DOWNSTREAM DEFECTS IN β -ADRENERGIC SIGNALING AND RELATION TO MYOCYTE CONTRACTILITY AFTER CARDIOPLEGIC ARREST

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Objective: Transient left ventricular dysfunction can occur after hypothermic, hyperkalemic cardioplegic arrest and is associated with decreased β -adrenergic receptor responsiveness. Occupancy of the β -adrenergic receptor activates adenylate cyclase, which phosphorylates the L-type Ca^{2+} channel—enhancing myocyte contractility. The goal of this study was to identify potential mechanisms that contribute to the defects in the β -adrenergic receptor signaling cascade after cardioplegic arrest. **Methods:** Isolated left ventricular porcine myocytes were assigned to one of two treatment groups: (1) cardioplegic arrest (24 mEq/L K^+ , $4^\circ\text{C} \times 2$ hours, then 5 minutes in 37°C cell media; $n = 130$) or (2) normothermic control (cell media, $37^\circ\text{C} \times 2$ hours; $n = 222$). Myocyte contractility was assessed at baseline and after either β -adrenergic receptor occupancy (25 nmol/L isoproterenol [INN: isoprenaline]), activation of adenylate cyclase (0.5 μmol forskolin), or direct activation of the L-type Ca^{2+} -channel (10 nmol/L or 100 nmol/L (–)BayK 8644). **Results:** Myocyte velocity of shortening ($\mu\text{m}/\text{sec}$) was increased with β -adrenergic receptor occupancy or direct adenylate cyclase stimulation compared with baseline in the normothermic group (187.3 ± 6.9 , 181.7 ± 10.2 , and 73.9 ± 2.9 , respectively; $p < 0.0001$) and after cardioplegic arrest (128.6 ± 8.9 , 124.3 ± 9.4 , and 46.1 ± 2.6 , respectively; $p < 0.0001$). However, the response after cardioplegic arrest was significantly reduced compared with normothermic values under all conditions ($p = 0.012$). Direct activation of the L-type Ca^{2+} -channel, which eliminates β -adrenergic receptor–dependent events, increased myocyte contractility in the normothermic group (161.90 ± 12.0 , $p < 0.0001$) and after cardioplegic arrest (92.78 ± 6.8 , $p < 0.0001$), but the positive inotropic response appeared reduced compared with normothermic control values ($p = 0.003$). **Conclusion:** These findings suggest that contributory mechanisms for the reduced β -adrenergic receptor–mediated response after hypothermic, hyperkalemic cardioplegic arrest lie downstream from these specific components of the transduction pathway and likely include defects in Ca^{2+} homeostasis, myofilament Ca^{2+} sensitivity, or both. (J Thorac Cardiovasc Surg 1998;115:190-9)

Hypothermic, hyperkalemic cardioplegic arrest is a common means of providing a quiescent heart during cardiac surgery. However, after reperfusion and rewarming, transient left ventricular (LV) dys-

function can occur.^{1,2} β -Adrenergic agonists are commonly administered to improve LV pump function in the early reperfusion-rewarming period. Stimulation of the β -adrenergic receptor (β -AR)

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activates adenylate cyclase with resultant production of cyclic adenosine monophosphate (cAMP). One of the intracellular events after cAMP production is phosphorylation of the L-type Ca^{2+} channel.^{3,4} Phosphorylation of this channel augments Ca^{2+} current during the excitation-contraction process, thereby enhancing myocyte contractility. However, diminished β -adrenergic responsiveness after cardioplegic arrest has been previously described.⁵⁻⁷ Schwinn and colleagues⁶ attributed this decrease to a desensitization of the β -AR with uncoupling of the β -AR from the G-protein complex after cardioplegic arrest. However, specific intracellular defects that contribute to this blunted β -adrenergic response after cardioplegic arrest and rewarming remain unclear.

Accordingly, the overall goal of this study was to examine the specific downstream intracellular defect(s) in the β -adrenergic signaling cascade after cardioplegic arrest. Well-documented changes in systemic loading conditions and neurohormonal system activity occur after cardioplegic arrest and separation from cardiopulmonary bypass.^{1,2,5-9} For example, a twofold increase in plasma norepinephrine has been described to occur in the early period after cardiopulmonary bypass.⁹ Thus defining the relationship between changes in contractile performance and specific defects in β -AR transduction processes in vivo can be problematic. This laboratory has previously described an isolated LV myocyte system that simulates cardioplegic arrest and rewarming.¹⁰ By use of this in vitro system, past studies have demonstrated that the contractile response to β -AR stimulation is reduced after cardioplegic arrest, consistent with clinical observations.^{5-7,10} Accordingly, this study used this myocyte model of cardioplegic arrest to examine the relationship between activation of specific components of the β -AR signaling pathway and contractile performance.

