Modulation of cardiac Na\(^+\),K\(^+\)-ATPase cell surface abundance by simulated ischemia-reperfusion and ouabain preconditioning

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Belliard A, Sottejeau Y, Duan Q, Karabin JL, Pierre SV. Modulation of cardiac Na\(^+\),K\(^+\)-ATPase cell surface abundance by simulated ischemia-reperfusion and ouabain preconditioning. Am J Physiol Heart Circ Physiol 304: H94–H1103, 2013. First published October 19, 2012; doi:10.1152/ajpheart.00374.2012.—Na\(^+\),K\(^+\)-ATPase and cell survival were investigated in a cellular model of ischemia-reperfusion (I/R)-induced injury and protection by ouabain-induced preconditioning (OPC). Rat neonatal cardiac myocytes were subjected to 30 min of substrate and coverslip-induced ischemia followed by 30 min of simulated reperfusion. This significantly compromised cell viability as documented by lactate dehydrogenase release and Annexin V/propidium iodide staining. Total Na\(^+\),K\(^+\)-ATPase \(\alpha_1\), and \(\alpha_3\)-polypeptide expression remained unchanged, but cell surface biotinylation and immunostaining studies revealed that \(\alpha_1\)-cell surface abundance was significantly decreased. Na\(^+\),K\(^+\)-ATPase-activity in crude homogenates and \(^{86}\)Rb uptake in live cells were both significantly decreased by about 30% after I/R, OPC, induced by a 4-min exposure to 10 \(\mu\)M ouabain that ended 8 min before the beginning of ischemia, increased cell viability in a PKC\(\varepsilon\)-dependent manner. This was comparable with the protective effect of OPC previously reported in intact heart preparations. OPC prevented I/R-induced decrease of Na\(^+\),K\(^+\)-ATPase activity and surface expression. This model also revealed that Na\(^+\),K\(^+\)-ATPase-mediated \(^{86}\)Rb uptake was not restored to control levels in the OPC group, suggesting that the increased viability was not conferred by an increased Na\(^+\),K\(^+\)-ATPase-mediated ion transport capacity at the cell membrane. Consistent with this observation, transient expression of an internalization-resistant mutant form of Na\(^+\),K\(^+\)-ATPase \(\alpha_1\) known to have increased surface abundance without increased ion transport activity successfully reduced I/R-induced cell death. These results suggest that maintenance of Na\(^+\),K\(^+\)-ATPase cell surface abundance is critical to myocyte survival after an ischemic attack and plays a role in OPC-induced protection. They further suggest that the protection conferred by increased surface expression of Na\(^+\),K\(^+\)-ATPase may be independent of ion transport.

In the heart, I/R-induced alteration of the Na\(^+\),K\(^+\)-ATPase enzyme is well documented (2, 3, 19, 21, 27, 31, 48), and elucidation of the mechanism involved is still considered key to the development of novel approaches for therapeutic intervention (29, 30). Surprisingly, however, the precise sequence of events remains incompletely understood, and the role played by surface abundance of the enzyme complex is not known. Contributing to our relative lack of understanding may be the technical limitations of intact heart preparations in which studies of Na\(^+\),K\(^+\)-ATPase subcellular expression and function are challenging. To address specifically the role of Na\(^+\),K\(^+\)-ATPase surface abundance in I/R injury and in cardiac protection, the present study uses a cell culture model, which provides complementary approaches to our previous studies in Langendorff-perfused hearts. Using primary cultures of rat NCM, we developed a model of simulated I/R injury and protection by ouabain preconditioning (OPC) induced by a short and transient exposure to a low concentration of ouabain (10 \(\mu\)M). OPC protection at this concentration has previously been characterized in the whole rat heart (28, 32, 38), in which Na\(^+\),K\(^+\)-ATPase affinity for cardiac glycosides is 2- to 3 orders of magnitude lower than that of the human heart. OPC is also effective at nanomolar concentration in the more sensitive rabbit heart (28). Using an in vitro model, we present evidence that cardiac Na\(^+\),K\(^+\)-ATPase cell surface abundance is modulated by simulated I/R and OPC, and can be targeted to increase myocytes survival during an ischemic attack.

