Effects of pinacidil on coronary Ca$^{2+}$-myosin phosphorylation in cold potassium cardioplegia model

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Matsuda, Naruto, Kathleen G. Morgan, and Frank W. Sellke. Effects of pinacidil on coronary Ca$^{2+}$-myosin phosphorylation in cold potassium cardioplegia model. Am J Physiol Heart Circ Physiol 279: H882–H888, 2000.—The effects of the potassium (K$^+$) channel opener pinacidil (Pin) on the coronary smooth muscle Ca$^{2+}$-myosin light chain (MLC) phosphorylation pathway under hypothermic K$^+$ cardioplegia were determined by use of an in vitro microvessel model. Rat coronary arterioles (100–260 μm in diameter) were subjected to 60 min of simulated hypothermic (20°C) K$^+$ cardioplegic solutions (K$^+$ = 25 mM). We first characterized the time course of changes in intracellular Ca$^{2+}$ concentration, MLC phosphorylation, and diameter and observed that the K$^+$ cardioplegia-related vasoconstriction was associated with an activation of the Ca$^{2+}$-MLC phosphorylation pathway. Supplementation with Pin effectively suppressed the Ca$^{2+}$ accumulation and MLC phosphorylation in a dose-dependent manner and subsequently maintained a small decrease in vasomotor tone. The ATP-sensitive K$^+$ (K$_{ATP}$)-channel blocker glibenclamide, but not the nitric oxide (NO) synthase inhibitor N$^ω$-nitro-l-arginine methyl ester, significantly inhibited the effect of Pin. K$^+$ cardioplegia augments the coronary Ca$^{2+}$-MLC pathway and results in vasoorstriction. Pin effectively prevents the activation of this pathway and maintains adequate vasorelaxation during K$^+$ cardioplegia through a K$_{ATP}$-channel mechanism not coupled with the endothelium-derived NO signaling cascade.

coronary vasoconstriction; vascular smooth muscle; potassium channel

COLD POTASSIUM CARDIOPLEGIA (usually a K$^+$ concentration of 15–25 mM), whether crystalloid or blood containing, is the most common means of myocardial preservation and has proved to be an essential component of modern cardiac operations. Although protection of cardiac function and myocardial perfusion has been improved, suboptimal cardiac performance still occurs postoperatively in a small set of patients, especially when operations are performed on acutely ischemic patients. A reason for this myocardial dysfunction may be related to coronary vasoconstriction during or after cardiac operations in which the administration of K$^+$ cardioplegia has been performed (18). Although the pathogenesis of vasospasm is not fully understood, altered vasomotor regulation in coronary smooth muscle associated with prolonged exposure to K$^+$ cardioplegia seems to be one of the essential events in this setting. Recent works have shown that K$^+$ cardioplegia causes a marked change in the coronary smooth muscle intracellular calcium concentration ([Ca$^{2+}$]) (8, 23). In addition, it has been reported that enhanced phosphorylation of the myosin light chain (MLC) in vascular smooth muscle could play a central role in the pathogenesis of coronary vasoconstriction (6).

In an effort to improve on the shortcoming of high-K$^+$ cardioplegic solutions, K$^+$ channel openers have received more attention. To date, several studies have demonstrated that supplementation of K$^+$ channel openers improved the cardioprotective effects afforded by K$^+$ cardioplegia (4, 7). One important factor that may contribute to this beneficial phenomenon is possibly related to the K$^+$ channel opener-evoked coronary vasorelaxation. However, there is little information available regarding the molecular and cellular mechanisms, particularly the role of Ca$^{2+}$, and the sequence of signaling events such as MLC phosphorylation underlying the development of vasoprotection. For this reason, the present study was designed to investigate the effects of supplementation of pinacidil, a widely used K$^+$ channel opener, on the coronary vasomotor regulation. Specifically, the relationship among coronary myoplasmic Ca$^{2+}$, phosphorylation of MLC, and lumen diameter was examined with the use of an in vitro coronary microvessel model.