Methods

The overall goal of this study was to examine the specific intracellular defect(s) in the β -adrenergic signaling pathway after cardioplegic arrest. This was accomplished through pharmacologic dissection of the β -adrenergic transduction pathway. Specifically, the contractile response of isolated myocytes was measured in the presence of either isoproterenol, a β -AR agonist, the naturally occurring diterpene, forskolin, shown to directly stimulate adenylate cyclase,^{11,12} or the L-type Ca^{2+} channel agonist, (-)BayK 8644^{13,14} under normothermic conditions and after cardioplegic arrest.

Fig. 1. The concentration-dependent effects of the L-type Ca^{2+} channel agonist, (-)BayK 8644, were determined with respect to changes in isolated myocyte velocity of shortening. After steady state measurements, myocyte function was measured in the presence of (-)BayK 8644 using concentrations of 0.1 nmol/L to 1 $\mu\text{mol/L}$. At concentrations of 1 to 100 nmol/L (-)BayK 8644, a concentration-dependent effect was observed and subjected to regression analysis. The computed effective concentrations of (-)BayK 8644 that yielded a 50% maximal response (EC_{50}) and a maximal or 100% response (EC_{100}) were 10 nmol/L and 100 nmol/L, respectively. The effects of these concentrations of (-)BayK 8644 on myocyte contractile performance under normothermic conditions and after simulated cardioplegic arrest are shown in Table I.

Myocyte isolation. Six age- and weight-matched Yorkshire swine were the source of myocytes in the study. All animals were treated and cared for in accordance with the National Institutes of Health "Guide for the Care and Use of Laboratory Animals" (National Research Council, Washington, D.C., 1996). On the day of the study, the individual animal was anesthetized with a combination of diazepam (Valium) (10 mg) and 2.5% isoflurane in oxygen, and a sternotomy was performed. The heart was then quickly excised and placed in cold (4°C) oxygenated Krebs solution. The LV free wall encompassing the left circumflex coronary artery ($5 \times 5 \text{ cm}$) was dissected free and the artery was cannulated. This was then used to

Table 1. Isolated myocyte contractile function in normothermic state and after simulated hypothermic hyperkalemic cardioplegic arrest effects of β -adrenergic stimulation, adenylate cyclase activation, and direct Ca^{2+} channel activation

Contractile parameter	Baseline	25 nmol/L isoproterenol	0.5 $\mu\text{mol/L}$ forskolin
Percent shortening (%)			
Normothermic control	5.46 $\pm$ 0.13	10.88 $\pm$ 0.27* $\leq$ 0.0001	9.49 $\pm$ 0.40* $\leq$ 0.0001
Cardioplegic arrest	3.42 $\pm$ 0.15‡ $\leq$ 0.0001	7.52 $\pm$ 0.53‡ $\leq$ 0.0001, * $\leq$ 0.0001	7.70 $\pm$ 0.49‡ $\leq$ 0.0045, * $\leq$ 0.0001
Shortening velocity ($\mu\text{m/sec}$)			
Normothermic control	73.92 $\pm$ 2.85	187.26 $\pm$ 6.85* $\leq$ 0.0001	181.73 $\pm$ 10.15* $\leq$ 0.0001
Cardioplegic arrest	46.07 $\pm$ 2.63‡ $\leq$ 0.0001	128.64 $\pm$ 8.94‡ $\leq$ 0.0001, * $\leq$ 0.0001	124.26 $\pm$ 9.40‡ $\leq$ 0.0001, * $\leq$ 0.0001
Relengthening velocity ($\mu\text{m/sec}$)			
Normothermic control	78.46 $\pm$ 3.87	163.99 $\pm$ 7.14* $\leq$ 0.0001	170.05 $\pm$ 10.81* $\leq$ 0.0001
Cardioplegic arrest	43.37 $\pm$ 2.95‡ $\leq$ 0.0001	102.89 $\pm$ 9.39‡ $\leq$ 0.0001, * $\leq$ 0.0001	90.76 $\pm$ 8.40‡ $\leq$ 0.0001, * $\leq$ 0.0001
Time to peak contraction (msec)			
Normothermic control	243.81 $\pm$ 5.17	179.27 $\pm$ 3.15* $\leq$ 0.0001	199.54 $\pm$ 5.93* $\leq$ 0.0001
Cardioplegic arrest	251.76 $\pm$ 5.29	183.50 $\pm$ 5.69* $\leq$ 0.0001	243.34 $\pm$ 20.12‡ $\leq$ 0.0489
Total contraction duration (msec)			
Normothermic control	502.50 $\pm$ 8.17	389.98 $\pm$ 8.66* $\leq$ 0.0001	469.43 $\pm$ 19.30
Cardioplegic arrest	512.70 $\pm$ 8.93	408.34 $\pm$ 16.82* $\leq$ 0.0001	540.26 $\pm$ 35.96
Number of observations (n)			
Normothermic control	222	108	43
Cardioplegic arrest	130	42	19