Na\(^+\),K\(^+\)-ATPase IS THE membrane-spanning enzyme complex that establishes and maintains the electrochemical gradient across the cell plasma membrane by coupling ATP hydrolysis to the transport of Na\(^+\) and K\(^+\) ions (20, 42). The Na\(^+\),K\(^+\)-ATPase consists of \(\alpha\)- and \(\beta\)-subunits, both existing as multiple isoforms and expressed in a tissue-specific manner. \(\alpha_1\) is the predominant isoform (about 80%) of the catalytic \(\alpha\)-subunit expressed in rat cardiac tissue (26). In rat neonatal cardiac myocytes (NCM), the only known \(\alpha\)-isoform expressed is \(\alpha_3\) (26). The Na\(^+\),K\(^+\)-ATPase protein complex is the pharmacological target of the digitalis drugs used in the treatment of heart failure and atrial arrhythmia (11). Cardiac glycosides such as digoxin and ouabain are well-known inhibitors of Na\(^+\),K\(^+\)-ATPase ion-pumping function. In the heart, this inhibition and the subsequent increase in intracellular Na\(^+\) result in an increased Na\(^+\)/Ca\(^{2+}\) exchange and increases contractility. Cardiac glycosides also initiate intracellular signaling cascades via stimulation of the Na\(^+\),K\(^+\)-ATPase receptor function. Activation of these cascades are the consequence of digitalis-induced interactions of Na\(^+\),K\(^+\)-ATPase with neighboring proteins such as Src and EGFR receptor (24, 37, 45). These interactions result in the activation of key downstream players such as Akt, PKC\(\varepsilon\), or ERK1/2 that are critical to additional cardiac effects of digitalis, including cardioprotection by preconditioning and physiological hypertrophy (5, 17, 25, 32, 38). Hence, integrity at the cell surface is important to both ion-pumping and signaling functions of the protein complex, and cell surface expression has been shown to vary in response to major physiological or pathophysiological stimuli, including ischemia-reperfusion (I/R) as shown recently in epithelial cells (35).

In the heart, I/R-induced alteration of the Na\(^+\),K\(^+\)-ATPase enzyme is well documented (2, 3, 19, 21, 27, 31, 48), and elucidation of the mechanism involved is still considered key to the development of novel approaches for therapeutic intervention (29, 30). Surprisingly, however, the precise sequence of events remains incompletely understood, and the role played by surface abundance of the enzyme complex is not known. Contributing to our relative lack of understanding may be the technical limitations of intact heart preparations in which studies of Na\(^+\),K\(^+\)-ATPase subcellular expression and function are challenging. To address specifically the role of Na\(^+\),K\(^+\)-ATPase surface abundance in I/R injury and in cardiac protection, the present study uses a cell culture model, which provides complementary approaches to our previous studies in Langendorff-perfused hearts. Using primary cultures of rat NCM, we developed a model of simulated I/R injury and protection by ouabain preconditioning (OPC) induced by a short and transient exposure to a low concentration of ouabain (10 \(\mu\)M). OPC protection at this concentration has previously been characterized in the whole rat heart (28, 32, 38), in which Na\(^+\),K\(^+\)-ATPase affinity for cardiac glycosides is 2- to 3 orders of magnitude lower than that of the human heart. OPC is also effective at nanomolar concentration in the more sensitive rabbit heart (28). Using an in vitro model, we present evidence that cardiac Na\(^+\),K\(^+\)-ATPase cell surface abundance is modulated by simulated I/R and OPC, and can be targeted to increase myocytes survival during an ischemic attack.
MATERIALS AND METHODS

NCM isolation. All animal experimentation was conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996), and protocols were approved by the University of Toledo Institutional Animal Use and Care Committee. One- to 2-day-old rats were euthanized by decapitation, and contracting NCM were isolated from the ventricles as described previously (34) with minor modifications. NCM were incubated in serum-free medium 48 h before experimentation.

\(\text{Na}^+,\text{K}^+\)-ATPase \(\alpha_1\) and \(\alpha_1\text{L}499\text{V} \) transient transfection. Transient transfections of native or mutated \(\text{Na}^+,\text{K}^+\)-ATPase, \(\alpha_1\)-YFP or \(\alpha_1\text{L}499\text{V}\)-YFP fused proteins were performed on NCM after 1 day of culture using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s recommendations. Transfection efficiency was verified by microscopic observation of intrinsic fluorescence property of YFP in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

\(\text{Na}^+,\text{K}^+\)-ATPase \(\alpha_1\) and \(\alpha_1\text{L}499\text{V} \) transient transfection. Details on experimental procedures related to vector construction, site-directed mutagenesis, and initial characterization of \(\text{Na}^+,\text{K}^+\)-ATPase \(\alpha_1\)-L499V enzyme properties can be found in Sottejeau et al. (43).

