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Remote Ischemic Preconditioning Cardio-protection via Enhanced Cell Volume Regulation Requires Activation of Swelling-Activated Chloride (Icl-Swell) Channels

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Abstract

Introduction

We have previously shown that remote ischemic preconditioning induced in cardiomyocytes by a brief direct incubation in rabbit blood dialysate, originated from other rabbits initially subjected to brief periods of limb ischemia/reperfusion, given prior exposure to a prolonged ischemia/reperfusion protects cardiomyocytes against necrosis. In this study, we examined the hypothesis that sarcolemmal protein kinase C epsilon interacts with the swelling-activated Cl- channel to both enhance cardiomyocyte volume regulation and protect against cardiomyocyte necrosis in limb ischemia/reperfusion remote ischemic preconditioning.

Methods

Cultured (forty-eight hours) rabbit cardiomyocytes (control and remote ischemic preconditioned dialysate treated) were, after stabilization, subjected to either thirty-minute hypo-osmotic stress or to seventy-five minutes of simulated ischemia (severe hypoxia plus metabolic inhibition) followed by sixty minutes of simulated reperfusion (in oxygenated media), with or without a specific swelling-activated chloride channel inhibitor or its vehicle given ten minutes prior and during the hypo-osmotic stress or the simulate ischemia, to measure peak cell swelling (between eight to twelve minutes of hypo-osmotic stress), regulatory volume decrease and cell necrosis (by trypan blue staining).

Results

Specific inhibition of swell-activated chloride channels not only substantially inhibited remote ischemic preconditioned dialysate induced protection against cardiomyocyte necrosis but it also significantly impaired cardiomyocyte volume regulation. PKCε was found to co-immunoprecipitate with ClC-3, consistent with this kinase influencing swell-activated chloride channel activity.

Conclusion

These findings indicate that swelling-activated chloride channels are essential for the cardioprotection by remote ischemic preconditioning.

Keywords: Remote ischemic preconditioning; Protein kinase C epsilon; Chloride channels; Clc-3; Cell volume regulation; Cardiomyocyte

Introduction

In remote ischemic preconditioning (rIPC), brief cycles of ischemia (I), followed by reperfusion (R), typically 5 minutes I/5 minutes R, are applied to one organ or region of tissue with the goal of protecting one or more distant tissues/organs from I/R injury. Most practically, rIPC is applied to a limb and the target organ for protection from I/R injury of most interest has been the heart although there is good evidence for protection of other organs such as the brain and kidneys as recently reviewed [1]. Clinically, limb ischemia/reperfusion (LI/R) based rIPC has been shown to protect the myocardium in coronary stenting as documented by significant reduction in major cardiac and cerebral events at 6 months [2] persisting to 6 years [3]. The most recent meta-analysis of LI/R-rIPC in adult cardiac surgery confirms myocardial protection [4].

We have established that LI/R-rIPC releases one or more blood borne substance(s) which circulate(s) to substantially reduce infarct size after coronary artery ligation/reperfusion, associated with upregulation of mitogen-activated protein (MAP) kinases p42/44 and PKCε redistribution from cytosol to particulate fraction [5] (both biochemical signatures also seen in classic local ischemic preconditioning (IPC) in the heart). This we achieved by pre-treatment of Langendorff perfused rabbit hearts with plasma, and dialysate (molecular weight <15 kDa) of plasma, from other rabbits subjected to 4 cycles of 5 minute ischemia/5 minute reperfusion followed immediately by circulating blood collection. Dialysate collected from healthy human volunteers with similar LI/R timing but applied to a forearm also conferred cross-species protection in the rabbit heart Langendorff model [5]. Furthermore, we demonstrated that the LI/R dialysate greatly reduced

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necrosis in a freshly isolated rabbit ventricular cardiomyocyte model of simulated ischemia (SI) followed by simulated reperfusion (SR), with equivalent protection to IPC in this model [5]. It had previously been demonstrated in pigs that LI/R-rIPC in the recipient protected a porcine heart transplanted into the same animal, indicating delivery of a signal through nerves to the heart is not necessary for protection [6]. The cardiomyocyte model further showed the protection to be independent of conceivable humoral effects on neural tissue still present in isolated hearts as Langendorff preparations.

