Chloride Channel Inhibition Blocks the Protection of Ischemic Preconditioning and Hypo-Osmotic Stress in Rabbit Ventricular Myocardium

Roberto J. Diaz,* Vito A. Losito,* Gou D. Mao, Meredith K. Ford, Peter H. Backx, Gregory J. Wilson

Abstract—The objective of this study was to examine the role of chloride (Cl–) channels in the myocardial protection of ischemic preconditioning (IP). Isolated rabbit ventricular myocytes were preconditioned with 10-minute simulated ischemia (SI) and 20-minute simulated reperfusion (SR) or not preconditioned (control). The myocytes then received 180-minute SI or 45-minute SI/120-minute SR. Indanyloxyacetic acid 94 (IAA-94, 10 μmol/L) or 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB, 1 μmol/L) was administered before IP or before SI or SI/SR to inhibit Cl– channels. Electrophysiological studies indicate that these drugs, at the concentrations used, selectively abolished Cl– currents activated under hypo-osmotic conditions (215 versus 290 mOsm). IP significantly (P<0.001) reduced the percentage of dead myocytes after 60-minute (30.8±1.3%, mean±SEM), 90-minute (35.3±1.3%), and 120-minute (39.2±1.7%) SI compared with controls (44.7±1.6%, 54.5±1.3%, and 58.9±1.8%, respectively) and after 45-minute SI/120-minute SR (36.3±0.6%) compared with control (56.6±2.2%). Hypo-osmotic stress also produced protection similar to IP. IAA-94 or NPPB abolished the protection of both IP and hypo-osmotic stress. In buffer-perfused rabbit hearts preconditioned with three 5-minute ischemia/10-minute reperfusion cycles given before the 40-minute long ischemia and 60-minute reperfusion, IP significantly (P<0.0001) reduced infarct size (IP+vehicle, 4.7±0.9%, versus control+vehicle, 26.6±3.3%; mean±SEM). Again, IAA-94 or NPPB abolished the protection of IP. Our results implicate Cl– channels in the IP protection of the myocardium against ischemic/reperfusion injury and demonstrate that hypo-osmotic stress is capable of preconditioning cardiomyocytes. (Circ Res. 1999;84:763-775.)

Key Words: ischemic preconditioning • hypo-osmotic stress • chloride channel • myocardial infarction • cardiomyocyte

During ischemia, metabolites accumulate in cardiomyocytes, thus increasing intracellular osmolarity.1 The development of an osmotic gradient between the intracellular and extracellular milieu, in turn, causes cell swelling. It is known that cell swelling and the loss of cell volume regulation play important roles in ischemic injury in the myocardium.2-5 Cell swelling activates a variety of transport pathways that result in the net efflux of K+, Cl–, organic anions, and organic osmolites.4 Cell swelling activates an outwardly rectifying conductance that has an anion selectivity sequence of CN– > I– > NO3– > Br– ≥ Cl– > F– > gluconate and is characterized by a single-channel conductance of 40 to 50 pS (at +120 mV).5 Duan et al6 have reported a tamoxifen-sensitive outwardly rectifying swell-activated Cl– current (Iclswell) in rabbit atrial myocytes. Iclswell has also been reported in dog7-8 guinea pig,7 chick,8 and human10 cardiomyocytes. Furthermore, Iclswell has been reported in a variety of noncardiac cells and demonstrates biophysical and pharmacological properties similar to those seen in cardiac myocytes.11,12

The regulation of Cl– channels has much in common with the controlling factors associated with the phenomenon of ischemic preconditioning (IP) of the myocardium. A Cl– channel conductance is activated by angiotensin II via AT1 receptors in sinoatrial cells13 and by adenosine in guinea pig ventricular myocytes,14 whereas activation of angiotensin II AT1 receptors15 and adenosine A1/A2 receptors16,17 is thought to trigger IP. In addition, transient exposure to phorbol 12-myristate 13-acetate, which has been shown to mimic the protection of IP via protein kinase C (PKC) activation in isolated cardiomyocytes,18 activates a Cl– current in feline19 and guinea pig ventricular myocytes20 but not in rabbit atrial myocytes.6 It is noteworthy that a transient increase in [Ca2+]i resulting from Ca2+ entry during the initial preconditioning ischemia is thought to mediate the protection of IP by

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triggering the activation of PKC and to activate a Ca
-dependent Cl conductance in rabbit ventricular myocytes. Furthermore, Sorota has shown that swell-activated Cl currents are triggered via protein tyrosine kinases that have recently been implicated in IP protection.

Because signaling pathways associated with IP also regulate the activity of Cl channels, we postulated that IP activates Cl channels, including swell-activated Cl channels, and thereby reduces myocardial necrosis during prolonged ischemia and reperfusion. In the present study, we demonstrated in the rabbit the participation of Cl channels in the protection of IP against myocardial necrosis.

Materials and Methods

Isolated ventricular myocytes and whole hearts were obtained from New Zealand White rabbits (weight range, 3.0 to 3.5 kg). Animal protocols conformed with the Guide for the Care and Use of Laboratory Animals published by NIH (NIH publication No. 85–23, revised 1996) and was approved by the Animal Care Committee of the Research Institute, The Hospital for Sick Children.