Coverslip-induced I/R in NCM. Ischemia was simulated in NCM by placement of a glass LifterSlip over the monolayers and removal of substrate, as modified from previously described procedures (35, 39, 40). Briefly, a 22 × 63 mm and two 22 × 44 mm LifterSlips (Thermo Scientific) were delicately placed over the NCM monolayer in a 100-mm diameter dish, resulting in coverage of about 57% of the dish. Reperfusion was simulated by gently removing the LifterSlips. In addition, substrate removal was performed to mimic I/R, by using Krebs-Henseleit (KH) buffer and PBS as detailed below. For confocal imaging studies, NCM were grown on square coverslip 22 × 22 mm in 6-well plates, and I/R was simulated using 18-mm diameter round glass coverslips with 57% of coverage. For \({}^{86}\text{Rb}^+\) uptake studies, NCM were cultured in collagen-coated 6-well plates and round coverslips (25 mm) were used to simulate I/R. The timing of interventions is detailed in the paragraph below as well as in Fig. 1. All assays were conducted on monolayers and/or media collected at the end of one of the 80-min protocols.

Protocols. All treatments were performed at 37°C under a 5% CO\(_2\) atmosphere. Six groups were studied as depicted in Fig. 1. The control group was incubated 80 min in KH solution containing (in mmol/l) 25 NaHCO\(_3\), 4.0 KCl, 1.2 MgSO\(_4\), 11 D-glucose, 118.0 NaCl, 1.3 KH\(_2\)PO\(_4\), 0.3 ethylene glycol bis (2-aminoethyl ether)-N,N,N’,N’-tetraacetic acid, and 1.8 CaCl\(_2\) (38) at 37°C. The I/R group was incubated 20 min in KH, subjected to coverslip ischemia in PBS for 30 min, and simulated reperfusion by gently removing the LifterSlips and changing the media back to fresh KH buffer during 30 min. The ouabain-preconditioned group (OPC + I/R) was incubated 8 min with KH buffer followed by 4 min of incubation with ouabain (10 \(\mu\)mol/l) and 8 min of KH before inducing 30 min of coverslip ischemia in PBS and reperfusion for 30 min. In some experiments, the PKCε translocation inhibitor peptide (TIP; 5 \(\mu\)mol/l) was added to the first 20 min of the protocol.

Measurement of lactate dehydrogenase activity. The amount of lactate dehydrogenase (LDH) released was used as an indicator of loss of cellular integrity. At the end of the simulated reperfusion, NCM media were collected and LDH activity was determined colorimetrically using a standard assay (Cytotoxicity Detection Kit; Roche Applied Science). To evaluate the extent of the injury as a percentage of the total cell population, LDH was also measured in a subset of experiments in lysates obtained by treatment of the monolayer with 0.1% Triton X-100 at 4°C for 30 min at the end of the protocol. Total LDH was obtained as the sum of LDH released in the media and LDH in the cell lysates.

Annexin V/propidium iodide staining. At the end of the experimental protocol, NCM were fixed in 2% paraformaldehyde for 10 min at 4°C and processed for Annexin V/propidium iodide labeling. Fig. 1. Experimental protocols. Ouabain was added at 10 \(\mu\)mol/l. The PKCε translocation inhibitor peptide (TIP; 5 \(\mu\)mol/l) was given before, during, and after ouabain preconditioning (OPC) treatment.
room temperature after a wash in PBS 1X. Cells were then incubated with Alexa Fluor 488 annexin V and red fluorescent propidium iodide (PI) (Vybrant Apoptosis Assay Kit no. 2; Invitrogen). The coverslips were mounted with ProLong Gold antifade reagent with 4,6-diamidino-2-phenylindole (Invitrogen). Confocal images were captured by sequential scanning with no overlap using a Leica TCS SP5 broadband confocal microscope system coupled to a DMI 6000CS inverted microscope equipped with multiple continuous wave lasers and a ×63/1.3 oil objective.

**Biotinylation and Western blotting.** \( Na^+\),K\(^{\text{-}} \) -ATPase \( \alpha_1 \) and \( \alpha_3 \) expressed at the cell surface were detected by biotinylation following the recommended procedures of Gottardi et al. (12). Briefly, 70% confluent NCM grown on 100-mm culture dishes were incubated for 80 min according to the protocols (Fig. 1). Cells were rinsed twice with ice-cold PBS and exposed once to 1.5 mg/ml alamethicin to allow for comparison of the maximal transport capacity of the enzyme (35). Samples were then solubilized in radioimmunoprecipitation assay (RIPA) buffer (1% Igepal CA630, 0.25% sodium deoxycholate, 150 mmol/l NaCl, 100 mmol/l EDTA, 50 mmol/l Tris, pH 7.4) for 60 min on ice, and the resulting lysates were cleared by centrifugation at 14,000 g for 10 min at 4°C. Supernatants were incubated overnight at 4°C with streptavidin-agarose beads (Pierce) with end-over-end rotation. The beads were then washed twice with each buffer: RIPA, high-salt RIPA (RIPA; 200 mmol/l NaCl), and no-salt wash buffer (10 mmol/l Tris, pH 7.4). Bound proteins were analyzed by electrophoresis and immunoblotting (using anti Na\(^+\),K\(^{\text{-}} \) -ATPase \( \alpha_1 \); 6F, from University of Iowa, Iowa City, IA; and anti Na\(^+\),K\(^{\text{-}} \) -ATPase \( \alpha_3 \); TED, from T. A. Pressley, Texas Tech University HSC, Lubbock, TX). For total expression, Na\(^+\),K\(^{\text{-}} \) -ATPase \( \alpha_1\), \( \alpha_2\), and \( \alpha_3\)-isoforms were probed using antibodies HERED, and TED primary-antibody, respectively (gifts from Dr T. A. Pressley, Texas Tech University HSC, Lubbock, TX). Actin was probed as a loading control using actin C-11 primary-antibody (Santa Cruz), as described previously (36, 41).