The end effector mechanisms by which LI/R-rIPC saves cardiomyocytes from I/R induced cell death have been little explored and isolated cardiomyocytes observed in primary cell culture together with our dialysate methodology provide an opportunity for important insights. In this study we demonstrate using mature rabbit ventricular cardiomyocytes in culture that the swell-activated Cl^- channel activity, which is most likely to be underlied by the ClC-3 channel [7], is essential to the humorally mediated cardioprotection of LI/R-rIPC as well as a substantial contributor to cell volume regulation in cardiomyocytes under physiological conditions. We also report the molecular association of ClC-3 channel with PKCε in the sarcolemma which is consistent with the well recognized importance of PKCε in mediating both IPC and rIPC [8].

Methods

All animal usage under our institutional animal protocol (No.12181, approved by our Institutional Animal Care Committee) referred in these studies conform to the Guide for the Care and Use of Laboratory Animals published by National Research Council (8th Edition, 2011, Washington, D.C.). All chemical were obtained from Sigma-Aldrich (Canada) unless stated otherwise. All chemical were obtained from Sigma-Aldrich (Canada) unless stated otherwise.

Isolation of cardiomyocytes

New Zealand White rabbits (weight range 3.0 to 3.5 kg) were used as heart donors to obtain ventricular cardiomyocytes using an enzymatic digestion method we have previously described [9-11].

Primary culture of cardiomyocytes

Following each cardiomyocyte isolation, cells were placed in primary culture in 35-mm bottom glass laminin-coated Petri dish and incubated, at 37ºC, in a humidified 5% CO_2-95% air mix environment for 48 hours, each dish containing 2 ml of culture medium 199 with Earle's salts (Gibco, Burlington, ON, Canada) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 10 μM cytosine β-D-arabinofuranoside, 100 mg/ml streptomycin, and 0.08 mg/ml gentamicin, as we have previously reported [12].

Experimental protocols

Cell necrosis studies in cultured cardiomyocyte: After 48 hours in culture, cardiomyocytes were initially stabilized for 30 min in media 199 supplemented only with 0.1% BSA and kept at 37ºC in an incubator. After stabilization, cardiomyocytes were remotely preconditioned (rIPC cardiomyocytes) by incubating them for 10 min in re-constituted rabbit blood dialysate obtained from rabbit donors which has been remotely preconditioned as we have previously described [5], then followed by a 10 min washout period (incubation in media 199 plus BSA only). Control (C) cardiomyocytes were similarly incubated in control (non-rIPC) dialysate. Next, all cardiomyocytes were subjected to 75 min of simulated ischemia (SI) followed by 60 min of simulated reperfusion (SR). Prolonged ischemia was simulated by incubating cardiomyocytes, at 37ºC in a 100% N_2 atmosphere, in a hypoxic HEPES-based buffer (37ºC) containing (in mM) 139 NaCl, 12 KCl, 0.5 $MgCl_2$, 0.9 $CaCl_2$, 5 HEPES, 10 2-deoxy-D-glucose, 20 DL-lactic acid, and 0.1% BSA, with pH adjusted to 6.5, for the purpose of simulating the advanced effects of ischemia in whole hearts [13]. We first characterized the protection of rIPC dialysate in our 48-hour culture cardiomyocyte model and then determined if protection against cell necrosis induced by rIPC dialysate pretreatment in cardiomyocytes can be abolished by concurrent treatment with the very selective blocker of native swelling-activated Cl- channel DCPIB (10 μM, 4-(2-Butyl-6,7-dichlor-2-cyclopentyl-indan-1-on-5-yl) oxybutyric acid) given 10 min prior and during the SI. Since swelling-activated Cl- channel is directly associated with the CLC-3 Cl- channel in cardiomyocytes [7], we use DCPIB to functionally impair CLC-3 mediated trans-sarcolemmal Cl- movement in our experiments. At the concentration we used in our experiments DCPIB has been reported to have no effect on the activity of $I_{Cl, Ca}$, or on cloned Cl- channels heterologously expressed in Xenopus oocytes (CLC-1, -2, -4, -5, CLC-K1 and hCFTR) or on native currents in guinea-pig cardiomyocytes (I_{KS}, I_{KR}, I_{K1}, I_{Na}, ICa and I_{Cl},P_{KA}) [7].

