 mmol/L KCl, 5.0 mmol/L Mg-ATP, 5.0 mmol/L creatine phosphate dipotassium salt, and 5.0 mmol/L HEPES, and pH was adjusted to 7.4 with KOH. Amphotericin B (Sigma-Aldrich, St. Louis, MO) was used in the pipette solution to achieve patch perforation. In Ca$^{2+}$ buffering experiments, we added 10 mmol/L EGTA to the pipette solution. We front-filled patch pipettes by dipping them into the pipette solution, and then back-filled with the pipette solution containing amphotericin B (600 µg/ml). External (bath) solution used for recording of action potentials was the Tyrode’s solution that consisted of 135 mmol/L NaCl, 0.33 mmol/L NaH$_2$PO$_4$, 5.4 mmol/L KCl, 1.8 mmol/L CaCl$_2$, 0.53 mmol/L MgCl$_2$, 5.5 mmol/L glucose and 5.0 mmol/L HEPES, and pH was adjusted to 7.4 with NaOH. External solution used for recording of $I_{\text{k}}$ was the K$^+$-free solution containing 135 mmol/L NaCl, 0.33 mmol/L NaH$_2$PO$_4$, 1.8 mmol/L CaCl$_2$, 0.53 mmol/L MgCl$_2$, 5.5 mmol/L glucose and 5.0 mmol/L HEPES (pH 7.4 with NaOH), which is known to suppress $I_{\text{Kr}}$ and enhance $I_{\text{k}}$ (Sanguinetti, M.C. & Jurkiewicz, N.K., Pflugers Arch. 1992;420:180-186). We added nisoldipine (3 µmol/L) and E-4031 (10 µmol/L), drugs that selectively block $I_{\text{Ca,L}}$ and $I_{\text{k}}$ channels, to the bath solution. External solution used for recording $I_{\text{Ca,L}}$ contained 140 mmol/L TEA-Cl, 0.53 mmol/L MgCl$_2$, 10 mmol/L glucose, 2 mmol/L CaCl$_2$, and 10 mmol/L HEPES (pH 7.4 with TEA-OH).

**Immunoprecipitation and immunoblotting**

Cardiomyocytes were isolated from adult guinea pigs (n=3). Cells were lysed in RIPA buffer (150 mmol/L NaCl, 20 mmol/L Tris-HCl [pH 7.4], 2 mmol/L EDTA, 1% (v/v) Nonidet P-40, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1 mmol/L phenylmethylsulfonyl fluoride, and 10 µg/ml of each aprotinin, leupeptin, and peptatin) at 4 °C for 20 min. Cell lysates were cleared by centrifugation at 15,000 rpm for 30 min at 4 °C and lysates containing 500 µg proteins were presorbed by incubation with 20 µl of protein G-Sepharose...
supernatant was incubated with 2 µg/ml of a monoclonal anti-NOS3 antibody (Zymed, South San Francisco, CA) and 15 µl of protein G-Sepharose overnight at 4 °C. Protein-antibody complexes were washed five times with the RIPA buffer, eluted with the SDS reducing sample buffer containing 62.5 mmol/L Tris-HCl (pH 6.8), 10% (v/v) glycerol, 2% (w/v) SDS, 0.64 mol/L 2-mercaptoethanol, and 0.05% (w/v) bromophenol blue, and subjected to immunoblot analysis by incubation with a 1:20,000-diluted anti-NOS3 antibody (Zymed), a 1:20,000-diluted polyclonal anti-caveolin-3 (Cav-3) antibody (N-18, Santa Cruz Biotechnology, Santa Cruz, CA), or a 1:20,000-diluted polyclonal anti-CaM antibody (FL-149, Santa Cruz Biotechnology) followed by incubation with a 1:40,000-diluted horseradish peroxidase-conjugated anti-mouse IgG (DAKO JAPAN Co. Ltd., Kyoto, Japan) or an anti-rabbit IgG (DAKO JAPAN Co. Ltd.). For pre-absorption experiments, membrane was incubated with the primary antibody that had been incubated with 1 µg of human partial caveolin-3 corresponding to 1Met–109Ile (Bio-Clone, San Diego, CA) or 1 µg of human full-length calmodulin (Merck, Munich Germany) overnight at 4 °C. Proteins were detected using the advance enhanced chemiluminescence system (Amersham Biosciences). Protein concentrations were determined using the bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL). Densitometric analysis was performed using a free software NIH Image.