Normothermic control = 37° C oxygenated cell media.

Cardioplegic arrest = 4° C 24 mEq K^+ Ringer's solution $\times$ 2 hr followed by 5 min rewarming in 37° C oxygenated cell media.* $p < 0.05$ versus baseline steady state.† $p < 0.05$ versus 10 nmol/L (–)BayK 8644 value.‡ $p < 0.05$ versus normothermic controls.

perfuse the tissue with a modified Krebs solution containing collagenase (0.5 mg/ml, type II; 273 U/mg; Worthington Biochemical Corp, Freehold, N.J.) for 20 minutes. The tissue was then minced, added to an oxygenated solution containing bovine serum albumin (2%, Sigma Chemical Co., St. Louis, Mo.), deoxyribonuclease (51 Kunitz units/ml, type IV, Sigma), 400 μmol CaCl_2 , and collagenase (0.5 mg/ml) and gently agitated. After 15 minutes, the supernatant was removed, filtered, and the cells allowed to settle. The liberated myocytes were then resuspended in standard culture medium (2 mmol/L Ca^{2+} ; medium M199, Gibco Laboratories, Grand Island, N.Y.) and plated on coverslips previously coated with a laminin/fibronectin matrix (Matrigel, Collaborative Research, Bedford, Mass.).

Isolated myocyte function and analysis. Isolated myocytes were placed into a thermostatically controlled chamber at 37° C and imaged with an inverted microscope (World Precision Instruments, Sarasota, Fla.). Myocyte contractions were elicited by field stimulation of the chamber at 1 Hz (S11, Grass Instruments, Quincy, Mass.) with a 5 msec pulse width. Contraction data for each myocyte were recorded from a minimum of 20 consecutive contractions. Myocyte contractions were imaged by use of a charge-coupled device, digitized, and input into a computer (80486; Zeos International, Minneapolis, Minn.) for subsequent analysis, as previously described.^{10, 15} Parameters computed from the digitized contraction profiles included percent shortening (%), velocity of shortening ($\mu\text{m/sec}$), velocity of relengthening ($\mu\text{m/sec}$), total contraction duration (msec), and time to peak contraction

(msec). All parameters were calculated for each contraction, and the results averaged for all contractions were observed. In past reports it has been demonstrated that changes in steady state isolated myocyte contractile function directly reflect changes in the intrinsic capacity of the LV myocardium to function against a given load.¹⁶ By means of this isolated myocyte system, this laboratory has demonstrated that myocytes respond in a predictable manner to incrementally increased external load in both normal and failing preparations.¹⁷ Thus, although the isolated myocyte function studies described in this study were performed under equivalent unloaded conditions, it is likely that these findings can be translated to intrinsic myocardial contractile capacity.

L-type Ca^{2+} channel activation with (–)BayK 8644-dose-dependent effects on myocyte function. Although the effects of the L-type Ca^{2+} channel agonist (–)BayK 8644 (Research Biochemicals International, Natick, Mass.) with respect to augmentation of myocyte Ca^{2+} current have been well described,^{13, 14} the concentration-dependent effects on myocyte contractile performance remained undefined. Thus initial dose-response studies were performed to determine appropriate concentrations of (–)BayK 8644 to be used in the subsequent cardioplegic arrest studies. After baseline measurements of contractile function, measurements were repeated in the presence of (–)BayK 8644 concentrations ranging from 0.1 nmol/L to 1 $\mu\text{mol/L}$. To avoid L-type Ca^{2+} channel rundown, myocytes were exposed to only one concentration of (–)BayK 8644, and a minimum of 15 myocytes were examined at each concentration. The dose-response relation for in-