The percentage of dead cardiomyocytes was used to measure cell death in cultured cardiomyocytes. Cell death was measured at two time points: prior to the long simulated ischemia (SI) and at the end of simulated reperfusion. We used trypan blue staining to distinguish dead cardiomyocytes (cells stained dark blue) from non-stained live cardiomyocytes. Using an inverted microscope equipped with a charge coupled device camera controlled by a computer, colour images from 4-5 randomly selected fields (200X magnification) were obtained and stored for subsequent analysis. In each experiment, at each time point, we counted at least 500 cardiomyocytes (dead + live) to determine the % of dead cardiomyocytes using the following formula: % of dead cardiomyocytes=((Total # of dead cardiomyocytes)/Total # of cardiomyocytes)) × 100.

Hypo-osmotic induced cell swelling and cell volume regulation studies: To examine the contribution that CLC-3 (swelling-activated Cl- channel) in the cardiomyocyte volume regulatory mechanism, cardiomyocytes were first stabilized for 30 minutes in oxygenated (95% O_2-5% CO_2) iso-osmotic Tyroid buffer (300 mOsm) and then subjected under oxygenated conditions (at 37ºC), to a 30-minute hypo-osmotic stress (HS, 200 mOsm). Changes in cell volume at peak swelling between (HS time=8 to 12 min) and at the end of 30-minute HS were measured using a method we have previously described [14,15].

Cardiomyocyte regulatory volume decrease (RVD) was calculated using the following formula: RVD=[(% change in cell volume at peak swelling)-(% change in cell volume at 30 min HS)]/(% change in cell volume at peak swelling). The swelling-activated Cl- channel blocker DCPIB (10 μM) or its vehicle (DMSO) was administered 10 min prior and during the entire hypo-osmotic stress period.

Co-Immunoprecipitation and Western blot analysis: While keeping all the reagents on ice at all times, freshly isolated cardiomyocytes were suspended in lysis buffer (100 mM NaCl, 50 mM TRIS (pH 7.5), 2.5 mM, EDTA (pH 7.5), 2.5 mM EGTA (pH 8), 10 mM benzamidine, 5 g/ml aprotinin (Roche Applied Sciences, USA), 5 g/ml leupeptin, 5 g/ml pepstatin A, 0.5 mM PMSF, 1 mM Na_3VO_4, 0.3% β-mercaptoethanol). The lysis buffer containing cardiomyocytes was then transferred into pre-chilled Eppendorff tubes and incubated at 4°C on a steady rotation for 30 min. Then, while on ice, the cell suspension was sonicated continuously (three times) for 7 seconds with 5-second intervals using a 60 Sonic Dismembrator (Fisher Scientific, USA). The lysis buffer with cardiomyocytes was then incubated again at 4°C on a steady rotation