Legends for supplementary figures

Fig. 1. Dose-dependency of APD shortening and I_Ks enhancement by A23187.

A. Left panel shows representative action potential traces in the absence of A23187 (●) or in the presence A23187 at 0.5 µmol/L (○), 2 µmol/L (▲), or 10 µmol/L (△). Right panel shows dose-response curves of shortening of APD20 and APD90 by A23187 in 5 experiments, respectively. Continuous line is the result of Hill’s fitting in the form of:

Relative current=A/{1+(EC_{50}×[A23187])^n}^{-1}

where A is the maximum response, and n is the Hill coefficient. The obtained EC_{50} value was
0.65±0.05 μmol/L for APD<sub>20</sub> and 0.52±0.06 μmol/L for APD<sub>90</sub>. <b>B</b>, Left panel shows representative I<sub>K<sub>s</sub></sub> traces in the absence of A23187 (●) or in the presence of A23187 at 0.5 μmol/L (○), 2 μmol/L (▲), or 10 μmol/L (△). Right panel shows a dose-response curve of I<sub>K<sub>s</sub></sub> enhancement by A23187 in 5 experiments. Continuous line is the result of Hill’s fitting, and the obtained EC<sub>50</sub> value was 0.45±0.55 μmol/L.

**Fig. 2.** Effects of EGTA on A23187-induced APD shortening and I<sub>K<sub>s</sub></sub> enhancement.

Addition of EGTA at 10 mmol/L to the pipette solution abolished shortening of APD (panel A), and enhancement of I<sub>K<sub>s</sub></sub> (panel B) by A23187.

**Fig. 3.** Effects of A23187 and SNP on the gating kinetics of I<sub>K<sub>s</sub></sub>.

**A,** To examine voltage-dependence of I<sub>K<sub>s</sub></sub> activation, the normalized peak tail I<sub>K<sub>s</sub></sub> amplitude was plotted against <i>V<sub>t</sub></i>, and was fit with the Boltzmann’s function in the form of:

\[
\frac{I_{K_s}}{I_{K_s,max}} = \frac{1}{1 + \exp \left[ \left( V_{1/2} - V_t \right)/s \right]}
\]

where <i>V<sub>1/2</sub></i> is the membrane voltage at which maximal activation is 50%, <i>V<sub>t</sub></i> is the test potential, and <i>s</i> is the slope factor. Panel <i>a</i> is the result for A23187, and panel <i>b</i> for SNP. A23187 (2 μmol/L) reversibly shifted <i>V<sub>1/2</sub></i> in the negative direction: <i>V<sub>1/2</sub></i> was +20.9±1.1 mV in the control condition, +16.2±0.8 mV after A23187 application (n=5, <i>p</i>&lt;0.05 versus control), and +19.5±0.7 mV after washout (n = 5, <i>p</i>=ns versus control) (panel <i>a</i>). Similarly, SNP (1 mmol/L) reversibly shifted <i>V<sub>1/2</sub></i> in the negative direction: <i>V<sub>1/2</sub></i> was +19.6±1.3 mV in the control condition, +14.8±1.1 mV after SNP application (n=5, <i>p</i>&lt;0.05 versus control), and +18.1±1.6 mV after washout (n = 5, <i>p</i>=ns versus control). <b>B,** The time courses of activation and deactivation of I<sub>K<sub>s</sub></sub> were examined as previously reported (Furukawa, T., et al. Circ. Res. 1992;70: 91-103.). The time course of activation was not significantly affected by A23187 or SNP (data not shown). The time course of I<sub>K<sub>s</sub></sub> deactivation was best described as a double exponential process (with a fast component [panels <i>a</i>, <i>c</i>] and a slow component [panels <i>b</i>, <i>d</i>]). Panel <i>a</i> and <i>b</i> are results for A23187 and panels <i>c</i> and <i>d</i> for SNP. *.<i>p</i>&lt;0.05. The <i>τ<sub>fast</sub></i> (at <i>V<sub>t</sub></i> between −70 mV and −30 mV)
and $\tau_{slow}$ (at $V_{t}$ between $-50$ mV and $-20$ mV) were similarly slowed by A23187 (panel a, b) and SNP (panel c, d). The $\tau_{fast}$ at $-40$ mV slowed from $137 \pm 21$ msec to $394 \pm 40$ msec by A23187 ($n=5, p<0.01$) and from $157 \pm 23$ msec to $415 \pm 27$ msec by SNP ($n=5, p<0.01$). The $\tau_{slow}$ at $-20$ mV slowed from $1.83 \pm 0.44$ s to $3.50 \pm 0.48$ s by A23187 ($n=5, p<0.01$) and from $2.17 \pm 0.30$ s to $3.53 \pm 0.31$ s by SNP ($n=5, p<0.01$).