**Immunocytochemistry and fluorescence imaging.** At the end of the experimental protocol, cells were fixed by 20 min incubation with ice-cold methanol, washed with PBS, and blocked with Signal Enhancer (Invitrogen). The cells were then incubated with a mouse monoclonal antibody (clone C464.6; Upstate) in PBS containing 1% BSA for 2 h at room temperature. After three PBS washes, cells were exposed to Alexa Fluor 488-conjugated anti-mouse secondary antibody for 2 h at room temperature, washed, and mounted onto glass slides. Image visualization was performed using a Leica TCS SP5 broadband confocal microscope system coupled to a DMI 6000CS inverted microscope (35).

**Active transport.** At the end of the experimental protocol, the medium was changed to DMEM (Sigma-Aldrich) with or without 1 mmol/l ouabain for 10 min; Na\(^+\),K\(^{\text{-}} \) -ATPase-mediated transport was then measured by using the ouabain-sensitive uptake of the K\(^{\text{+}} \) -congener, \( ^{86}\text{Rb}^+ \) (1 \( \mu\)Ci) over 10 min at 37°C, as described (34). To allow for comparison of the maximal transport capacity of the enzyme in all groups, the intracellular Na\(^{\text{+}} \) concentration in the NCM was increased with the ionophore monensin at a concentration of 20 mmol/l NaCl, and no-salt wash buffer (10 mmol/l Tris·HCl, 1 mmol/l MgCl\(_2\), 100 mmol/l NaCl, 20 mmol/l KCl, 1 mmol/l EGTA-Tris, and 5 mmol/l Na\(_2\)SO\(_4\)). After addition of 2 mmol/l Mg\(^{2\text{+}} \) and 1 ATP, the enzymatic reaction was allowed to run for 10 min before the addition of 1 ml cold 8% TCA and rapid placement of each test tube in ice to terminate the reaction. Released phosphate was determined using an inorganic phosphate detection kit (Biomol Green; AK-111). Ouabain insensitive activity was measured in a separate reaction in the presence of 1 mmol/l ouabain in the same buffer. Ouabain sensitive Na\(^+\),K\(^{\text{-}} \) -ATPase activity was then determined by subtraction of ouabain insensitive from total ATPase activity (46).

**Statistical analysis.** Statistical analysis was performed using t-test or one-way ANOVA followed by Tukey’s multiple comparison post hoc test. \( P < 0.05 \) was considered statistically significant.

**RESULTS**

**Cell viability.** In vitro-simulated I/R was induced in NCM by removing the metabolic substrates from the culture medium and by placing and removing coverslips over the monolayer, according to a protocol modified from Pitts and Toombs (39). After treatment, cell viability was assessed quantitatively (LDH release) and qualitatively (AnnexinV/PI labeling). To evaluate the extent of injury, we first assessed the released LDH-to-total LDH ratio, where released LDH represents the population of cells with a loss of membrane integrity and total LDH represents the total cell population. As shown in Fig. 2, I/R resulted in a sevenfold increase of the ratio, indicative of a loss of viability of the I/R-exposed cells. OPC significantly reduced the released LDH-to-total LDH ratio, by about 24% (\( P < 0.001 \)). To test whether OPC-induced protection was PKCe-dependent as previously found in the whole heart (38), a PKCe TIP was added in a subset of experiments. Because intragroup variation in the released LDH-to-total LDH ratio was found to be minimal in all conditions, released LDH only was examined in these series of experiments. As shown in Fig. 3, TIP did not affect LDH release in control or I/R-exposed groups, but completely abolished OPC-induced protection. Next, staining with Alexa Fluor 488 annexin V was used to label apoptotic cells.