for another 30 min and cell membrane fraction obtained by differential sucrose gradient removal of mitochondrial fraction and further spins to separate endosomes from the sarcolemma as previously reported by Fuller et al. [16]. Briefly, the lysis buffer with cardiomyocytes was first centrifuged for 10 min at 10,000 g (at 4°C) to sediment down cellular organelles, nuclei and cell debris. The resulting pellet was discarded and the supernatant was centrifuged at 40,000 g. The resulting supernatant was discarded and the pellet incubated for 30 min, while on ice, in 200 µl lysis buffer containing 0.5% Triton X-100. Sonication was performed, followed by the sucrose gradient centrifugation at 40,000 g (at 4°C) and the resulting pellet from this centrifugation was stored (at -80°C) and represented the final detergent soluble cell membrane fraction that was used for the co-immunoprecipitation assays. Protein concentration was measured by the Bradford method using BioRad Protein Assay (Bio-Rad, USA). A standard curve was constructed with varying concentrations of BSA and each absorbance measured by a spectrophotometer (at 595 nm) was determined. Protein sample concentration was determined by plotting each measurement on a standard curve. In immunoprecipitation studies, enough cell membrane fraction volume was placed into pre-chilled microcentrifuge tubes each containing a protein concentration of 3 mg/ml. Each sample was then topped up to a total volume of 1 ml with cold lysis buffer containing 0.5% Triton X-100, pre-cleared by adding Protein G PLUS-agarose beads (Santa Cruz, USA) and incubating for 1 hr (at 4°C), on a steady rotation. Next, samples were centrifuged at 10,000 g to collect the agarose beads in the pellet. The pre-cleared supernatant was then transferred to a fresh pre-chilled Eppendorff tube and incubated with either anti-PKCε (BD Transductions, USA) or anti-ClC-3 antibodies (Sigma, Canada) overnight at 4°C on a steady rotation. The next day, antibody/protein complexes were then allowed to form on the surface of newly added protein G agarose beads during 3 hours incubation at 4°C. Agarose beads were then precipitated by centrifugation (1000 g for 10 minutes). Once the supernatant was discarded, the agarose beads (pellet) containing the pulled down protein complexes were washed 3 times with lysis buffer to remove unbound antibodies and proteins, boiled for about 7 minutes to denature proteins and centrifuged (1000g for 10 minutes) to remove agarose beads (pellet). The resulting supernatant containing the pulled down proteins was loaded onto a Tris-Glycine 4-12% gel and separated by SDS-polyacrylamide gel electrophoresis (20 minutes at 80 V followed by 2 hours at 120 V) along with a 10 µl of molecular weight protein marker (Fermentas, USA). Proteins were then transferred onto polyvinylidene fluoride (PVDF) membranes with both agarose gel and PVDF membrane equilibrated in transfer buffer (25 mM Tris, 192 mM 40 glycine, 20% methanol, pH 8.42). The transfer continued for 1 hr at 100 V at room temperature or overnight at 30 V in the cold room. After the transfer, each PVDF membrane was then blocked (as per in the Western blot assay) for 1 hour at room temperature and incubated overnight (at 4°C) with appropriate primary antibodies against the protein of interest, followed by a suitable secondary antibody (either goat anti-mouse or goat-anti rabbit IgG conjugated to horseradish peroxidase) incubation for 1 hour at room temperature. Between each step (blocking, primary and secondary antibody incubation), each PVDF membrane was washed 3 times for 10 minutes with washing buffer. The success of each protein transfer was demonstrated by staining each PVDF membrane with Ponceau S (0.5% Ponceau S staining solution, 1% glacial acetic acid (Fisher Scientific. USA). Gels were also stained after transfer by incubation in 25 ml Coomassie Brilliant Blue (0.25% Coomassie Brilliant Blue (w/v) (Bio-Rad, USA), 45% methanol, 2% glacial acetic acid) for 30 min. Next, PVDF membranes were incubated in 10 ml of 5% w/v TBS Blotto A (Santa Cruz, USA) blocking solution for 1 hr, room temperature, on a rotating platform at 2.5 rpm. Each membrane was quickly rinsed in Tris-buffered saline, TBST (49.3 mM Tris, 149 mM NaCl, 0.1% Tween-20) before it was incubated in a heat sealed plastic pouch in 2 ml of 5% w/v TBS Blotto A solution with 1:1000 anti-PKCε antibodies (BD Transductions, USA) or 1:000 Anti-ClC-3 antibodies (Alomone, Israel) for 1 hr at room temperature or overnight at 4°C. Then, each membrane was washed three times in 25 ml of TBS and then immersed in 10 ml of 5% w/v TBS Blotto A containing the secondary antibodies (goat anti-mouse IgG for anti-PKCε antibodies, goat anti-rabbit IgG for anti-ClC-3 antibodies) conjugated to horseradish peroxidase for 1 hr at room temperature on a rotating shaker. Each membrane was then washed three times in TBST and protein bands were visualized by enhancing the chemiluminescence of horse radish peroxidase using the ECL Plus Western Blotting Detection System (Amersham GE Healthcare) and exposing membranes to films to show the protein bands. Furthermore, a careful distinction between the expression observed with the anti-PKCε antibody and the expression observed with anti-ClC-3 antibody was made by stripping PVDF membrane from one antibody and then again incubating with the other antibody. We carefully assessed for the efficiency of the stripping process by exposing each film to a PVDF membrane immediately after completing the stripping procedure to confirm the success of the stripping procedure (absence of protein expression on film) before incubation with the other antibody was performed.

Statistical analysis

Data were first analyzed for normal distribution (Kolmogorov-Smirnov test) and equality of variance (F-test). Where data was found to be suitable for analysis by parametric testing we performed an analysis of variance (ANOVA) followed by a post-hoc test (Scheffe's test). If data were appropriate for analysis by non-parametric tests we performed a Kruskall Wallis test followed by a post-hoc test (Mann-Whitney U test). Significant difference was achieved with $P<0.05$.