MATERIALS AND METHODS

Experimental Techniques

Isolated arteriole preparations. Male Sprague-Dawley rats (250–350 g) were anesthetized with chloroform. Heparin sodium (1,000 IU/kg) was then administered intravenously. The heart was removed into cold (4°C) Krebs physiological saline solution (Krebs-PSS), which consisted of the following ionic concentrations (in mM): 119.0 NaCl, 25.0 NaHCO$_3$, 5.0 KCl, 1.2 NaH$_2$PO$_4$, 1.2 MgSO$_4$, 1.8 CaCl$_2$, and 11.0 glucose.

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Immediately, coronary arterioles (100–260 µm in diameter) were dissected from the left anterior descending artery-dependent subepicardial region in the left ventricle with the use of a ×10–60 dissecting microscope (Olympus Optical, Tokyo, Japan). During dissection, care was taken to remove as much of the surrounding myocardium as possible, and the endothelium was intentionally preserved to maintain the vascular integrity. Vessels were transferred to an experimental chamber in which both ends of the microvessel were cannulated with dual glass micropipettes (tip interior diameter ~60 µm) and secured with 10-0 nylon monofilament suture (Ethicon, Somerville, NJ). The chamber was mounted on a transillumination system, and oxygenated (95% O₂-5% CO₂) Krebs-PSS (37°C) was continuously circulated through the vessel chamber. All of the animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Research Council.

**Measurements of intraluminal diameter.** The vessels were pressurized to 40 mmHg in a no-flow state with the use of a burette manometer filled with Krebs-PSS. The internal luminal diameter was measured with a microscopic image system (Zeiss IM35 and Hitachi CCD TV camera, model KP-115). The calibration of the measurement was performed by use of an 80-µm tungsten wire. The changes in internal luminal diameter were expressed as percent contraction of the baseline diameter.

**Measurements of [Ca²⁺]i.** Coronal smooth muscle was measured by the method described by Meisinger et al. (9), an approach we have previously used (8, 23). Coronary arterioles in the vessel chamber were loaded with 5 µM fura 2-AM (acetoxymethyl ester of fura 2) in Krebs-PSS containing 0.05% DMSO and 0.01% pluronic-F-127. In the preliminary experiments, we confirmed that these loading vehicles do not affect the coronary microvascular reactivity. The loading time was 45 min at room temperature (21–23°C) followed by a 30-min wash period at 37°C. The objective lens used was a Nikon Fluor ×20 (numerical aperture 0.75). Optimal focus was adjusted by the viewing of smooth muscle layer under bright-field microscopy. Excitation light at 350 ± 5 and 390 ± 6 nm was used. Emission at 510 ± 24 nm was monitored with a photomultiplier tube (Hamamatsu R928), digitized by a data acquisition analog-to-digital converter, and processed by use of DTView version 3.0 (Data Translation). [Ca²⁺]i was estimated from the ratio (R) of measured fluorescence signals (F) elicited at two wavelengths according to the following equation: 

\[
R = \frac{F_{350}(mv)}{F_{390}(mv)}
\]

where \(F_{350}(mv)\) and \(F_{390}(mv)\) are the total measured fluorescence of the vessels at wavelengths of 350 and 390 nm, respectively, and \(F_{350}(bg)\) and \(F_{390}(bg)\) are the background fluorescence signals at each respective wavelength. Background signals were measured on vessels before the loading of fura 2-AM. In the preliminary experiments, the background autofluorescence intensities measured in the absence of fura 2 were not significantly altered by any of our experimental interventions, including administration of drugs and hypoxia. Despite the undoubted benefit of fura 2 in evaluating relative changes in [Ca²⁺]i, it has become clear that there are a number of uncertainties in precise quantification of absolute [Ca²⁺]i, (2), and its dissociation constant (\(K_d\)) value is temperature sensitive (17). Therefore, we used the ratio of measured fluorescence signals elicited at wavelengths of 350 and 390 nm (R(350/390)) as an index of [Ca²⁺]i. As is well known, a ratioimetric analysis of fura 2 is not linear to the absolute [Ca²⁺]i; however, this makes it possible to evaluate relative changes in [Ca²⁺]i, independently of dye concentration, path length, excitation intensity, and photobleaching. Thus the