10 nmol/L (-)BayK 8644	100 nmol/L (-)BayK 8644
7.68 ± 0.40* ^{<0.0001}	9.45 ± 0.50* ^{<0.0001, †0.0079}
5.34 ± 0.40‡ ^{0.0001, *^{<0.0001}}	5.96 ± 0.35‡ ^{<0.0001, *^{0.0001}}
119.41 ± 9.01* ^{<0.0001}	161.90 ± 11.98* ^{<0.0001, *^{0.0064}}
75.08 ± 7.82‡ ^{0.0004, *^{+0.0010}}	92.78 ± 6.77‡ ^{<0.0001, *^{<0.0001}}
113.92 ± 12.72* ^{0.0104}	158.15 ± 16.97* ^{0.0001, †0.0417}
68.14 ± 9.21‡ ^{0.0048, *^{0.0140}}	71.32 ± 6.27‡ ^{<0.0001, *^{0.0002}}
236.18 ± 10.83	219.57 ± 8.23* ^{0.0159}
273.66 ± 13.93‡ ^{0.0373}	256.12 ± 14.77‡ ^{0.354}
649.99 ± 29.68* ^{<0.0001}	639.65 ± 27.39* ^{<0.0001}
642.13 ± 23.22* ^{<0.0001}	637.21 ± 20.87* ^{<0.0001}
41	28
37	34

creasing concentrations of (-)BayK 8644 with respect to myocyte shortening velocity is presented in Fig. 1. These dose-response data were subjected to log-linear regression analysis to determine the effective concentration of (-) BayK 8644, which elicited 50% of maximal response (EC₅₀) and a maximal or 100% response (EC₁₀₀). These computations revealed an EC₅₀ and EC₁₀₀ for (-)BayK 8644 with respect to myocyte velocity of shortening of 10 nmol/L and 100 nmol/L, respectively. Accordingly, these concentrations of the L-type Ca²⁺ channel agonist were used in the experimental protocols described in the following section.

Experimental protocol. Myocytes were randomly assigned to one of three treatment groups, hypothermic cardioplegic arrest, hypothermia alone, or normothermic control animals. The simulated cardioplegic arrest protocol, which has been previously described,¹⁰ was performed by incubating myocytes in a conventional hyperkalemic crystalloid cardioplegic solution (lactated Ringer's, 24 mEq/L K⁺, 30 mEq/L HCO₃⁻, oxygen tension > 300) for 2 hours at 4° C. The myocytes were then incubated in 37° C cell media for a 5-minute rewarming period before study. Normothermic control animals were maintained in 37° C cell media and used for comparison. In a final group, the potential effect of temperature on contractile performance was examined by maintaining myocytes in 4° C cell media for 2 hours followed by a 5-minute rewarming period. Myocyte contractile function was examined at baseline and then measured in the presence of either 25 nmol/L isoproterenol (INN: isoprenaline), 0.5 μmol/L forskolin, 10 nmol/L (-)BayK 8644, or 100 nmol/L (-)BayK 8644. Previous dose-response studies have demonstrated that 25 nmol/L isoproterenol (Sigma) and 0.5 μmol/L forskolin (Sigma) elicit maximal effects on myo-

cyte contractility under control conditions.¹⁸ Thus these concentrations were chosen for this study.

Data analysis. In this study, two main treatment effects were used: cardioplegic arrest and drug treatment. Accordingly, the initial data analysis was performed using a two-way analysis of variance (ANOVA) to identify the main treatment effects and any potential interaction between the main effects with respect to myocyte contractility. A multiway ANOVA was then carried out to identify the specific treatment interventions (i.e., β-adrenergic occupancy, adenylate cyclase stimulation) on contractile performance under both normothermic control conditions and after simulated cardioplegic arrest. If the multiway ANOVA revealed significant differences, pairwise tests of individual group means were compared using Bonferroni's probabilities. All statistical analyses were performed using standard statistical software programs (BMDP Statistical Software, Inc., Los Angeles, Calif.). Results are presented as mean ± standard error of the mean. Values *p* < 0.05 were considered statistically significant.

Results

Indices of steady state myocyte contractile function under normothermic control conditions and after β-AR occupancy, adenylate cyclase stimulation, or L-type Ca²⁺ channel activation are summarized in Table I. Myocyte contractility was increased after β-AR occupancy with isoproterenol and after direct adenylate cyclase stimulation with forskolin. For example, myocyte velocity of shortening was increased by 153% after β-AR occupancy and by 146% after adenylate cyclase stimulation. Direct activation of the L-type Ca²⁺ channel with (-)BayK 8644 increased myocyte contractility. For example, velocity of shortening was increased by 62% with 10 nmol/L and 120% with 100 nmol/L, reflecting the EC₅₀ and EC₁₀₀ concentrations, respectively. In myocytes maintained in hypothermic cell culture media for 2 hours (*n* = 59), baseline contractile function was unchanged after rewarming compared with normothermic control values. For example, myocyte velocity of shortening was 79.5 ± 4.1 μm/sec in the hypothermic control group compared with 81.8 ± 2.1 μm/sec in the normothermic control group (*p* = 0.6237). Therefore it did not appear that hypothermic conditions significantly affected myocyte contractility. These results are consistent with a past report from this laboratory, which demonstrated that prolonged hypothermia alone had minimal effects on myocyte contractile function.¹⁰