![Fig. 2. Characterization of the proportion of cell death in the model.](http://ajpheart.physiology.org/)
totic cells in combination with red-fluorescent PI to label late apoptotic/necrotic cells. Pictures representative of the observations made at the center of the area covered by the coverslip (core) show that I/R resulted in a marked increase in the annexin V/PI population (3B) that was completely prevented by OPC. This suggested that although it did not completely prevent LDH release in response to I/R injury, OPC afforded a strong protection against cell death at 30 min of reperfusion, especially in the ischemic core. Furthermore, this protection was PKCε-dependent, as suggested by the TIP-induced abolition of OPC protection (Fig. 3, A and B). Taken together, these data suggested that our previous findings of I/R-induced injury and protection by OPC in the Langendorff-perfused rat heart could be simulated in this in vitro model.

Na⁺,K⁺-ATPase function and expression. Surface expression of the Na⁺,K⁺-ATPase α₁ polypeptide was analyzed using cell-surface biotinylation. As shown in Fig. 4A, α₁ surface abundance in the I/R-exposed cells was decreased by about

![Graph](http://ajpheart.physiology.org/)

**Fig. 3.** Effect of I/R and OPC on cell viability. Viability of neonatal cardiac myocytes (NCM) was measured after exposure to the protocols shown in Fig. 1. The PKCε TIP was added as described in Fig. 1. A: effect of PKCε inhibition. Values are means ± SE (n = 5). ***P < 0.001 vs. I/R without TIP; #P < 0.05 vs. OPC without TIP. B: annexin V/propidium iodide (PI) staining. Pictures are representative of at least 6 fields observed in the center of the area under the coverslip in 3 different preparations for each condition. Scale bar = 25 μm. DAPI, 4,6-diamidino-2-phenylindole.
30% compared with control (P < 0.05, top graph), and this decrease was completely prevented by OPC (bottom graph). Immunofluorescent labeling of Na\textsuperscript{+},K\textsuperscript{+}-ATPase \(\alpha_1\) in NCM was also consistent with an increased intracellular signal after I/R (Fig. 4C) that was prevented by OPC treatment. As shown in Fig. 4B, I/R and OPC did not induce any detectable change in total Na\textsuperscript{+},K\textsuperscript{+}-ATPase \(\alpha_1\) expression. In addition to \(\alpha_1\), \(\alpha_3\) but not \(\alpha_2\) was detected in control NCM (not shown), in
agreement with a previous report (47). I/R or OPC did not induce any detectable change in α3 surface or total protein levels (Fig. 5). Taken together, these results suggest that although I/R had no effect on overall Na\(^{+},K^{+}\)-ATPase α-subunit expression, it resulted in a decreased number of α1-polypeptides at the plasmalemma. The results also suggest that OPC prevented I/R-induced modulation. To assess functionally the impact of I/R- and OPC-induced modulations of surface abundance on cellular Na\(^{+},K^{+}\)-ATPase-mediated ion transport capacity, \(^{86}\)Rb\(^{+}\) uptake was evaluated in the presence of the ionophore monensin to minimize the possible effect of variations in intracellular sodium. The results presented in Fig. 6 indicated that I/R significantly reduced the uptake (27 \pm 3%; \(P < 0.001\)) compared with control. In the OPC group, Na\(^{+},K^{+}\)-ATPase-mediated \(^{86}\)Rb\(^{+}\) uptake was also significantly decreased by about 25\% (\(P < 0.001\)). Because OPC prevented the I/R-induced decrease in cell surface abundance, this suggested that some of the Na\(^{+},K^{+}\)-ATPase units at the cell surface were not transporting ions in the OPC group. To explore the possibility that OPC prevented I/R-induced internalization but not I/R-induced inactivation of the enzyme function, we measured maximal ouabain-sensitive Na\(^{+},K^{+}\)-ATPase activity in the presence of saturating concentrations of substrates and inhibitor. Alamethicin pretreatment was performed to insure access of substrates and inhibitor to their respective binding sites on the enzyme despite potentially closed membrane vesicles, as reported previously (46). As shown in Fig. 7, I/R decreased Na\(^{+},K^{+}\)-ATPase activity by about 28\% (\(P < 0.001\)), and this decrease was completely prevented by OPC (\(P < 0.01\)), in a PKCe-dependent manner.