Preparation of Ventricular Myocytes

Ventricular myocytes were isolated by enzymatic dissociation using a method previously reported. Briefly, donor rabbits were anesthetized with a mixture of domperidone (60 mg/kg) and heparin (200 IU/kg). The heart was excised and immediately perfused on a nonrecirculating Langendorff apparatus at 75 mm Hg of perfusion pressure with oxygenated (95% O2–5% CO2) Joklik-modified minimal essential medium solution (S-MEM, Gibco) at 37°C, pH 7.4, and supplemented with (in mmol/L) CaCl2 1.2, MgSO4 0.2, 2, creatine 20, taurine 60, NaHC03 24, and HEPES 10 (pH 7.4 with NaOH). The perfusate solution contained, in mmol/L: potassium phosphate 100, KCl 1, MgCl2 1, EGTA 5, and HEPES 10 (pH 7.4 with KOH). To activate the spontaneous dihydropyridine-sensitive L-type Ca2+ current (ICaL), 2-deoxyglucose (10 mmol/L) and cyanide (2 mmol/L) were added to the external solution. To isolate L-type Ca2+ current (ICaL), we used a bath solution containing the following (in mmol/L): NaCl 130, KC1 5, MgCl2 1, CaCl2 1.8, MgCl2 1, CdCl2 0.3, and HEPES 10 (pH 7.4 with NaOH). The pipette solution contained, in mmol/L: potassium phosphate 100, KCl 1, EGTA 5, and HEPES 10 (pH 7.4 with KOH). To activate ICaL, 2-deoxyglucose (10 mmol/L) and cyanide (2 mmol/L) were added to the external solution. To isolate L-type Ca2+ current (ICaL), we used a bath solution containing the following (in mmol/L): NaCl 130, CsCl 5, MgCl2 1, CaCl2 1.8, HEPES 10, and glucose 10 (pH 7.4 with NaOH). The pipette solution contained, in mmol/L: CsCl 130, MgCl2 1, EGTA 5, and HEPES 10 (pH 7.4 with CsOH).

Isolated Ventricular Myocyte Studies

Simulated Ischemia (SI) and Simulated Reperfusion (SR)

Ischemia was simulated in myocytes with the use of a previously described method. Briefly, 1.5 mL of the cell suspension was placed in a 1.8-mL Eppendorff tube and centrifuged (45g for 2 minutes) to form an 8- to 10-mm-thick cell pellet. The supernatant was discarded, except for a volume equivalent to about one third of the pellet thickness. The cell pellet and supernatant were covered by a 3- to 4-mm–thick mineral oil layer and incubated at 37°C.

Reperfusion was simulated using a method we have previously characterized. Briefly, 100 to 150 mL of the ischemic cell pellet was resuspended in 1.0 mL of oxygenated calcium-containing buffer supplemented with 0.1% BSA and incubated on a Nunclon multidish cell culture plate (Irvine Scientific, Inc) at 37°C with agitation in an O2 atmosphere.

Experimental Protocol for Ventricular Myocytes

Isolated ventricular myocytes (n=7 hearts in each set) were studied to determine the role of Cl channels in the protection of IP against cell death caused by ischemia and reperfusion. After an initial 30-minute stabilization period (incubation in a 95% O2–5% CO2 atmosphere at 30°C without agitation), myocytes were either pre-conditioned using a 10-minute period of SI (37°C) followed by 20-minute SR or not pre-conditioned (control). Next, both control and pre-conditioned myocytes were subjected to either 180-minute SI (ischemia protocol) or 45-minute SI/120-minute SR (ischemia/reperfusion protocol) (Figure 1).