Cardioplegic arrest and rewarming group. Steady state myocyte contractile function after cardioplegic arrest and rewarming and after β-AR occupancy, adenylate cyclase stimulation, or L-type Ca²⁺ channel activation is summarized in Table I.

Fig. 2. Myocyte contractile function in both the normothermic state and after cardioplegic arrest was measured by absolute change in myocyte shortening velocity after β -AR (B-AR) occupancy with 25 nmol/L isoproterenol, stimulation of adenylate cyclase with 0.5 μ mol/L forskolin (AC), or direct activation of the L-type Ca^{2+} channel with both 10 nmol/L and 100 nmol/L (–)BayK 8644. The absolute change in shortening velocity after β -receptor occupancy, stimulation of adenylate cyclase, or direct activation of the L-type Ca^{2+} channel after cardioplegic arrest was reduced compared with normothermic values ($p = 0.012$, $p = 0.005$, $p = 0.0006$, respectively). The response to 100 nmol/L (–)BayK 8644 was greater than that to 10 nmol/L (–)BayK 8644 in the normothermic group ($p = 0.02$) and after cardioplegic arrest ($p = 0.0015$) as previously predicted by the dose-response study (* $p < 0.05$ vs normothermic control, # $p < 0.05$ vs 10 mol/L (–)BayK 8644; actual p values provided in text).

Baseline myocyte contractile function was reduced after cardioplegic arrest. For example, myocyte velocity of shortening was reduced by 38% compared with normothermic control values. The two-way ANOVA revealed that cardioplegic arrest was a significant main effect with respect to contractile function. For example, the F values for myocyte percent shortening and velocity of shortening were significant for the main treatment effect of cardio-

plegia ($F = 132.57$, $F = 108.35$, $p < 0.0001$, respectively). The two-way ANOVA also demonstrated that the drug treatment interventions represented a main effect with respect to contractile performance. For example, the F values for myocyte percent shortening and velocity of shortening were significant ($F = 118.93$, $F = 108.88$, $p < 0.0001$, respectively). More important, this analysis identified a significant interaction between these two main effects; specifically, F values of 2.87 and 3.82 were obtained for myocyte percent shortening and velocity of shortening ($p = 0.0225$ and $p = 0.0044$, respectively). In light of the significant interaction between the main treatment effects, individual pairwise comparisons for indices of myocyte contractility were performed using Bonferroni confidence intervals constructed from the mean square error obtained from a multiway ANOVA. The results from these pairwise contrasts and the individual p values obtained are shown in Table I. Myocyte contractility increased after β -AR occupancy or adenylate cyclase stimulation, but these effects appeared reduced compared with normothermic control values. For example, myocyte velocity of shortening was reduced by more than 30% after either β -AR occupancy or adenylate cyclase stimulation compared with respective normothermic control values. Direct activation of the L-type Ca^{2+} channel increased myocyte contractility after cardioplegic arrest but was reduced when compared with the normothermic control values. For example, myocyte velocity of shortening was reduced by 37% and 43% compared with the normothermic control values at the 10 nmol/L and 100 nmol/L (–)BayK 8644 concentrations, respectively.

In light of the significant differences in baseline contractile function between the normothermic control myocytes and myocytes after cardioplegic arrest and rewarming, the effect of β -adrenergic occupancy, adenylate cyclase stimulation, or direct L-type Ca^{2+} channel activation on myocyte contractile function may be difficult to interpret. Therefore the absolute change from baseline for velocity of shortening was computed in each myocyte after each treatment (Fig. 2). Cardioplegic arrest and rewarming were associated with a significant decrease in absolute change from baseline in myocyte velocity of shortening after β -adrenergic occupancy, adenylate cyclase stimulation, or direct L-type Ca^{2+} channel activation. Another approach for expressing the relative contractile response to the individual drug interventions would be as a percent change from

steady state basal values. By means of this strategy, myocyte velocity of shortening was increased by 231% and 304% with β -AR occupancy, by 110% and 109% with adenylate cyclase stimulation, and by 90% and 85% with Ca^{2+} channel activation (100 nmol/L BayK) in the normothermic control and hypothermic cardioplegia groups, respectively. Both analyses revealed that the myocytes retained the capacity to respond to an inotropic stimulus after hypothermic cardioplegic arrest. Nevertheless, the absolute value for indices of contractility remained significantly blunted in myocytes after cardioplegic arrest compared with controls after any of the three drug interventions.