**Impact of Na\(^{+},K^{+}\)-ATPase α3 L499V expression.** The above-mentioned results suggested that preservation of Na\(^{+},K^{+}\)-ATPase cell surface abundance, rather than ion transport, correlated with protection against I/R-induced cell death by OPC. This is consistent with our previous observation that expression of a L499V mutant of the Na\(^{+},K^{+}\)-ATPase α3-polypeptide in epithelial cells resulted in increased surface expression without change in \(^{86}\)Rb\(^{+}\) uptake (43) and conferred resistance to I/R injury (35). To directly evaluate the impact of cell surface expression on NCM viability during I/R, we...
transiently transfected NCM with the native $\alpha_1$-polypeptide or the L499V mutant, both YFP-tagged at the COOH terminus. The tag did not induce any detectable change in enzymatic activity and allowed us to immunologically discriminate the exogenous $\alpha_1$-constructs from the endogenous $\alpha_1$. Full length expression of both constructs was verified by Western blot using an anti-GFP antibody, and transfection efficiency was estimated by visualization under a fluorescent microscope before exposure to I/R (not shown). A similar efficiency of at least 40% of fluorescent myocytes was estimated for both $\alpha_1$-YFP and $\alpha_1$L499V-YFP constructs. As shown in Fig. 8, I/R-induced LDH release was significantly decreased in NCM transiently expressing the $\text{Na}^+,\text{K}^+\text{-ATPase}$ $\alpha_1$L499V-YFP mutant compared with NCM expressing $\alpha_1$-YFP, by about 45% ($P < 0.01$).

**DISCUSSION**

In an in vitro model (35, 40) of simulated I/R in NCM, the present study revealed that OPC protects cardiac myocytes against I/R-induced damage through mechanisms that do not require the presence of other cell types. The use of this model allowed us to obtain evidence that cardiac $\text{Na}^+,\text{K}^+\text{-ATPase}$ cell surface abundance is modulated by simulated I/R and OPC, in a $\text{Na}^+,\text{K}^+\text{-ATPase}$ $\alpha$ isoform-specific manner. Because the protection occurred without restoration of $\text{Na}^+,\text{K}^+\text{-ATPase}$-mediated ion-transport and transient expression of the internalization-resistant mutant $\text{Na}^+,\text{K}^+\text{-ATPase}$ $\alpha_1$L499V reduced I/R-induced cell injury, we propose that maintenance of one or more of the signaling functions of $\text{Na}^+,\text{K}^+\text{-ATPase}$ at the cell surface may play a key role.

**Characterization of the in vitro model.** Pitts and Toombs (39) first proposed a coverslip-based method for creating regional ischemic conditions in NCM. In our modified version of their protocol, the coverslip was carefully removed after 30 min, and fresh media was added back for an additional 30 min, to simulate reperfusion. These interventions were designed to mimic the combination of metabolic disturbances and hypoxic conditions of our previous studies of I/R and OPC in Langendorff-perfused rat hearts (38). The results presented in Fig. 2 indicated that the extent of the injury produced was indeed comparable. Another indication that this in vitro model was well suited was the protection provided by OPC (Figs. 2 and 3). This was a critical result for two reasons. First, it confirmed microscopic observations and total LDH release measurements (not shown) indicating that the injury observed in the I/R group was not the result of a mechanical insult induced by manipulation of the coverslip. Second, it showed for the first time that OPC signaling occurs in and directly protects the cardiac myocytes. Indeed, because of the ubiquitous expression of the $\text{Na}^+,\text{K}^+\text{-ATPase}$ receptor complex, ouabain signaling may occur in any cardiac cell and contribute to the protection observed in the whole heart in earlier studies. Without excluding other effects of fibroblastic or vascular origin for example, the results of the present study clearly show that OPC occurs in cardiac myocytes independently of the presence of any other cell type. This is hardly a unique case among preconditioning mechanisms, and in fact many forms of protection have been shown in cardiac myocyte monolayers. Such examples include ischemic-, hypoxic-, and opioid-induced preconditionings (6, 13, 33). The results presented in Fig. 3, A and B, also show that OPC-induced protection requires PKCε activation, consistent with our previous finding in whole hearts (32, 38).

**I/R-induced alteration of $\text{Na}^+,\text{K}^+\text{-ATPase}$ and protection by OPC.** Because Beller et al. (2) first correlated postischemic alterations in cardiac glycoside binding to decreased $\text{Na}^+,\text{K}^+\text{-ATPase}$ activity in dog myocardium in 1976, numerous studies have examined the mechanisms leading to cardiac $\text{Na}^+,\text{K}^+\text{-ATPase}$ alteration during I/R (2, 3, 19, 21, 27, 31, 48). Indeed, alteration of $\text{Na}^+,\text{K}^+\text{-ATPase}$ during myocardial infarction may render the myocardial tissue more sensitive to the arrhythmogenic effect of digitalis, and uncertainties surrounding their safety in heart failure patients with an ischemic origin have contributed to the decrease in digitalis use despite their unique hemodynamic and neurohumoral benefits (11). However, although the elucidation of the mechanism underlying I/R-induced alteration of $\text{Na}^+,\text{K}^+\text{-ATPase}$ is still considered key to the development of novel approaches for therapeutic intervention (29, 30), the precise mechanism remains unclear. The use of a cellular model allowed us to observe a number of previously unknown characteristics.