Control, pre-conditioned, and oxygenated baseline (240-minute incubation in an O2 atmosphere at 37°C) myocytes were simultaneously treated with either of 2 selective Cl channel blockers that have distinct molecular structures and inhibitory mechanisms. 10 mmol/L IAA-94 or 1 mmol/L 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) (Research Biochemicals International Inc.) or they were treated with the vehicle of either drug (0.03% and 0.09% ethanol for IAA-94 and NPPB, respectively). The blocker was initially added to the myocyte suspension either 10 minutes before IP or just before the long SI (IAA-94 only). When added before IP, neither blocker was washed out before the long SI. The concentrations of IAA-94 and NPPB were selected to be 10 times the IC50.
whether the concentrations of IAA-94 and NPPB used in these
buffer supplemented with 80 mmol/L mannitol. To determine
myocytes were also incubated in the same taurine- and creatine-free
myocytes transiently subjected to hypo-osmotic stress. Control
MgSO4 0.83, glucose 5.55, and amylobarbitone 3.0; 0.5% glutaral-
validating the assay. Therefore, Cl– channel inhibitors have no
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All histological examinations were performed with a light micro-
Assessment of Ventricular Myocyte Viability
60 myocytes per group) were initially subjected to 15-minute aerobic
Experimental Protocol for Isolated Hearts
The stability of our preparation has been previously demonstrated
using isolated buffer-perfused rabbit hearts in which LVDP, heart
systolic minus diastolic pressure) and heart rate. A probe was also
left ventricle to assess left ventricular developed pressure (LVDP; oxygenation with 95% O2 –5% CO2) at 37°C and constant pressure of
2.5, NaHCO3 24.8, KH2PO4 1.2, and glucose 10 (pH 7.4 by
ratus, and immediately perfused with Krebs-Henseleit buffer solu-
Experimental protocols used in myocyte viability studies. Isolated ven-
tricular myocytes were initially subjected to 30-minute stabilization (myocytes sus-
pended in oxygenated buffer at 30°C) followed either by additional 30-minute stabilization (control myocytes) or by IP consisting of 10-minute SI (pelleting under oil at 37°C) followed by 20-minute SR (resuspension in oxygenated buffer).
Then, myocytes were subjected to either 180-minute SI (ischemia-alone protocol) or 30-minute SI followed by 120-minute SR (ischemia/reperfusion protocol). IAA-94 was added either 10 minutes before IP or just before the long SI, whereas NPPB was added before IP only. Myocyte viability was assessed at the following time points (): end of stabi-
ligation, before long SI, during long SI alone (30, 60, 90, 120, and 180 minutes), and at the end of SI (45 minutes) and during SR (15, 60, and 120 minutes) in the ischemia/reperfusion protocol.
Isolated Heart Studies
Surgical Preparation
Rabbits were prepared as previously reported.15 Briefly, hearts were
excised, mounted on a modified nonrecirculating Langendorff appa-
ratus, and immediately perfused with Krebs-Henseleit buffer solution
containing (in mmol/L) NaCl 118.5, KCl 4.7, CaCl2 2.5, NaHCO3 24.8, KH2PO4 1.2, and glucose 10 (pH 7.4 by
oxygenation with 95% O2 –5% CO2) at 37°C and constant pressure of
75 mm Hg. To induce regional ischemia, a branch of the left
coronary artery was intermittently occluded. An intraventricular
latex balloon connected to a pressure transducer was placed into the
left ventricle to assess left ventricular developed pressure (LVDP; systolic minus diastolic pressure) and heart rate. A probe was also
placed in the heart to monitor myocardial temperature. Once instru-
mented, hearts were placed in a water-jacketed chamber and stabi-
lized before each experiment began.
Experimental Protocol for Isolated Hearts
The stability of our preparation has been previously demonstrated
using isolated buffer-perfused rabbit hearts in which LVDP, heart
rate, and coronary flow were measured.15 Using the same model, 30
hearts (n=5 per group) were initially subjected to 15-minute aerobic
perfusion (stabilization period) followed by 40-minute normothermic
(37°C) regional ischemia and 60-minute reperfusion. Control hearts
were also subjected to an additional 45-minute aerobic perfusion (total stabilization period, 60 minutes) before the long ischemia so as to equalize the total length of the experimental protocol in all groups. Preconditioned hearts were also subjected to 3 cycles of 5-minute regional ischemia followed by 10-minute reperfusion before the long ischemia. To determine whether Cl–
96% retention of PKC activity, respectively. PKC inhibition by 10,
25, and 50 μmol/L chelerythrine resulted in 55%, 40%, and 10% retention of PKC activity, respectively, whereas inhibition by 10 and 25 μmol/L polymyxin B resulted in 16% and 0%, respectively, validating the assay. Therefore, Cl channel inhibitors have no
significant effect on PKC activity at the concentrations used.
Because cell swelling is known to activate swell-activated Cl–
channels, we also assessed whether activation of these channels by
transient hypo-osmotic stress could mimic the protection of IP. After
stabilization, myocytes were incubated in a hypo-osmotic (80-mOsm
osmotic gradient) buffer identical to the iso-osmotic buffer except for
the removal of creatine (20 mmol/L) and taurine (60 mmol/L) for 10
minutes, in place of IP, and then resuspended in iso-osmotic buffer
for 20 minutes just before the long SI. IAA-94 (10 μmol/L) and
NPPB (1 μmol/L) were given 10 minutes before the long SI to
myocytes transiently subjected to hypo-osmotic stress. Control
myocytes were also incubated in the same taurine- and creatine-free
buffer supplemented with 80 mmol/L mannitol. To determine whether the concentrations of IAA-94 and NPPB used in these studies have a nonspecific effect on mitochondrial KATP channels, the
effects of IAA-94 and NPPB on protection in myocytes by diazox-
ide33 each Cl– channel inhibitor (IAA-94 or NPPB) was also coadministered with diazoxide before SI in separate groups of
myocytes.
Assessment of Ventricular Myocyte Viability
All histological examinations were performed with a light micro-
scope at ×100 magnification. Myocyte viability was assessed in each
group at different time points (Figure 1). Hypotonic (85 mOsm)
trypan blue–modified Tyrode staining solution containing
(1:1 but
3:1. We counted 250 to 300 myocytes per
sample in <15 minutes to minimize variability associated with
changes in the ratio of stained/unstained cells over time. From these
data, the percentage of dead cells was calculated and compared. Only experiments with >70% of viable myocytes (rod-shaped, square, and
round myocytes) observed at the end of the stabilization period were
considered acceptable for this study. Viable round myocytes (cells
excluding trypan blue, ~5%) were only found at the end of stabilization or before the long SI.

Figure 1. Experimental protocols used in myocyte viability studies. Isolated ven-
tricular myocytes were initially subjected to 30-minute stabilization (myocytes sus-
pended in oxygenated buffer at 30°C) followed either by additional 30-minute stabilization (control myocytes) or by IP consisting of 10-minute SI (pelleting under oil at 37°C) followed by 20-minute SR (resuspension in oxygenated buffer). Then, myocytes were subjected to either 180-minute SI (ischemia-alone protocol) or 30-minute SI followed by 120-minute SR (ischemia/reperfusion protocol). IAA-94 was added either 10 minutes before IP or just before the long SI, whereas NPPB was added before IP only. Myocyte viability was assessed at the following time points (): end of stabi-
ligation, before long SI, during long SI alone (30, 60, 90, 120, and 180 minutes), and at the end of SI (45 minutes) and during SR (15, 60, and 120 minutes) in the ischemia/reperfusion protocol.
channels play a role in the protection of IP during the long ischemia, hearts were subjected to the same control and IP protocols with a 10-minute exposure to IAA-94 or NPPB, at the same concentrations used in the isolated myocyte model, before the long ischemia. As in isolated myocyte studies, we explored whether IAA-94 (10 μmol/L) could block the protection induced by stimulation of mitochondrial K<sub>ATP</sub> channels by diazoxide (100 μmol/L) in 21 additional buffer-perfused hearts. The following 3 groups of hearts were studied: those treated with control vehicle (0.05% DMSO; n=5), those treated with diazoxide (n=8), and those treated with diazoxide + IAA-94 (n=8) hearts. Diazoxide was given 15 minutes before the long ischemia. IAA-94 was concurrently given with diazoxide 10 minutes before the long ischemia.

Infarct Size Measurements

At the end of each experiment, the coronary artery subjected to occlusions was reoccluded. The heart was then perfused with 5- to 10-μm zinc-cadmium sulfide yellow fluorescent particles (Duke Scientific, Inc) to identify the area at risk (areas without particles). Next, hearts were cross-sectioned into 4 or 5 slices and incubated in a 1.25% solution of triphenyl tetrazolium chloride, made with 0.2 mol/L Tris buffer (pH 7.4), at 37°C for 10 minutes. Using this staining method, viable tissue stains brick-red, and necrotic tissue looks white or tan. The necrotic, risk, and total areas from each heart slice were then traced onto an acetate sheet and computer planimetered to calculate the percentage of the biventricular area that was at risk (risk area/total area) and the area at risk that was necrotic (necrotic area/risk area).