Discussion

Transient LV dysfunction can occur after hypothermic, hyperkalemic cardioplegic arrest and is associated with decreased β -adrenergic responsiveness. The overall goal of this study was to define the contributory intracellular mechanism(s) potentially responsible for the defects in the β -adrenergic signaling cascade that occur after cardioplegic arrest. Under normal conditions, occupancy of the β -adrenergic receptor stimulates adenylate cyclase with subsequent activation of the L-type Ca^{2+} channel. Activation of this channel increases Ca^{2+} current thereby enhancing myocyte contractility. Accordingly, this study examined myocyte contractile performance after β -AR occupancy, direct stimulation of adenylate cyclase, or direct activation of the L-type Ca^{2+} channel under normothermic conditions and after simulated cardioplegic arrest. The significant findings of this study were twofold. First, β -AR occupancy or direct adenylate cyclase stimulation increased myocyte contractility; but the effect of either was reduced after simulated cardioplegic arrest. Second, direct activation of the L-type Ca^{2+} channel, which eliminates β -AR dependent events, increased myocyte contractile performance under both conditions. However, the myocyte inotropic response to L-type Ca^{2+} channel activation was reduced after cardioplegic arrest. These findings suggest that contributory mechanisms for the reduced β -adrenergic response after cardioplegic arrest are downstream from this transduction pathway.

A schematic representation of the β -adrenergic signaling cascade is shown in Fig. 3, with emphasis on the rationale for the experimental design used in this study. Under normal conditions, occupancy of the β -AR initiates a signaling cascade involving a

G-protein complex, through which adenylate cyclase is stimulated to increase production of cAMP. Increased intracellular cAMP activates protein kinase A (PKA), which mediates three important intracellular events. First, PKA phosphorylates the L-type Ca^{2+} channel, augmenting Ca^{2+} flux across the sarcolemma and thereby enhancing the release of intracellular Ca^{2+} stores from the sarcoplasmic reticulum (SR).^{4, 19, 20} Second, PKA phosphorylates the SR-bound regulatory protein phospholamban, which augments intracellular Ca^{2+} resequestration by the SR.^{4, 19, 20} Third, PKA phosphorylates troponin-I, which directly influences myofilament Ca^{2+} sensitivity and therefore cross-bridge formation.^{4, 21} In this study, myocyte contractile response to β -adrenergic stimulation was reduced after cardioplegic arrest. Additionally, the inotropic response failed to return to normal levels with either direct stimulation of adenylate cyclase or direct activation of the L-type Ca^{2+} channel. These findings suggest that the decreased myocyte inotropic response to β -AR stimulation after cardioplegic arrest may be the result of three intracellular defects. First, diminished PKA activation may occur with subsequent defects in phosphorylation of the L-type Ca^{2+} channel, phospholamban, and troponin-I. Second, the intracellular homeostatic mechanisms responsible for release and resequestration of SR Ca^{2+} stores may be altered. Third, a decrease in the sensitivity of the contractile apparatus to Ca^{2+} with a subsequent reduction in cross-bridge formation may occur. Using this isolated myocyte model of cardioplegic arrest, future studies that more carefully focus on these potential intracellular defects with emphasis on PKA-mediated events are warranted.

The administration of β -AR agonists is a common means of improving LV pump function after cardioplegic arrest and separation from cardiopulmonary bypass.²² However, diminished inotropic response to β -adrenergic stimulation after cardioplegic arrest has been reported.⁵⁻⁷ Several contributing factors for the reduced inotropic effect of β -AR stimulation after cardioplegic arrest have been identified.⁵⁻¹⁰ For example, Schwinn and colleagues⁶ demonstrated that an uncoupling of the β -AR with a reduction in adenylate cyclase activity occurs after cardioplegic arrest.⁶ Thus in this study contributory mechanisms for the reduction in contractile response with β -adrenergic occupancy or adenylate cyclase stimulation may be an uncoupling of the β -AR with a resultant reduction in adenylate cyclase activity. Furthermore, this study builds on