$\text{Na}^+,\text{K}^+\text{-ATPase} \alpha_1$- and $\alpha_2$-protein abundance. First, this study revealed a decrease in $\text{Na}^+,\text{K}^+\text{-ATPase}$ $\alpha_1$-cell surface abundance without change in total $\alpha_1$-expression, suggestive of an increased internalization or decreased delivery to the surface without changes of synthesis or degradation of the polypeptide in this model (Fig. 4). On the basis of our recent data in epithelial cells (35), we propose that the mechanism is likely to involve I/R-induced internalization. Figure 4 clearly shows that OPC prevented this I/R-induced decrease in $\text{Na}^+,\text{K}^+\text{-ATPase}$ $\alpha_1$ cell surface abundance. Further analysis of the expression of $\text{Na}^+,\text{K}^+\text{-ATPase} \alpha_2$, the only other known $\alpha$-isoform present in rat NCM (26), revealed no I/R-induced change in surface expression (Fig. 5), suggesting that I/R-induced internalization is isoform specific. The elucidation of the respective roles played by clathrin-coated pits and caveolar-based signalosomes (9) in I/R and OPC-induced effects on the $\text{Na}^+,\text{K}^+\text{-ATPase}$ $\alpha_1$-polypeptide was beyond the scope of the present study, but would be the next logical step. Likewise, the question of the specific roles of PKC isoforms (4) in the regulation of $\text{Na}^+,\text{K}^+\text{-ATPase}$ surface expression during I/R remains to be addressed, along with the possibility of a direct regulation of $\text{Na}^+,\text{K}^+\text{-ATPase}$ surface expression by PKCε during OPC.
Although such PKCe would most likely be distinct from the ones involved in the cardioprotective intra-mitochondrial signaling reviewed by Garlid et al. (9), PKCe associated with the ouabain signalosome itself (9) may in turn affects Na⁺,K⁺-ATPase surface expression during OPC.

**Na⁺⁺,K⁺⁺-ATPase activity in crude homogenates.** In agreement with previous reports (2, 3, 19, 21, 27, 31, 48), we observed an I/R-induced alteration of Na⁺⁺,K⁺⁺-ATPase-enzyme catalytic properties (Fig. 7). It is important to note that maximal Na⁺⁺,K⁺⁺-ATPase was measured in crude homogenates from the myocytes that remained attached to the culture dish at the end of the protocols. Hence, the I/R-induced decrease that was observed is unlikely to be a simple consequence of an increased number of proteins from dead cells in the homogenates, and more likely to reflect an inactivation of the enzyme structural properties. Inserte et al. (19) have shown in Langendorff-perfused rat hearts that such Na⁺⁺,K⁺⁺-ATPase alteration can actually be detected before cell death occurs. The data presented here also suggest that the use of cellular models may prove useful in understanding the mechanism of I/R-induced Na⁺⁺,K⁺⁺-ATPase alteration. At least theoretically, the Na⁺⁺,K⁺⁺-ATPase activity results combined with the results from Fig. 4 suggest that I/R induces Na⁺⁺,K⁺⁺-ATPase α₁ internalization, resulting in altered intracellular ion homeostasis and inactivation of the enzyme properties according to a mechanism that could be comparable with the one occurring during lung hypoxia (16). Because rat α₁-isozyme-specific structural determinants (reviewed in Ref. 35) have been involved in hypoxia-induced effects on lung Na⁺⁺,K⁺⁺-ATPase, the involvement of at least some of the players identified in the hypoxia model is also consistent with the apparent lack of change in α₁ surface expression in the present study. Among such key players, previously involved in hypoxia-induced Na⁺⁺,K⁺⁺-ATPase alteration in the lungs and likely to be involved in an I/R-induced isoform-specific alteration of Na⁺⁺,K⁺⁺-ATPase in the heart, are reactive oxygen species (16, 23, 49). Taken together, data from the studies mentioned above and the present findings suggest that the fate of individual cardiac Na⁺⁺,K⁺⁺-ATPases during I/R is likely to differ from one α₁-isozyme to another, based on predicted difference in surface expression and known differences in their sensitivities to oxidative stress in sarcolemmal preparations. Figure 7 also shows for the first time that OPC, which is triggered through activation of Na⁺⁺,K⁺⁺-ATPase signaling function by a low concentration of ouabain (32, 38), is capable of protecting the catalytic properties of the enzyme itself against subsequent I/R-induced damage. This Na⁺⁺,K⁺⁺-ATPase protective effect is not specific to OPC and has in fact been previously demonstrated for IPC by Inserte et al. (18).