Results

To determining if I_{Cl}-Swell channel activity is essential for remote ischemic preconditioning protection against cell necrosis caused by a long period of simulated ischemia (75 min)/simulated reperfusion (60 min), we inhibited ICl-Swell channels before treatment and during the SI/SR period in an attempt to block protection by rIPC dialysate treatment. Treatment of cardiomyocytes with rIPC dialysate, administered for 10 min and followed 10 min of washout prior to SI/SR significantly ($P<0.0001$) reduced the % of dead cardiomyocyte, compared to cardiomyocytes pretreated with control dialysate (Figure 1). DCPIB (10 µM), administered 10 min prior and during SI/SR completely abolished protection by rIPC dialysate (rIPC+DCPIB) while it has not effect on cell necrosis in control dialysate treated cardiomyocytes (C+DCPIB).

In order to confirm that the intended effect of DCPIB (inhibition of $I_{Cl\text{-}Swell}$ channels) is directly associated with impairment of cell volume control in cardiomyocytes, we tested whether 10 µM DCPIB is able to impair substantially cell volume regulation in cardiomyocytes, under oxygenated conditions, while exposed to 200 mOsm hypo-osmotic stress (HS). As expected, untreated cardiomyocyte volume increased substantially reaching a peak between 8-12 min of HS and substantially recovered by 30 min of HS. DCPIB treatment during HS did not affect peak swelling change in volume but significantly impaired the recovery after 30 min of HS. This impairment in cell volume control is shown in Figure 2 where RVD is significantly ($P<0.025$) inhibited by DCPIB, compared to vehicle treated cardiomyocytes. The RVD analysis was generated from the % change in cell volume presented in the Table 1.

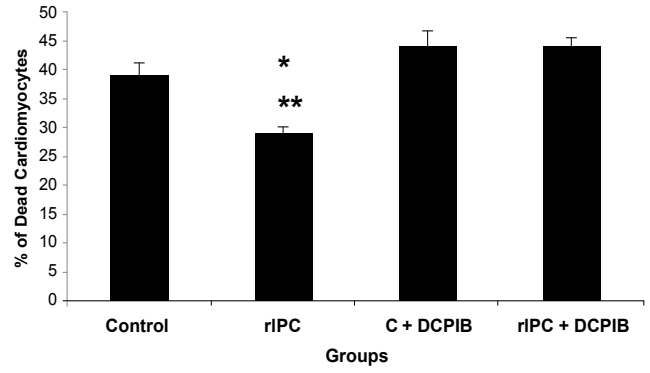

Figure 1: Blockade of rIPC cardioprotection by $I_{Cl\text{-}Swell}$ **inhibition.** This graph shows the effect of selective $I_{Cl\text{-}Swell}$ inhibition with 10 μM DCPIB on protection by rIPC against cell necrosis caused by 75 min SI/60 min SR in 48-hour cultured cardiomyocytes. Treatment of cardiomyocytes with rIPC dialysate, administered for 10 min and followed 10 min of washout prior to SI/SR significantly (*) reduced the % of dead cardiomyocyte (rIPC 28.9±2.9%), compared to cardiomyocytes pretreated with control dialysate treated (control 39.0±2.4%). DCPIB (10 μM), administered 10 min prior and during SI/SR completely abolished protection by rIPC dialysate (rIPC+DCPIB 44.0±2.7%) while it has not effect on cell necrosis in control dialysate treated cardiomyocytes (C+DCPIB 44.1±2.4%, P=0.2822 vs control). Data are mean±SEM (n=4 hearts/group). *P<0.030 vs control dialysate and rIPC+DCPIB; **P=0.034 vs rIPC+DCPIB.

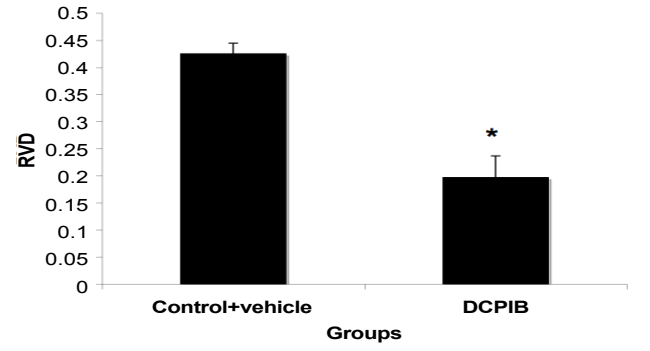

Figure 2: Impairment of cardiomyocyte cell volume regulation by inhibition of $I_{Cl\text{-}Swell}$. This graphs shows the effect of $I_{Cl\text{-}Swell}$ inhibition with 10 mM DCPIB on the regulatory volume decrease (RVD) in oxygenated cardiomyocytes calculated from the changes measured in cell volume shown in graph A. DCPIB significantly impaired the ability of cardiomyocytes to regulate cell volume, as demonstrated by the magnitude of RVD inhibition. All data are mean±SEM (N= 3 hearts, n= 50 cells/group). *P<0.025 vs untreated control.