Fig. 3. A schematic representation of the β -adrenergic signaling cascade, with emphasis on the rationale for the experimental design used in this study. Under normal conditions, occupancy of the β -AR initiates a signaling cascade involving a G-protein complex, through which adenylate cyclase is stimulated to increase production of cAMP. Increased intracellular cAMP activates protein kinase A, which mediates three important intracellular events: (A) phosphorylation of the L-type Ca^{2+} channel, which augments Ca^{2+} flux across the sarcolemma, enhancing the release of intracellular Ca^{2+} stores from the SR; (B) phosphorylation of troponin-I, which directly influences myofilament Ca^{2+} sensitivity and thereby cross-bridge formation; and (C) phosphorylation of the SR-bound regulatory protein phospholamban, which enhances intracellular Ca^{2+} resequestration by the SR. In this study the β -adrenergic signaling cascade was examined by pharmacologic dissection at three specific points. First, the β -AR was stimulated with isoproterenol. Second, adenylate cyclase was stimulated with forskolin. Third, the L-type Ca^{2+} channel was activated with (-)BayK 8644. After simulated cardioplegic arrest, significant blunting of the inotropic response to all three treatments compared with normothermic control values was demonstrated. These results suggest that defects in intracellular Ca^{2+} homeostasis, myofilament Ca^{2+} sensitivity, or both contribute to the diminished inotropic response after cardioplegic arrest.

these past findings by demonstrating that signaling pathway components essential for the β -adrenergic response (i.e., the L-type Ca^{2+} channel) may also be affected after cardioplegic arrest. Additional studies have demonstrated that with the onset of cardiopulmonary bypass, catecholamine levels increase several fold and remain elevated throughout the bypass

period.^{8,9} It has been suggested that the persistently elevated catecholamine levels associated with cardiopulmonary bypass cause desensitization of the β -AR with diminished adenylate cyclase activation.⁶⁻⁸ In this study, an isolated myocyte system of cardioplegic arrest, which is independent of neuro-hormonal influences, was used. After simulated

cardioplegic arrest and rewarming, defects in myocyte β -AR responsiveness were observed and are consistent with past *in vivo* studies.¹⁰ Therefore increased plasma catecholamine levels alone cannot explain the decreased β -AR responsiveness that occurs after cardioplegic arrest and separation from cardiopulmonary bypass.

Forskolin, a naturally occurring diterpene, has been shown to directly stimulate adenylate cyclase, which increases intracellular cAMP,^{11,12} effectively bypassing the β -AR and G-protein complex of the β -adrenergic signaling cascade. Several past studies have demonstrated that forskolin produces a positive inotropic effect in myocardial preparations.^{11,12} For example, Bristow and colleagues¹¹ demonstrated that forskolin increased adenylate cyclase activity and produced a potent positive inotropic response in human right ventricular papillary muscle. Hearse and colleagues,¹² using an isolated rat heart model of cardioplegic arrest, demonstrated that while contractile function was improved and adenylate cyclase activity was increased in the presence of forskolin, contractile performance remained lower than normal control values. Consistent with these past observations, this study found that the inotropic response to forskolin was blunted after cardioplegic arrest. Other past studies have demonstrated that phosphodiesterase inhibitors, which prevent the degradation of cAMP, have a positive inotropic effect after cardiopulmonary bypass with cardioplegic arrest.²³ However, this laboratory has previously demonstrated that although phosphodiesterase inhibition increased isolated myocyte contractility after cardioplegic arrest, the inotropic response was blunted compared with normothermic control values.²⁴ Taken together the findings of this study and evidence from past reports suggest that the defect(s) resulting in reduced responsiveness to β -adrenergic stimulation after cardioplegic arrest occur downstream from cAMP production in the signaling cascade. Because one of the important events after adenylate cyclase activation includes phosphorylation of the L-type Ca^{2+} channel, this study next examined the effect of direct activation of this channel on myocyte contractile function under normothermic conditions and after simulated cardioplegic arrest.