Statistical Analysis

All data shown are expressed as mean±SEM. The isolated myocyte and buffer-perfused heart data were first tested for normality (Kolmogorov-Smirnoff test) and homogeneity of variance (Levene test). Because the criteria for parametric analysis were not met, we performed a nonparametric analysis using the multigroup-comparison Kruskal-Wallis test to assess for differences among the groups, followed by the post hoc Dunn procedure to determine whether a statistically significant difference (P<0.01) existed between 2 groups. ANOVA was performed in the whole-heart studies, in which the data were found to meet the criteria for parametric tests to assess for differences among the groups. Where appropriate, the Scheffe test was then applied to determine whether a statistically significant difference (P<0.05) existed between 2 groups. A regression analysis was performed to assess for any association between 2 measurements in each study. For all electrophysiological studies, the paired Student t test was used.

Results

Single-Cell Electrophysiology Studies

In Figure 2A, the magnitude of the outward current recorded from a representative myocyte is shown. Initially, the myocyte was superfused with the iso-osmotic external solution (290 mOsm). After 5 minutes, the hypo-osmotic external solution (215 mOsm) was applied, and this evoked a 2-fold increase in outward current after 4 minutes. Application of

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Figure 2. Effect of NPPB and IAA-94 on I<sub>Cl,swell</sub> and I<sub>Cl,b</sub>. A, Under iso-osmotic conditions (solid bar), I<sub>Cl,b</sub> is observed. Hypo-osmotic conditions (open bar) resulted in the activation of a time-dependent Cl– current (ie, I<sub>Cl,swell</sub>). Application of 1 μmol/L NPPB under hypo-osmotic conditions (left thin solid bar) resulted in inhibition of both I<sub>Cl,b</sub> and I<sub>Cl,swell</sub>. On removal of NPPB (right thin solid bar, ie, washout), both I<sub>Cl,b</sub> and I<sub>Cl,swell</sub> were normalized. B, Raw traces show that under iso-osmotic conditions (O in panel A), a modest Cl– current (ie, I<sub>Cl,b</sub>) was observed. Hypo-osmotic conditions resulted in a 2-fold increase in Cl– current (● in panel A). NPPB (1 μmol/L; + in panel A) blocked both Cl– currents. Blockade by NPPB was completely reversed by washout of Cl– channel inhibitor (□ in panel A). Dotted line represents 0 current. C and D, Mean (±SEM) I-V relationship curves for NPPB and IAA-94, respectively. Iso-osmotic conditions (C) produced I<sub>Cl,b</sub>. Under hypo-osmotic conditions (D), the Cl– current increased. NPPB (1 μmol/L) or IAA-94 (10 μmol/L) dramatically reduced Cl– current (●, in panels C and D, respectively). Washout of Cl– channel inhibitor with iso-osmotic solution (□) normalized current to basal level, whereas washout with hypo-osmotic solution (●) normalized current to basal plus swell-activated level.
NPPB significantly ($P<0.001$) reduced outward current to below that observed in iso-osmotic conditions. Washout of NPPB with hypo-osmotic external solution resulted in complete normalization of swell-activated outward current. Raw traces are shown in Figure 2B and mean ($\pm$SEM) current-voltage ($I$-$V$) plots in Figure 2C. Under iso-osmotic conditions, the currents were outwardly rectifying, as reported previously for Cl$^-$ currents. $^6$ The current reversed at $-27.8$ mV, close to the calculated Cl$^-$ reversal potential of $-30.4$ mV. After a 10-minute hypo-osmotic period, the current increased at all voltages, continued to outwardly rectify, and reversed at $-25.2$ mV. The outward current density increased by $46.3\%$ at $-60$ mV ($n=4$, $P<0.01$). This swell-activated current was time dependent, taking $5.6\pm2.2$ minutes ($\pm$SEM) to activate, consistent with a previous report.$^6$ Application of 1 $\mu$mol/L NPPB inhibited both iso-osmotic and hypo-osmotic currents. Washout of NPPB with iso-osmotic external solution restored outward current to baseline, while washout with hypo-osmotic solution restored outward current to a swel-activated level. Similar results were observed when IAA-94 was applied (Figure 2D). These results are consistent with the presence of a basal Cl$^-$ current ($I_{\text{Cl,b}}$) and $I_{\text{Cl,swell}}$, as previously reported in cardiac myocytes.$^6,7,10,36$

Previous studies have reported that NPPB (10 to 40 $\mu$mol/L) activates ATP-sensitive K$^+$ currents ($I_{\text{KATP}}$),$^{37}$ whereas NPPB (100 $\mu$mol/L) and IAA-94 (200 $\mu$mol/L) partially block Ba$^{2+}$ current through Ca$^{2+}$ channels,$^{38}$ albeit at concentrations much higher than those used in this study. The mean $I$-$V$ plots shown in Figure 3A and 3B summarize the effects of NPPB and IAA-94 on sarcolemmal $I_{\text{KATP}}$. In the absence of glucose and exogenous ATP, typical inward rectifier-like K$^+$ currents$^{40}$ were observed. External application of NPPB (C) or IAA-94 (D) did not activate $I_{\text{KATP}}$ (□). E, External application of 1 $\mu$mol/L NPPB (●) or 10 $\mu$mol/L IAA-94 (△) did not affect $I_{\text{Ca}}$ under control conditions (○).