**Measurements of MLC phosphorylation.** Phosphorylation of 20-kDa MLC was measured by the method using glycerol-urea minigels (22). At specific time points during the experimental protocol, the vessels were rapidly removed from the experimental apparatus and immediately frozen (within 3 s) by immersion for 60 min in an acetone dry-ice slurry containing 10% TCA and 10 mM dithiothreitol (DTT). Frozen vessels were gradually warmed up to room temperature, followed by three rinses with acetone containing 5 mM DTT to remove TCA, and then stored at ~80°C before use. In general, pooled segments of three vessels (6–8 mm in total length) subjected to a given protocol were combined for one phosphorylation measurement. The samples were suspended in 20 µl of urea sample buffer [8.0 M urea, 20 mM Tris base, 23 mM glycine (pH 8.6), 10 mM DTT, 10% glycerol, and 0.04% bromphenol blue], applied to glycerol-urea minigels (10% acrylamide-0.8% bisacrylamide, 40% glycerol, 20 mM Tris base, and 23 mM glycine), and subjected to electrophoresis at a constant voltage of 400 V until the dye front ran off. Electrophoretic transfer of proteins from the gels onto polyvinylidene difluoride membranes was carried out. The membrane was blocked in 5% milk solution for 30 min and then incubated overnight with a 20-kDa MLC antibody (Sigma). We preliminarily confirmed that this monoclonal antibody could not show any reactivity to myocardial tissue in the absence of coronary vessels. The blot was then placed in an anti-mouse IgG (goat) conjugated with horseradish peroxidase (Calbiochem) and visualized with chemiluminescence (Super Signal; Pierce, Rockford, IL). The MLC bands were quantitated densitometrically with the use of NIH Image, and the MLC phosphorylation levels were expressed as the area of phosphorylated MLC divided by the total area of 20-kDa MLC times 100%. Care was taken to ensure that bands subjected to densitometry were not saturated.

**Experimental Design.** In an attempt to simulate the clinically relevant condition with nonoxygenated hypothermic crystalloid hyperkalemic cardioplegia in the operating room, an in vitro microvessel model was designed. Normal Krebs solution was maintained within the vessels during exposure to cardioplegia. All cardioplegic solutions were applied into the vessel chamber. To confirm that vasoactive agent administered extraluminally has a direct effect on the endothelium smooth muscle interactions of isolated coronary microvessels, we preliminarily performed vascular function studies. Microvessels (100–180 µm in diameter, n = 6 in each group) were divided into two groups according to the drug administration routes: 1) extraluminal alone and 2) both intraluminal and extraluminal. All vessels were studied in a pressurized (40 mmHg), static (no-flow), normothermic (37°C) state. After baseline diameter measurements were taken, a stable thromboxane A₂ analog U-46619 (10⁻⁷ M), which is a receptor-mediated direct smooth muscle constrictor, was applied by each route. The contractile responses to U-46619 measured after stabilization (generally 5–10 min) were 32.5 ± 3.1 and 31.1 ± 4.2%, respectively (% contraction of baseline diameter, P > 0.05 between groups). An endothelium-dependent vasodilator, ADP (10⁻⁴ M), was then coadministered with U-46619 by each route. ADP effectively reduced these U-46619-elicited contractions to 7.3 ± 3.0 and 5.1 ± 2.1%, respectively (% contraction of baseline diameter, P > 0.05 between groups). These findings indicated that extraluminally administered
Data Analysis

The responses of coronary vessels to only one protocol were examined once in each vessel. Six vessels were studied for the simultaneous [Ca$$^{2+}$$], diameter measurements in each group, and separate vessels not loaded with fura 2 were prepared for the MLC phosphorylation. Results were expressed as means ± SE. ANOVA combined with a multiple comparison Fisher’s test was used to test the differences among groups with different interventions (Abacus Concepts, Berkeley, CA). The probability was considered to be significant at P < 0.05.