These findings establish a mechanistic explanation to the blockade of rIPC protection against cell necrosis in our cardiomyocyte primary culture model.

Finally, to explore a possible mechanistic link between cell volume regulation via ClC-3 channels and participation of PKCεin remote ischemic preconditioning, we investigated if, in our cardiomyocytes, an association between ClC-3 channel protein and PKCε exists in the sarcolemma. For this purpose, we immunoprecipitated PKCε from cell membrane fractions obtained from isolated rabbit cardiomyocytes using specific monoclonal anti-PKCε antibodies. Following separation of proteins from the precipitate, ClC-3 presence was confirmed by selective anti-ClC-3 antibody screening (Figure 3). Reverse pulldown with immunoprecipitation of ClC-3 confirmed the presence of PKCε in the precipitate.

Discussion

In this study, we demonstrate for the first time that specific

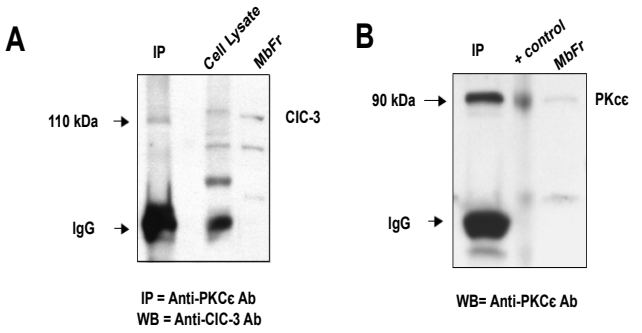

Figure 3: Co-immunoprecipitation of PKCε with ClC-3 Cl^- channel**.** The images show proteins in the immunoprecipitate (IP) resolved by Western blot (WB) and co-immunoprecipitation of ClC-3 with PKCε in cell membrane fractions (MbFr) from freshly isolated adult rabbit cardiomyocytes. A- Immunoblotting of PKCε precipitate probed with anti-ClC-3 antibodies demonstrated a protein band at the expected molecular weight for ClC-3 (100-110 kDa) in the immunoprecipitate (IP), cell lysate, and purified cell membrane (sarcolemmal) fraction (MbFr) samples. B- Re-probing the polyvinylidene fluoride (PVDF) membrane, after stripping, with anti-PKCε antibody showed a protein band at the expected molecular weight of 90 kDa for PCKε.

pharmacological blockade of the swelling-activated Cl- channel by DCPIB abrogates protection of humorally mediated LI/R-rIPC on mature rabbit ventricular cardiomyocytes under conditions of simulated ischemia and simulated reperfusion in cell culture.

Cardiomyocyte survival under conditions of I/R requires avoiding both necrosis and apoptosis and it is increasingly recognized that necrosis is by far the dominant mode of cell death following a single I/R episode [17]. Necrosis, according to the nomenclature committee on cell death, "is morphologically characterized by a gain in cell volume (oncosis), swelling of organelles, plasma s rupture and subsequent loss of intracellular contents" [18]. During ischemia, metabolites accumulate on cardiomyocytes, increasing intracellular osmolarity which tends to draw in osmotically obligated water from the extracellular space promoting cell swelling [19]. As ischemic cardiomyocytes approach irreversible injury they develop cell membrane fragility such that they do not tolerate mechanical forces imposed on the sarcolemma by cell swelling (osmotic fragility). On reperfusion, unlimited water is available to enter cardiomyocytes as osmolytes accumulated in the extracellular space are washed out which can lead to explosive cell swelling and necrotic (oncotic) cell membrane rupture [20].