Figure 3. Nonspecific effects of NPPB and IAA-94 on $I_{\text{KATP}}$ and $I_{\text{Ca}}$. A and B, In the absence of metabolic inhibition an inward rectifying current was observed (ie, $I_{\text{K1}}$). Metabolic inhibition resulted in activation of $I_{\text{KATP}}$ (●). External application of 1 $\mu$mol/L NPPB or 10 $\mu$mol/L IAA-94 (△, in panels A and B, respectively) did not have an effect on $I_{\text{KATP}}$. In contrast, application of 10 $\mu$mol/L glibenclamide (▲) significantly reduced $I_{\text{KATP}}$. C and D, In the absence of metabolic inhibition, $I_{\text{K1}}$ was observed (■). External application of NPPB (○) or IAA-94 (△) did not activate $I_{\text{KATP}}$. F, Application of 1 $\mu$mol/L NPPB (○) or 10 $\mu$mol/L IAA-94 (△) did not affect $I_{\text{Ca}}$ under control conditions (○).
modest traces from a typical cell. Iso-osmotic conditions resulted in a 4-fold inhibition of both $I_{Cl}$ and $I_{Cl,swell}$ (Figure 3C; $n=5$). Replacement of external iso-osmotic solution with a hypo-osmotic solution again activated $I_{Cl}$, resulting in complete inhibition of both $I_{Cl}$ and $I_{Cl,swell}$ in the absence of glucose and ATP. External application of NPPB (Figure 3C; $n=5$) had no effect on the current even after 20 minutes, which suggests that these blockers do not activate sarcolemmal $I_{KATP}$ nor block $I_{K1}$ currents at the concentrations used in this study. Also, NPPB or IAA-94 did not affect calcium current ($I_{Ca}$), as demonstrated by the mean I-V relations in Figure 3E ($n=4$).

To determine whether the $Cl$- channel blockers could directly activate sarcolemmal $I_{KATP}$, $K^+$ current (ie, $I_{K1}$) was recorded in the absence of glucose and ATP. External application of NPPB (Figure 3C; $n=4$) or IAA-94 (Figure 3D; $n=4$) had no effect on the current even after 20 minutes, which suggests that these blockers do not activate sarcolemmal $I_{KATP}$ nor block $I_{K1}$ currents at the concentrations used in this study. Also, NPPB or IAA-94 did not affect calcium current ($I_{Ca}$), as demonstrated by the mean I-V plots in Figure 3E ($n=4$).

Whether $I_{Cl,swell}$ is regulated by PKC in rabbit ventricle has not been previously determined. The plot in Figure 4A shows the time course of current magnitude in a typical cell under iso-osmotic conditions, whereas Figure 4B shows corresponding raw traces in response to voltage steps from –80 to +60 mV. Replacement of external iso-osmotic solution with a hypo-osmotic solution again activated $I_{Cl,swell}$. Application of chelerythrine (20 μmol/L) in the continued presence of hypo-osmotic solution, a concentration sufficient to block PKC, inhibited both $I_{Cl}$ and $I_{Cl,swell}$ in ventricular myocytes by 85% and 88%, respectively, at +60 mV ($n=4$, $P<0.01$). The effects of chelerythrine were observed at all of the voltages tested (Figure 4C).

**Isolated Ventricular Myocyte Studies**

Myocyte viability expressed as percentage (mean±SEM %) of dead myocytes for each group of cells during 180-minute SI and during 45-minute SI combined with 120-minute reperfusion are presented in Figures 5 through 8. There was no difference in the percentage of dead myocytes among untreated control myocyte groups or among untreated IP myocyte groups, with or without the vehicle, in all cell experiments at any time point (Figures 5 through 8). Because there was no difference in the percentage of dead myocytes between untreated and treated (with drug) oxygenated baseline myocytes, in each set of experiments, we have pooled the data for oxygenated baseline and presented it in a single line graph (Figures 5 through 8).

Furthermore, there was no difference between untreated control and untreated IP myocytes, in terms of percentage of dead myocytes, either before the long SI or after 30-minute SI in each set of experiments (Figures 5 through 8). In the first set of experiments (Figure 5A), IP significantly ($P<0.001$) limited the percentage of dead myocytes after 60-, 90-, or 120-minute SI, when compared with controls. A similar protective effect was also observed in the second set of experiments (Figure 5B). This protective effect of IP was not detected after 180-minute SI (Figure 5A and 5B). Inhibition of $Cl$- channels with 10 μmol/L IAA-94 or 1 μmol/L NPPB, each drug given before IP, completely abolished ($P>0.05$) the protection of IP against myocyte death caused by SI alone, whereas it did not increase myocyte mortality in treated controls, as shown in Figure 5A and 5B.

Next, we tested IAA-94 and NPPB using a model of SI combined with SR for isolated ventricular myocytes. In this model, IP significantly ($P<0.001$) reduced the percentage of dead myocytes caused by a combined 45-minute SI/120-minute SR (Figure 6A and 6B). Furthermore, this IP protection was completely abolished ($P>0.05$) when either 10 μmol/L IAA-94 or 1 μmol/L NPPB was added into the cell suspension 10 minutes before IP (Figure 1) to inhibit $Cl$- channels (Figure 6A and 6B). IAA-94 and NPPB had no effect on treated controls (Figure 6A and 6B).

To investigate the specific role of Cl- channels during the maintenance phase of IP and rule out the possibility that Cl- channel inhibition might have blocked the induction of IP, we added IAA-94 10 minutes before the long SI in both the SI-alone and combined SI/SR protocols. When the SI-alone protocol was used (Figure 7A), IP significantly ($P<0.0001$) reduced the percentage of dead myocytes after 60-, 90-, or 120-minute SI as compared with untreated controls. Similarly, IP significantly ($P<0.0001$) reduced the percentage of dead myocytes after 120-minute SR when the combined SI/SR protocol was used (Figure 7B). Complete inhibition of Cl- channels by IAA-94 produced a total blockade of the protective effect of IP (Figure 7A and 7B). In the same group of myocytes in which a combined SI/SR protocol was used, we assessed the morphology of myocytes (rod, square, and round shape) in each group. The percentage of rod-shaped myocytes was significantly ($P<0.05$) higher after 45-minute SI/120-minute SR in the preconditioned group (IP+vehicle, 58.8±3.3%, mean±SEM) as compared with the control group (C+vehicle 27.8±4.1%). The percentages of square...
and round myocytes were significantly (P<0.0001) lower in preconditioned groups (IP+vehicle 6.9±1.8% and 34.0±2.6%, respectively) as compared with control groups (C+vehicle 17.3±4.1% and 53.9±2.6%, respectively). This IP effect on myocyte morphology was completely abolished by 10 μmol/L IAA-94 (IP+IAA-94 19.0±3.8% square and 56.3±1.5% round myocytes) as compared with untreated IP (IP+vehicle).