Fig. 4. Effects of digoxin on APD and $I_{Ks}$.

A, Effects of digoxin at 0.2 $\mu$mol/L on APD, and its reversal by L-NAC (1 mmol/L). B, Effects of digoxin at 2 $\mu$mol/L on APD, and its reversal by L-NAC (1 mmol/L). C, Effects of digoxin at 0.2 $\mu$mol/L on $I_{Ks}$, and its reversal by L-NAC (1 mmol/L). D, Effects of digoxin at 2 $\mu$mol/L on $I_{Ks}$, and its reversal by L-NAC (1 mmol/L).

Fig. 5. Effects of rise in $[Ca^{2+}]_{o}$ on APD and $I_{Ks}$.

A, Effects of rise in $[Ca^{2+}]_{o}$ from the control state (1.8 $\mu$mol/L) to 2.4 $\mu$mol/L, and to 3.0 $\mu$mol/L on APD, and its reversal by L-NAC (1 mmol/L). B Effects of rise in $[Ca^{2+}]_{o}$ from the control state (1.8 $\mu$mol/L) to 2.4 $\mu$mol/L, and to 3.0 $\mu$mol/L on $I_{Ks}$, and its reversal by L-NAC (1 mmol/L).

Fig. 6. Time course of changes in $[Ca^{2+}]_{i}$ after application of A23187.

$[Ca^{2+}]_{i}$ in the basal state (closed circles) and at the peak of Ca$^{2+}$ transient at various times after application of A23187 at 2 $\mu$mol/L. Numbers in parentheses indicate number of experiments. *: $p<0.05$ versus before A23187 application.

Fig. 7. Effects of a NO-scavenger, L-NAC, and a CaM inhibitor, W7, on $I_{Ca,L}$ suppression by A23187.

A, Application of A23187 (2 $\mu$mol/L) suppressed peak inward currents of $I_{Ca,L}$, which was not affected by a NO scavenger, L-NAC (1 mmol/L) (●), but was reversed by a CaM inhibitor, W7 (10 $\mu$mol/L) (○). B, The % changes in $I_{Ca,L}$ in the presence of A23187 alone (black bars) or in the presence of A23187 with L-NAC or W7 (hatched bars). Numbers in parentheses indicate number of experiments. *$p<0.05$, #.$p=ns$. C, D, Representative recordings of $I_{Ca,L}$ in the control state (trace a), in the presence
of A23187 (trace \textit{b}), and in the presence of A23187 with L-NAC (panel \textit{C}) or W7 (panel \textit{D}) (trace \textit{e}).
Supplementary figure 1
Supplementary figure 2
Supplementary figure 4
Supplementary figure 5
Supplementary figure 6
Supplementary figure 7