Cell swelling activates a variety of transport pathways that result in a net efflux of Cl^-, K^+, H^+, organic anions and organic osmolytes with individual Cl^- and K^+ channels as the key contributors [21]. We have demonstrated that pan chloride channel inhibition blocked the protection of IPC against I/R necrosis in both Langendorff perfused hearts and isolated ventricular myocytes [11]. Furthermore, we have determined that the osmotic equivalent for IPC protection in cultured cardiomyocytes is 50-60 mOsm [15] and by quantitative fluorescence imaging of Cl- in the same model observed that IPC causes a net Cl^- efflux of ~30 mOsm [10]. To achieve charge balance across the sarcolemma an osmotic load of ~60mOsm should also require an efflux of about 30 mM of positive ions of which K^+ are a component. Using viral transfection of cardiomyocytes to overexpress dominant negative Kir 2.1 and 2.2 components in the IK1 channel we have demonstrated its essential role in IPC. Also, we have determined that ion channels which remove Cl- from cardiomyocytes are necessary for cell volume control under normoxic conditions and that IPC causes a striking reduction in cardiomyocyte swelling under conditions of simulated ischemia [15]. Our findings further support that cell volume dynamics

	Duration of Hypo-Osmotic Stress (min)		
Groups	**10**	**20**	**30**
Control	26.8 ± 1.5%	19.5 ± 1.8%	15.4 ± 1.2%
DCPIB	23.9 ± 1.6%	21.8 ± 1.5%	19.2 ± 1.4%

Cardiomyocytes (untreated controls) subjected to 30 min hypo-osmotic stress initially increased their cell volume by 10 min exposure with subsequent substantial reduction in cell swelling (RVD, Regulatory volume decrease). This RVD was blunted by treatment of cardiomyocytes with 10 μM DCPIB.

Table 1: Percent change in cardiomyocyte volume following 10, 20 and 30 min of hypo-osmotic (200 mOsm) stress under oxygenated conditions.

play an important role in the transition from reversible to irreversible I/R cardiomyocyte injury.

Multiple Cl- channels exist as end effector candidates for IPC and LI/R-rIPC cardioprotection. These are ClC-2, ClC-3, the CFTR Cl- channel and the swell-activated Cl- channels which are all well characterized [22-26]. It is known that CFTR -/- mice do not show IPC protection against myocardial infarction on I/R [27]. The CFTR Cl- channel is not volume regulated [28] but is regulated by PKC [29,30] as are both IPC and rIPC [8]. ClC-2 channels, as expressed in Xenopus oocytes, not only export Cl- in response to osmotic swelling but also due to low pH [25,26,31] both of which are relevant to ischemic conditions. Thus, in addition to CFTR Cl- channel, ClC-2 might be a player in cardioprotection.

However, we have been particularly drawn to the swell-activated Cl- channel because of our previous work showing the importance of cardiomyocyte swelling in cardioprotection by classic, local ischemic preconditioning [14,15]. It is known that ClC-3 most likely underlies swell-activated Cl- channel activity. It has been established that cardiac-specific inducible ClC-3 gene deletion eliminates native volume sensitive Cl- channel in mice [26]. Of note, in conventional ClC-3 knockout mice there is a compensatory increase in volume sensitive (i.e., swell-activated) Cl- channel activity [31] and this observation was previously interpreted as suggesting that ClC-3 was not the swell-activated Cl- channel. The combination of our forward and reverse co-immunoprecipitation (pulldown) assays on ClC-3 in this study indicates an association of PKCε with ClC-3 in the cell membrane fraction of rabbit ventricular cardiomyocytes. Given the demonstrated importance PKCε in both IPC and rIPC [32], the co-localization of PKCε with ClC-3 seems unlikely to be only coincidental. ClC-3 has not been proven to be the swell-activated Cl- channel [33]. The protein and gene for the swell-activated Cl- channel have not been identified. However, the ion current which defines a swell-activated Cl- channel is merely a trans-sarcolemmal ion current measured under particular biophysical conditions. Thus, the fact that when ClC-3 gene expression is selectively knocked out in mouse hearts this current disappears [30] does seem compelling evidence that ClC-3 underlies the swell-activated Cl- channel.

The findings of the present study not only demonstrate that cardioprotection by remote ischemic preconditioning requires activation of swell-activated Cl- channels but, most importantly, they define an end-effector role for the swell-activated Cl- channel in LI/R-rIPC. These results open the way for more comprehensive investigations of the relative contributions of different Cl- channels to cardiomyocyte volume regulation and cardioprotection in the context of ischemia/reperfusion.

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