RESULTS

Time-Course Studies

First, we sought to clarify the time-course changes in the coronary myoplasmic [Ca$$^{2+}$$]i, MLC phosphorylation, and vascular diameter in the control and 200 μM Pin-supplemented groups as a reference of this model for subsequent experiments. As shown in Fig. 1A, [Ca$$^{2+}$$]i in the control group increased gradually in a time-dependent manner, reached a peak at 30 min, and maintained the peak value through the end point of a 60-min cardioplegic period. It returned to the baseline value after reperfusion. As shown in Fig. 1B, the phosphorylation levels of MLC in the control group rapidly increased, reached the peak at 3 min, and thereafter decreased but remained at suprabasal levels during the cardioplegic period. The time-course dynamics of the [Ca$$^{2+}$$]i and MLC phosphorylation in the 200 μM Pin group were similar to those of the control group, but Pin significantly decreased the sustained levels of these two indexes during the cardioplegic period. As depicted in Fig. 1C, at the start of the hypothermic cardioplegic period, control vessels showed a transient relaxation that was followed by a gradual contraction; Pin vessels maintained the initial relaxation.

Dose Dependency of Pin

On the basis of the observations described above, in subsequent studies we focused on the responses at the end of the 60-min cardioplegic period. The data are summarized in Fig. 2. Pin supplementation at concentrations of 10, 50, and 200 μM significantly decreased the myoplasmic Ca$$^{2+}$$ accumulation in a dose-dependent manner. Pin at 50 or 200 μM significantly decreased the level of MLC phosphorylation (both P < 0.05 vs. control) and consequently inhibited the cardioplegia-induced contraction (both P < 0.05 vs. control). Nic (10 μM) produced findings similar to those of ≥50 μM Pin.

Antagonism by Glib

Coadministration of a specific KATP channel blocker Glib (10 μM) with Pin (200 μM) significantly blocked those effects of Pin supplementation (all indexes P < 0.05 vs. 200 μM Pin group), suggesting that KATP channels are involved in the mechanism(s) mediating the vasodilative action of Pin.

Minimal Effect of L-NAME

Coadministration of an NO synthase inhibitor, L-NAME (10 μM), with Pin (200 μM) did not make any significant changes on the [Ca$$^{2+}$$]i, the MLC phosphorylation, or the relaxation observed in the 200 μM Pin group (all indexes P > 0.05 vs. 200 μM Pin group), indicating that, in this setting, the contribution of the endothelium-derived NO to the Pin-evoked relaxation is minimal.

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DISCUSSION

The major findings of the present study were that 1) K⁺ cardioplegia-induced coronary constriction is associated with an elevation of \([\text{Ca}^{2+}]_i\) and an increase in the level of MLC phosphorylation; 2) this potentiation of the \(\text{Ca}^{2+}\)-MLC phosphorylation pathway was inhibited by supplementation of Pin in a dose-dependent manner; 3) a selective blocker of K$_{\text{ATP}}$ channel, Glib, antagonized the effects of Pin; 4) the ability of Pin to attenuate the \(\text{Ca}^{2+}\)-MLC phosphorylation cascade was noted, regardless of the activity of NO synthase; and 5) Nic produced findings similar to those of Pin.

Alteration of the \(\text{Ca}^{2+}\)-MLC Phosphorylation Pathway in Coronary Arterioles

It is generally accepted that the major determinant of vascular smooth muscle contractility is the increase in \([\text{Ca}^{2+}]_i\) via sarcolemmal channels or sarcoplasmic reticulum, which binds calmodulin and subsequently increases MLC kinase activity and phosphorylation of the 20-kDa regulatory MLC (3, 10, 19, 25). MLC phosphorylation results in an increased actomyosin Mg$^{2+}$-ATPase activity and provides the chemical energy for force production under a variety of stimulus conditions (15). The present results provided experimental evidence that K⁺ cardioplegia activates this \(\text{Ca}^{2+}\)-MLC phosphorylation pathway and causes vascular contraction.