**Na⁺⁺,K⁺⁺-ATPase-mediated ion transport in live cells.** In addition to the assessment of Na⁺⁺,K⁺⁺-ATPase enzyme catalytic properties in crude homogenates, the use of rat NCM monolayers allowed us to assay Na⁺⁺,K⁺⁺-ATPase ion-transporting activity at the surface of live cells without homogenization or partial purification (Na⁺⁺,K⁺⁺-ATPase-mediated ⁸⁶⁶Rb⁺⁺ uptake; Fig. 6). It is important to note that the assay was performed in the presence of monensin, a particular condition designed to measure maximal Na⁺⁺,K⁺⁺-ATPase-mediated ⁸⁶⁶Rb⁺⁺ uptake and minimize the impact of I/R-induced changes in intracellular ion concentrations. The assay revealed an alteration of Na⁺⁺,K⁺⁺-ATPase-mediated ion transport by I/R, as could be expected given the combined decrease in α₁-surface abundance (Fig. 4) and Na⁺⁺,K⁺⁺-ATPase function (Fig. 7). Interestingly, Na⁺⁺,K⁺⁺-ATPase ion-transporting activity was also lower in the OPC group than in the control group (Fig. 6). This decrease cannot be explained by a decreased surface expression (Figs. 4 and 5) or an alteration of catalytic function (Fig. 7), but is consistent with residual ouabain binding to the α₁-containing isozyme, which has a high affinity and a very slow dissociation rate compared with α₁ in the rat species, and contributes to about 20% of the total activity (15, 26, 44). Whether the inhibition of catalytically competent Na⁺⁺,K⁺⁺-ATPases by the labile cytosolic compound described by Fuller et al. (8) could also play a role will remain to be specifically addressed, especially since the stability of such compound after 30 min of reperfusion in a cellular model is not known. Irrespective of the potential mechanism involved, failure to maintain adequate ion homeostasis alone does not appear to be responsible for I/R-induced myocyte cell death or OPC-induced protection. This unforeseen finding is another key characteristic that this in vitro model revealed. Combined with the results shown in Fig. 7 and discussed in the next paragraph, these results suggest for the first time a correlation between Na⁺⁺,K⁺⁺-ATPase α₁-surface abundance and cell survival in face of an ischemic attack in the cardiac tissue. These results also show that, like ischemic preconditioning, OPC occurs in cardiac myocytes and protects cardiac Na⁺⁺,K⁺⁺-ATPase itself against I/R injury (6, 7, 18). These add to the list of shared properties between the two forms of preconditioning, which includes the production of reactive oxygen species, activation of Src and PKCe, and mitoK-ATP channel opening (1, 10, 13, 32, 38).

**Role of Na⁺⁺,K⁺⁺-ATPase cell surface abundance.** The results presented in Figs. 2–4 show that an increased number of Na⁺⁺,K⁺⁺-ATPase α₁ units at the cell surface correlates with an increased tolerance to simulated I/R in the OPC group. To test whether there was more than a correlative association between these observations, we assessed the effect of a specific increase of Na⁺⁺,K⁺⁺-ATPase cell surface abundance on NCM survival after I/R, using a modified Na⁺⁺,K⁺⁺-ATPase α₁-polypeptide (α₁L499V) with increased cell surface abundance compared with the native form (43). As shown in Fig. 8, α₁L499V-expressing cells have increased viability compared with cells expressing the native α₁-polypeptide. A direct comparison of the extent of the protection provided and the number of Na⁺⁺,K⁺⁺-ATPase α₁ units at the cell surface would not be possible given the differences in model used and the nonquantitative nature of the studies. Nevertheless, this result is consistent with our previous findings in epithelial cells (35) and with the protection observed with OPC in the present study. Mechanistically, a relatively straightforward explanation for the increased viability would be that additional Na⁺⁺,K⁺⁺-ATPase ion-pumping capacity at the cell surface in the mutant group helped preserve intracellular ion homeostasis during I/R. However, the results of the present study suggest otherwise, and we propose that one or several signaling functions served by the Na⁺⁺,K⁺⁺-ATPase complex at the plasma membrane may be involved in the modulation of myocytes survival during I/R. Future studies may reveal whether one such function lies in survival signaling or preservation of the integrity of intracellular structures.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: A.B., Y.S., and S.V.P. edited and revised manuscript; A.B., Y.S., Q.D., and S.V.P. interpreted results of experiments; A.B., Y.S., Q.D., J.L.K., and S.V.P. prepared Q.D., J.L.K., and S.V.P. analyzed data; A.B., Y.S., Q.D., and S.V.P. inter-

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