Moreover, we found no correlation (P>0.05) between the percentage of dead myocytes for either IAA-94–treated or NPPB-treated control and IP myocytes as compared with oxygenated baseline myocytes at 60-, 90-, and 120-minute SI in the ischemia-alone protocol, and at 15-, 60-, and 120-minute SR in the ischemia/reperfusion protocol. Thus, the increase in the percentage of dead myocytes in IP cells treated either with IAA-94 or NPPB, was, in all likelihood, due to the inhibition of the IP protective effect rather than the consequence of increased myocyte mortality rate with the preparation.

We explored the effect of IAA-94 and NPPB at concentrations that completely blocked IP, on diazoxide-induced protection against myocyte death during ischemia.33,34 Diazoxide

**Figure 5.** A, Effect of Cl− channel inhibition, with IAA-94 (10 μmol/L) administered before brief SI, on the protective effect of IP against myocyte death caused by SI alone. IP (●) significantly reduced myocyte death after 60-, 90-, and 120-minute SI as compared with controls (■). Inhibition of Cl− channel with IAA-94 completely inhibited the protection of IP (■), whereas it did not have an effect on controls (□). B, Effect of Cl− channel inhibition, with NPPB administered before brief SI, on the protective effect of IP against myocyte death caused by SI alone. IP (●) significantly reduced myocyte death after 60-, 90-, and 120-minute SI as compared with controls (■). Inhibition of Cl− channels with NPPB completely inhibited the protection of IP (■), whereas it did not have an effect on controls (□). In both series of experiments, the percentage of dead myocytes did not increase significantly after an incubation period in oxygenated buffer at 37°C equivalent to control and IP protocols in time (oxygenated baseline, +). All data are expressed as mean±SEM. *P<0.0001.
significantly \( P < 0.001 \) reduced myocyte mortality after either 90 \( (24.7 \pm 2.9\%) \) or 120 \( (27.7 \pm 1.4\%) \) minutes of SI when compared with controls \( (50.0 \pm 2.9\% \text{ and } 49.0 \pm 2.0\%, \text{ respectively}) \). Blockade of Cl\(^{-}\) channels either with IAA-94 or NPPB did not inhibit diazoxide protection \( \text{diazoxide} + \text{IAA-94, } 24.8 \pm 3.8/26.6 \pm 1.5\% \text{ [90-/120-minute SI]; diazoxide} + \text{NPPB, } 27.1 \pm 3.1/27.8 \pm 1.5\%, \text{ respectively}) \), indicating that IAA-94 and NPPB have no effect on mitochondrial K\(_{ATP}\) channels.

The results above show that Cl\(^{-}\) channels blockers can inhibit preconditioning, whereas Cl\(^{-}\) channels can be activated by hypo-osmotic stress. Therefore, we examined whether hypo-osmotic stress could induce protection. Suspending myocytes in hypo-osmotic buffer \( (80\text{-mOsm osmotic gradient}) \) for 10 minutes and then resuspending them in iso-osmotic buffer for 20 minutes before the long SI produced protection similar to IP. The percentage of dead myocytes was significantly \( P < 0.0001 \) reduced in myocytes subjected to hypo-osmotic stress after 60-, 90-, or 120-minute SI compared with control \( (P > 0.0001) \). In both series of experiments, the percentage of dead myocytes did not increase significantly after an incubation period in oxygenated buffer at \( 37^\circ\text{C} \) equivalent to control and IP protocols in time \( \text{oxygenated baseline, +} \). All data are expressed as mean \( \pm \text{SEM.} \quad * P < 0.0001. \)
and 120-minute SR compared with control (Figure 8B). Blockade of Cl– channels either with IAA-94 or NPPB completely abolished the protection induced by hypo-osmotic stress (Figure 8A and 8B).

### Isolated Heart Studies

There was no difference among all groups, in terms of LVDP, heart rate, and coronary flow, after 15-minute stabilization or before ischemia or after 40-minute regional myocardial ischemia. Preconditioned hearts treated with IAA-94 or control (with vehicle) hearts showed significantly higher heart rate and lower coronary flow, respectively, at the end of reperfusion, when compared with preconditioned (with vehicle) hearts (data not shown). However, a regression analysis performed on the function (LVDP and heart rate) and the infarct size data between these groups indicated that there was no association (P>0.05) between either heart rate or coronary flow and infarct size.

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**Figure 7.** A, Effect of Cl– channel inhibition, with IAA-94 (10 μmol/L) administered just before long SI, on the protective effect of IP against myocyte death caused by SI alone. IP (■) significantly reduced myocyte death after 60-, 90-, and 120-minute SI as compared with controls (○). Inhibition of Cl– channels with IAA-94 completely blocked the protection of IP (■), whereas it did not increase cell mortality in controls (□). B, Effect of Cl– channel inhibition, with IAA-94 administered just before long SI, on the protective effect of IP against myocyte death caused by combined 45-minute SI and 120-minute SR. IP significantly reduced myocyte death after SR (■) as compared with controls (□). Inhibition of Cl– channels with IAA-94 (10 μmol/L) completely blocked the protection of IP (■), whereas it did not increase cell mortality in control myocytes (□). In both experiments, the percentage of dead myocytes did not increase significantly after an incubation period in oxygenated buffer equivalent to the control and IP protocols in time (oxygenated baseline, +) in both cell models. All data are expressed as mean±SEM. *P<0.0001.
IP significantly ($P<0.0001$) reduced infarction within the myocardium at risk (control+vehicle versus IP+vehicle), as shown in Figure 9. Administration of the Cl– channel inhibitors, either IAA-94 (10 μmol/L) or NPPB (1 μmol/L), before the long ischemia, completely blocked the protection against infarction by IP, whereas they did not alter infarct size in controls (Figure 9). The area at risk did not differ among the groups (data not shown).