Several factors probably modulate the coronary myoplasmic \(\text{Ca}^{2+}\) regulation during hypothermic K⁺ cardioplegia. It has been suggested that extracellular high K⁺-induced membrane depolarization promotes \(\text{Ca}^{2+}\) influx through voltage-operated \(\text{Ca}^{2+}\) channels, which can also lead to the release of \(\text{Ca}^{2+}\) from intracellular \(\text{Ca}^{2+}\) stores (1). In fact, in the present study, we demonstrated the potential role of the voltage-operated \(\text{Ca}^{2+}\) channels in the response to K⁺ cardioplegia using Nic, a selective L-type \(\text{Ca}^{2+}\) channel blocker. In addition, during surgical cardioplegia, especially nonoxygenated crystalloid cardioplegia, the coronary smooth muscle is exposed to conditions of insufficient oxygen supply, with a lower production of ATP compared with a normal state. It is widely recognized that hypoxia causes \([\text{Ca}^{2+}]_i\) elevation in various cell types. A detailed discussion of the mechanism of hypoxia-related
Ca\textsuperscript{2+} accumulation is beyond this brief review, but several intracellular organelles that have ATP-regulated Ca\textsuperscript{2+}-handling mechanisms, i.e., the sarcoplasmic reticulum and mitochondria, may be considered as candidates for the Ca\textsuperscript{2+} release (3). Moreover, a number of thermosensitive Ca\textsuperscript{2+} transport systems may be modified in a hypothermic condition (11, 20, 24). We previously evaluated the individual effects of extracellular K\textsuperscript{+} concentration and temperature on the intracellular Ca\textsuperscript{2+} homeostasis during cardioplegia by use of this model (23). We observed that high-K\textsuperscript{+} concentration (25 mM) itself caused a marked increase in [Ca\textsuperscript{2+}]i, and this high K\textsuperscript{+}-induced intracellular Ca\textsuperscript{2+} accumulation was attenuated in Ca\textsuperscript{2+}-free hypothermia (4°C) but was minimally affected in hypothermia in the presence of extracellular Ca\textsuperscript{2+} (1 mM). Thus the
net effects of cold K$^+$ cardioplegia on the intracellular Ca$^{2+}$ regulation seem to be complex and interactive and need to be clarified in further studies.

Exposure to the K$^+$ cardioplegia resulted in a transient elevation of MLC phosphorylation levels, which remained at significantly suprabasal levels during the 60-min cardioplegic period. This pattern of MLC phosphorylation is similar to that previously reported in swine carotid arteriolar smooth muscle with high-K$^+$ stimulation (14), which might be consistent with the muscle entering the "latch" state, a condition whereby force generation is maintained by attached, slowly cycling cross bridges (15). The lower, but sustained, phosphorylation level during cardioplegia might be explained by the fact that the increase in [Ca$^{2+}$], is maintained and will continue to increase the MLC kinase activity relative to phosphatase activity (22).

**Effects of Pin Supplementation on the Vasomotor Regulation**

Pin effectively prevented the cardioplegia-induced Ca$^{2+}$ accumulation in a dose-dependent manner at concentrations between 10 and 200 $\mu$M. Furthermore, at concentrations >50 $\mu$M, Pin attenuated the MLC phosphorylation, which resulted in prevention of the cardioplegia-induced vasoconstriction. K$^+$ channels are known to be important in regulating the membrane potential of arterial smooth muscle (12, 13). Although we have not examined the membrane potential of vascular smooth muscle, the present findings are consistent with the previous report that opening of K$^+$ channels shifts the resting membrane potential of vascular smooth muscle to the negative direction (hyperpolarization), leading to suppression of the inward Ca$^{2+}$ current through voltage-operated Ca$^{2+}$ channels and subsequently to vasorelaxation.

In line with the membrane potential-regulated influences on the Ca$^{2+}$ influx, it has been reported that membrane hyperpolarization induced by a K$^+$ channel opener, cromakalim, inhibits not only the sarcolemmal Ca$^{2+}$ influx but also the inositol 1,4,5-trisphosphate-induced Ca$^{2+}$ release from sarcoplasmic reticulum in porcine coronary artery (26). In addition, there is also a report that demonstrated that a K$^+$ channel opener, nicorandil, directly stimulated the synthesis of cGMP in vascular smooth muscle, which consequently reduces [Ca$^{2+}$], because of acceleration of Ca$^{2+}$ extrusion via the sarcolemmal Ca$^{2+}$ pump, and may prevent the MLC phosphorylation (21). Thus several mechanisms may underlie the effect of K$^+$ channel openers on preventing the cardioplegia-induced potentiation of the Ca$^{2+}$-MLC phosphorylation pathway.