The dihydropyridine derivative, (-)BayK 8644, binds to the α subunit of the L-type Ca^{2+} channel, induces prolonged channel opening, and thereby increases Ca^{2+} flux through the channel.^{4,14} Past studies have demonstrated that (-)BayK 8644

increased contractile function in myocardial preparations.^{13,14} However, to our knowledge, this study is the first to examine the direct effects of (-)BayK 8644 on the contractile performance of the isolated myocyte under normothermic conditions and after simulated cardioplegic arrest. In this study, (-)BayK 8644 elicited a dose-dependent positive inotropic response consistent with specific activation of the L-type Ca^{2+} channel in isolated myocytes under normothermic conditions. After simulated cardioplegic arrest, an apparent similar dose-dependent increase in myocyte contractility was noted. Under both normothermic conditions and after simulated cardioplegic arrest, total contraction duration was increased by more than 25%. A likely contributory mechanism for the increased contraction duration in the presence of (-)BayK 8644 was alterations in Ca^{2+} homeostatic processes. (-)BayK 8644 increases intracellular Ca^{2+} levels without elevating cAMP levels.¹⁴ Thus, while intracellular Ca^{2+} levels rise, basal phospholamban activity and rate of Ca^{2+} resequestration by the SR remain unchanged. This net effect would potentially result in increased cross-bridge release time and prolonged elevation in the cytoplasmic Ca^{2+} concentration that would be translated as an increase in total contraction duration. Although direct activation of the L-type Ca^{2+} channel elicited a positive inotropic response after cardioplegic arrest, this response was significantly blunted compared with normothermic control values. A recent study by Lopez and colleagues²⁵ demonstrated that a hyperkalemic environment increased resting intracellular Ca^{2+} levels in isolated myocytes. A past study from this laboratory demonstrated that the positive inotropic response to increased extracellular Ca^{2+} in the isolated myocyte is diminished after cardioplegic arrest.²⁶ Taken together, results from our study and past reports suggest that the decrease in myocyte inotropic response after cardioplegic arrest may involve defects in intracellular Ca^{2+} handling, use, or both. Increased intracellular Ca^{2+} accumulation has been shown to contribute to reoxygenation injury and decreased recovery of contractile performance with reperfusion.²⁷ Alterations in intracellular Ca^{2+} compartmentalization and sequestration may occur during and immediately after prolonged periods of cardioplegic arrest. These persistent defects in Ca^{2+} homeostatic processes within the myocyte after cardioplegic arrest may contribute to the diminished contractile responsiveness with direct L-type Ca^{2+} channel activation. The specific abnor-

malities in intracellular Ca^{+2} compartmentalization after cardioplegic arrest, particularly with respect to the function of the SR warrant further study.

Although the isolated myocyte system used in this study provided a means to pharmacologically dissect the β -adrenergic signaling cascade to examine specific defects in contractile mechanisms, several limitations to this in vitro system exist. The model excludes environmental factors present in vivo, including changes in systemic loading conditions and neurohormonal influences. This model of cardioplegic arrest differs with respect to in vivo conditions in that the myocytes are not subject to variations in temperature and are continuously exposed to the hyperkalemic environment. This model of simulated cardioplegic arrest causes a decrease in myocyte contractility. However, this decrease in contractile performance is a transient phenomenon. Specifically, 30 minutes after reperfusion/rewarming, indices of myocyte contractile function approach normothermic values.²⁸ Although this is a limitation of the model, it does appear to recapitulate the transient LV dysfunction that may be encountered after cardioplegic arrest in the clinical setting. Our study makes the assumption that the response elicited by (-)BayK 8644 after cardioplegic arrest arises from a functionally intact L-type Ca^{2+} channel. Whether cardioplegic arrest causes direct negative effects to the L-type Ca^{2+} channel with respect to functional state is unknown. Future studies measuring the Ca^{2+} current through the L-type Ca^{2+} channel in the presence and absence of (-)BayK 8644 in the normothermic state and after cardioplegic arrest would be necessary to address this issue. It should also be understood that this study was conducted using asanguineous cardioplegia and cell media and that results in a blood-perfused in vivo preparation may result in differential findings with respect to the β -adrenergic signaling cascade.

The findings of this study open avenues for future studies aimed at identifying other possible sites in the β -adrenergic signaling cascade that may be affected by cardioplegic arrest. Specifically, future studies examining the role of PKA-mediated intracellular events on myocyte contractile performance after cardioplegic arrest are warranted. An increasing number of patients are presenting for cardiac surgery with preexisting LV dysfunction,²⁹ a condition that has been associated with decreased sensitivity and inotropic response to β -adrenergic stimulation.³⁰ Thus direct activation of the L-type Ca^{2+} channel may prove beneficial as a therapeutic mo-

dality in this subset of patients. However, future in vitro and in vivo studies would be required to examine this hypothesis. Nevertheless, the findings of this study support the conclusion that defect(s) contributing to decreased inotropic responsiveness in isolated myocytes after cardioplegic arrest may include defects in the β -AR signaling cascade, disturbances in intracellular Ca^{2+} homeostasis, changes in myofilament Ca^{2+} sensitivity, or any combination of the above.

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