Pretreatment with 100 μmol/L diazoxide for 15 minutes before ischemia significantly ($P<0.001$) reduced infarct size as shown in Figure 9. This protective effect of diazoxide was not inhibited by blockade of Cl– channels with 10 μmol/L IAA-94. No significant differences were observed in LVDP and heart rate after 60-minute reperfusion between diazoxide-treated and control hearts. Diazoxide significantly ($P<0.001$) increased coronary flow, as previously reported by Garlid et al. in the presence or absence of Cl– channel inhibition by IAA-94 or NPPB (data not shown).

Figure 8. A, Effect of Cl– channel inhibition on the hypo-osmotic (80-mOsm osmotic gradient) protective effect against myocyte death caused by SI alone. Hypo-osmotic stress (■), given in place of IP (○), significantly reduced myocyte death to the same extent as IP after 60-, 90-, and 120-minute SI as compared with controls (○). Inhibition of Cl– channels with either 10 μmol/L IAA-94 (■) or 1 μmol/L NPPB (□) completely blocked the protection induced by hypo-osmotic stress. B, Effect of Cl– channel inhibition on the hypo-osmotic stress protective effect against myocyte death caused by combined 45-minute SI and 120-minute SR. Hypo-osmotic stress (■), in place of IP (■), significantly reduced myocyte death to the same extent as IP after combined SI/SR as compared with controls (○). Inhibition of Cl– channels with either 10 μmol/L IAA-94 (■) or 1 μmol/L NPPB (□) completely blocked the protection induced by hypo-osmotic stress. In both experiments, percentage of dead myocytes did not increase significantly after an incubation period in oxygenated buffer at 37°C equivalent to each protocol (control, IP, and hypo-osmotic stress) in time (oxygenated baseline, +) in both cell models. All data are expressed as mean±SEM. *$P<0.0001$. 

(A) ISCHEMIA PROTOCOL

(B) ISCHEMIA/REPERFUSION PROTOCOL
The principal aim of the present study was to assess the participation of Cl– channels in the protection of IP against ischemic injury alone and combined ischemic/reperfusion injury. In isolated rabbit cardiomyocytes IP reduced myocyte death during a 180-minute SI period (Figure 5). These results are in agreement with previously published reports by other investigators in the same model.18,40 A reduction in cell death after 45-minute SI/120-minute SR was also observed with IP (Figure 6A and 6B). Similarly, in buffer-perfused isolated rabbit hearts, IP (3 cycles of 5-minute ischemia followed by 10-minute reperfusion) significantly reduced necrosis after 40-minute ischemia and 60-minute reperfusion, as reported previously.15 Our results show that the protection of IP was abolished by Cl– channel inhibition with IAA-94 or NPPB in isolated cardiomyocytes (Figures 5 through 7) and isolated hearts (Figure 8).

Chloride channel blockers were shown in our experiments to abolish the protection of IP when added either before IP or before the long ischemia. Previous studies have separated the protection of IP into an initiation phase, which involves the activation of signal transduction during the short IP ischemia, and a maintenance phase, involving those events related to IP activation during the short IP ischemia, protection of IP into an initiation phase, which involves the participation of Cl– channels in the protection against necrosis.41 It is not clear whether Cl– channels are involved in the initiation. To test this, Cl– channels must be blocked only during the initiation phase, requiring complete washout of blockers during the 20-minute reperfusion period before the long ischemia. Unfortunately, the ability of NPPB and IAA-94 to block the initiation of IP could not be tested, because washout of Cl– channel blockers required at least 20 minutes as assessed in our electrophysiological experiments with continuous superfusion. Nevertheless, the protection achieved by a single brief episode of hypo-osmotic stress in place of IP supports a role for Cl– channel activation in protection during the initiation phase of IP, since this hypo-osmotic stress results in Cl– channel activation (Figure 2). In addition, Cl– channels clearly play a role in the maintenance phase, since Cl– channel inhibition before and during the long ischemic period abolished the protection of IP in both isolated cardiomyocytes (Figure 7) and buffer-perfused hearts (Figure 9).

In the present study, we used the whole-cell patch-clamp technique to demonstrate that rabbit ventricular myocytes possess currents that were inhibited by the specific Cl– channel blockers NPPB and IAA-94. Under iso-osmotic conditions designed to isolate Cl– currents, the current recorded reversed close to the reversal potential for Cl–, did not inactivate, and was outwardly rectifying, as reported previously.6,7,36 Under hypo-osmotic conditions, the Cl– current, with properties otherwise similar to those of the I_{Cl,b}, increased 2-fold, as previously reported in cardiac myocytes.6,7,36 The recorded currents were largely abolished by IAA-94 (10 μmol/L) or NPPB (1 μmol/L), as expected if the basal and swell-activated conductances are Cl– currents (I_{Cl,b} and I_{Cl,swell}, respectively).

IAA-94 and NPPB have been used to selectively inhibit Cl– channels in different cell types.47,48 It is noteworthy that NPPB and IAA-94 applied at concentrations much greater than those used in this study affect sarcolemmal I_{KATP}38 and I_{KATP}.39 This is important, because inhibition of these channels has been shown to block IP.42,43 However, at the concentrations used in this study, NPPB and IAA-94 had no effect on sarcolemmal I_{KATP} and I_{KATP}. Furthermore, these blockers had no effect on I_{K1}, PKC activity, and the protection produced by selective stimulation of mitochondrial KATP channels with diazoxide (Figures 3, 4, and 9), which supports the notion that the abolition of IP protection against necrosis was the result of Cl– channel blockade.