**Inhibitory Effects of Glib**

To provide a perspective for the mechanistic role of K$_{ATP}$ channels on the cardioplegia-related vascular events, an inhibitory effect of Glib, a selective antagonist of K$_{ATP}$ channels (12, 13), was addressed. As expected, the present study showed that Glib significantly antagonized the effects of Pin on the Ca$^{2+}$-MLC phosphorylation cascade. Therefore, it is reasonable to assume that K$_{ATP}$ channels are involved in the mechanisms mediating the effects of Pin on this signal transduction. These in vitro findings are consistent with a previous in vivo study with dogs that demonstrated that a Pin-evoked increase in the coronary blood flow was reversed by Glib (5). Furthermore, we tested the effects of Glib in the absence of Pin. The lack of effect of Glib alone on the K$^+$ cardioplegia-induced potentiation of the Ca$^{2+}$-MLC phosphorylation pathway suggests that K$_{ATP}$ channels are not efficiently activated in the absence of a K$_{ATP}$ channel-opening drug during cardioplegia.

**Role of Endothelium-Derived NO**

The effect of K$^+$ cardioplegia on the endothelial function has been an unresolved topic. We have previously observed that an endothelium-dependent coronary vasorelaxation is markedly impaired after exposure to K$^+$ cardioplegia (16). Therefore, it would be tempting to speculate that sufficient Pin supplementation could afford endothelial protection. In the present study, we tested whether the vasodilative effect of Pin was mainly mediated through the endothelium-derived NO (EDNO) mechanism. Interestingly, there was no significant effect on the vasodilative responses to Pin in the presence of the NO synthase inhibitor l-NAME compared with those in the absence of l-NAME. Thus our experiments suggest that the action of Pin is not mediated through the EDNO pathway in this setting. However, the endothelium-dependent relaxation and contraction are known to be attributable to a variety of different factors, including not only EDNO but also vasodilative and vasoconstrictive metabolites of arachidonic acid and endothelium-derived hyperpolarizing factor(s), and the nature of these factors has not yet been conclusively identified. Therefore, the possibility still remains that sufficient Pin supplementation could afford endothelial protection. Because the main purpose of this study was not to provide direct evidence about functional implications of the endothelium but to identify the overall effects of Pin supplementation on the vasomotor regulation in the integrated coronary microvasculature, we did not examine the effects of Pin on endothelium-denuded coronary vessels. Thus the coronary endothelium smooth muscle interactions under K$^+$ cardioplegia remain to be elucidated.

**Clinical Implications and Limitations**

The state of myocardial perfusion is mainly regulated by the coronary microvascular tone. The present study was designed to elucidate the mechanisms for the functional alterations in coronary smooth muscle undergoing hypothermic K$^+$ cardioplegia. The findings presented in this model provide some novel information to better understand the cellular mechanisms of cardioplegia-related functional changes in the coronary vasculature and fill the gap between the studies on the perfused heart preparations and those on the isolated vascular rings. In addition, the current study...
may further support a therapeutic approach for cardiac surgery with the K\textsuperscript{+} channel opener Pin to improve coronary vasoprotection.

We acknowledge the potential limitation inherent in our in vitro microvascular model, which is isolated from blood, neural activity, and hormonal influences. It is possible that the effect of Pin on the coronary Ca\textsuperscript{2+}-MLC phosphorylation pathway may be different from warm or tepid cardiopleptic solutions, because temperature substantially modulates ion-channel activity, membrane ATPases, and the activity of a number of enzymes regulating contractile protein interactions (24).

In conclusion, this study clarified that K\textsuperscript{+} cardioplegia augments the Ca\textsuperscript{2+}-MLC pathway in coronary smooth muscle and results in vasoconstriction. Supplementation of >50 µM Pin effectively reduces the intracellular Ca\textsuperscript{2+} accumulation and maintains adequate vasorelaxation during the cardioplegic period through a K\textsubscript{ATP} channel mechanism.

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