The conclusions from the isolated cardiomyocyte studies depend on correctly distinguishing viable from necrotic cardiomyocytes. The trypan blue staining solution used to assay cell death was hypo-osmotic (85 mOsm) to compensate for the lack of mechanical forces on cardiomyocytes generated by intercellular attachments in the heart that play a major role.
role in ischemia/reperfusion injury.\textsuperscript{35,44} Because an osmotic gradient is used to test the fragility of the cell membrane during the trypan blue viability assay, it could be argued that cardiomyocytes exposed to Cl\textsuperscript{–} channel blockers might be more susceptible to hypo-osmotic stress. This, being distinct from relative injury to the cell membrane during the long SI, might therefore influence the results. However, in the present studies, the cardiomyocytes were immediately (<1 minute) fixed in glutaraldehyde and rendered metabolically inactive by amylobarbitone at the same time as exposure to trypan blue. In contrast, Cl\textsuperscript{–} channel activation by hypo-osmotic stress typically requires \(\approx\)5 minutes (Figure 2). This suggests that the Cl\textsuperscript{–} channel blockers are affecting events occurring during the ischemia, and these results are not artifacts of the cellular model. This is consistent with the results in the isolated hearts, which mirrored the findings in isolated ventricular myocytes.

Our inclusion of SR after the long SI in the isolated cardiomyocyte model is novel. In the absence of IP, the extent of cardiomyocyte death seen after 120-minute SI after 45-minute SI was similar to that observed in rabbit hearts on reperfusion in vivo after 30-minute regional ischemia.\textsuperscript{45} In the presence of IP, cardiomyocyte death during SR as assessed by trypan blue staining was significantly reduced compared with control (Figures 6, 7B, and 8B). Rounded cardiomyocytes are generally not viable, and the IP protection corresponded to a significant reduction in the proportion of round cells on SR. Interestingly, IP also promoted the recovery during SR from square-shaped myocytes after 45-minute SI to rod-shaped myocytes, resulting in a significantly higher proportion of rod-shaped myocytes after 120-minute SR. This was not observed either under control conditions or when IP was blocked by Cl\textsuperscript{–} channel inhibition.

In the present study cardiomyocyte swelling, which occurs during ischemia, activates PKC, whereas IP is abolished by PKC inhibition. Consistent with this, the specific PKC inhibitor chelerythrine inhibited \(I_{\text{Cl,swell}}\) in rabbit ventricular myocytes, as in previous reports in noncardiac tissue,\textsuperscript{46,47} but in contrast to rabbit atrial myocytes.\textsuperscript{48} Differences in PKC regulation of this current between atrial and ventricular myocytes may be accounted for by regional differences within the heart.

During ischemia, cardiomyocytes swell\textsuperscript{1} and respond by extruding \(K^+\), Cl\textsuperscript{–}, organic solutes, and water, in an attempt to restore cell volume.\textsuperscript{49} Three major ion-transport mechanisms have been postulated to contribute to cell volume regulation, including cation-coupled cotransport (\(K^+$/Cl\textsuperscript{–} or Na\textsuperscript{+}/K\textsuperscript{+}/2Cl\textsuperscript{–}), K\textsuperscript{+}/H\textsuperscript{+} exchange coupled to Cl\textsuperscript{–}/HCO\textsubscript{3}\textsuperscript{–} exchange, and both K\textsuperscript{+} and Cl\textsuperscript{–} diffusion pathways.\textsuperscript{4,50,51} Furthermore, given that activation of sarcolemmal \(I_{\text{K,ATP}}\) results in \(K^+\) movement, this channel might also play a role in volume regulation of the myocyte and mitochondria. Swell-activated and other Cl\textsuperscript{–} channels are known to play an important role in cell volume regulation.\textsuperscript{4,52} However, if volume regulation depends on Cl\textsuperscript{–} channels alone, it would be anticipated that ischemic and/or reperfusion-induced necrosis would be greater in tissue treated with Cl\textsuperscript{–} channel inhibitors compared with nontreated tissue. In this study, this was not the case either in isolated cardiomyocytes or isolated hearts. This does not necessarily mean that Cl\textsuperscript{–} channels are not involved in cell volume regulation. Rather, it would be advantageous for cells to have several volume-regulatory mechanisms working in concert or independently.

In the rabbit ventricle, protein kinase A–activated\textsuperscript{53} and Ca\textsuperscript{2+}-activated\textsuperscript{22,23} Cl\textsuperscript{–} currents are known to exist. Furthermore, we demonstrate the presence of \(I_{\text{Cl,swell}}\). Since available Cl\textsuperscript{–} channel blockers are not specific for any 1 type of Cl\textsuperscript{–} channel, specific Cl\textsuperscript{–} channel involvement in IP cannot be distinguished pharmacologically. However, we demonstrate that hypo-osmotic stress activated \(I_{\text{Cl,swell}}\) in rabbit ventricular myocytes and resulted in protection from ischemic and reperfusion injury (Figures 2 and 8). This implies that \(I_{\text{Cl,swell}}\) in particular, might play an important role in IP. Given that cardiomyocyte swelling is associated with ischemic injury,\textsuperscript{1–3} Cl\textsuperscript{–} channels are known to play an important role in cell volume regulation,\textsuperscript{4} and both swell-activated Cl\textsuperscript{–} channels (Figure 4) and IP\textsuperscript{54} are linked to PKC activity, we postulate that IP affects swell-activated Cl\textsuperscript{–} channels such that they are activated earlier and/or to a greater extent during the long ischemia. If this is correct, swell-activated Cl\textsuperscript{–} channels may act as an end effector of IP through improved cell volume control during the long ischemia. We believe that cell swelling may prove to be a useful unifying perspective from which to examine IP.

In summary, the present study demonstrates the presence of IAA-94–sensitive and NPPB-sensitive swell-activated and basal Cl\textsuperscript{–} currents, which are inhibited by PKC blockade, in ventricular myocytes. Inhibition of these Cl\textsuperscript{–} currents completely abolishes IP protection against necrosis induced by ischemia and reperfusion in isolated rabbit ventricular myocytes and buffer-perfused rabbit hearts. Conversely, Cl\textsuperscript{–} channel activation by hypo-osmotic stress mimics the protection of IP, and this protection is also abolished by Cl\textsuperscript{–} channel blockade.

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Chloride Channel Inhibition Blocks the Protection of Ischemic Preconditioning and Hypo-Osmotic Stress in Rabbit Ventricular Myocardium

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