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Abstract—Altered gap junction coupling of cardiac myocytes during ischemia may contribute to development of lethal arrhythmias. The phosphoprotein connexin 43 (Cx43) is the major constituent of gap junctions. Dephosphorylation of Cx43 and uncoupling of gap junctions occur during ischemia, but the significance of Cx43 phosphorylation in this setting is unknown. Here we show that Cx43 dephosphorylation in synchronously contracting myocytes during ischemia is reversible, independent of hypoxia, and closely associated with cellular ATP levels. Cx43 became profoundly dephosphorylated during hypoxia only when glucose supplies were limited and was completely rephosphorylated within 30 minutes of reoxygenation. Similarly, direct reduction of ATP by various combinations of metabolic inhibitors and by ouabain was closely paralleled by loss of phosphoCx43 and recovery of phosphoCx43 accompanied restoration of ATP. Dephosphorylation of Cx43 could not be attributed to hypoxia, acid pH or secreted metabolites, or to AMP-activated protein kinase; moreover, the process was selective for Cx43 because levels of phospho-extracellular signal regulated kinase (ERK)1/2 were increased throughout. Rephosphorylation of Cx43 was not dependent on new protein synthesis, or on activation of protein kinases A or G, ERK1/2, p38 mitogen-activated protein kinase, or Jun kinase; however, broad-spectrum protein kinase C inhibitors prevented Cx43 rephosphorylation while also sensitizing myocytes to reoxygenation-mediated cell death. We conclude that Cx43 is reversibly dephosphorylated and rephosphorylated during hypoxia and reoxygenation by a novel mechanism that is sensitive to nonlethal fluctuations in cellular ATP. The role of this regulated phosphorylation in the adaptation to ischemia remains to be determined. **(*Circ Res*. 2004;95:726-733.)**

Key Words: connexin43 ■ ischemia ■ gap junctions ■ protein phosphorylation ■ AMP kinase ■ protein phosphatases ■ okadaic acid ■ glycolysis

Altered electrical coupling of cardiac myocytes is widely assumed to promote the development of lethal arrhythmias during myocardial infarction. Electrical coupling in excitable tissues is mediated by gap junctions, plasma membrane intercellular channels that underpin synchronous electrical activation, and coordination of cardiac myocyte contraction. Connexins, the major protein components of gap junctions, range from 26 to 57 kDa in size; most cell types express at least 1 connexin isoform.[1,2] Mammalian ventricular gap junctions contain predominantly 43 kDa connexin (Cx43); atrial and conduction tissue express additional isoforms.[3–5] Cx43 is essential for normal electrical conduction in the heart; mice with cardiac-restricted inactivation of Cx43 exhibit slower ventricular conduction and experience lethal arrhythmias,[6–8] and heterozygous deficiency of Cx43 predisposes mice to arrhythmia under ischemic stress.[9] Cx43 may also play a role in transmission of cell fate signals during myocardial ischemia: mice with Cx43 deficiency have smaller myocardial infarctions after experimental coronary artery occlusion,[10] and administration of the gap junction blocker heptanol reduces infarct size in ischemic pig and rabbit hearts.[11] Whether modification of Cx43 itself contributes to the outcome of myocardial infarction remains to be defined.

Under normal conditions, myocardial Cx43 is highly phosphorylated at multiple carboxy-terminal serine and threonine residues. However, neither the effectors nor the physiological significance of Cx43 phosphorylation have been fully resolved. Recently, using a Langendorff model of global cardiac ischemia, Beardslee et al demonstrated a fall in phosphorylated Cx43 (phosphoCx43), accumulation of nonphosphorylated Cx43 (dephosphoCx43), and loss of gap junction coupling;[12] loss of phosphoCx43 correlated with inability of the myocardium to recover contractility after ischemia. The mechanism for dephosphorylation was not identified, although hypoxia, build-up of toxic metabolites,

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and ATP depletion were suggested as possible factors. In support of this view, Vera et al reported a close correlation between ATP levels and gap junction coupling in astrocytes as measured by Lucifer yellow dye transfer.[13] Metabolic inhibition can induce Cx43 dephosphorylation;[14] and prolonged acidification causes Cx43 membrane dissociation and internalization.[15] None of these studies determined whether Cx43 dephosphorylation and gap junction uncoupling was a result of catastrophic cell damage or a regulated and reversible process. Similarly, the protein kinases controlling the phosphorylation of Cx43 have not been fully characterized but may include protein kinase C (PKC),[16,17] protein kinase A (PKA),[18] and extracellular signal–regulated kinases (ERKs).[19]

To address these questions, we studied the individual contributions of hypoxia, extracellular factors, glucose, and ATP depletion to ischemic dephosphorylation of Cx43 in a well-defined cardiac myocyte model of hypoxia and ischemia. We show here that the phosphorylation state of Cx43 is reversibly regulated by specific mechanisms that are independent of hypoxia but are tightly linked to cellular levels of ATP.

Materials and Methods

Reagents

PD98059, SB203850, bisindolmaleimide, chelerythrine, antimycin A, potassium cyanide, deoxyglucose (DOG), phorbol 12-myristate 13-acetate (PMA), KT5720, KT5823, staurosporine, cycloheximide, 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR), brefeldin A, okadaic acid, and FK506 were from Calbiochem and were used at established effective concentrations. The recombinant adenovirus encoding a dominant-negative Jun kinase mutant was described previously.[20,21] Dr Daria Mochly-Rosen (Stanford University, Stanford, Calif) generously provided peptide inhibitors and activators of PKC δ and ϵ isoforms.

Cell Culture

Cardiac myocytes were prepared from 1- to 3-day-old neonatal rat hearts as described previously,[22] maintained in minimal essential medium (MEM)+5% FCS for 3 days to allow formation of synchronously contracting monolayers, and then transferred to defined serum-free medium (MEM+transferrin+insulin+vitamin B_{12}) for the duration of the experiments. Some cells were maintained in high glucose (HG) media (3 g/L glucose) at a volume of 2 mL per 10^6 cells and replenished with fresh hypoxic media after 10 hours. All other experiments used low glucose (LG) media (0.2 g/L glucose); 1 mL of this medium was added per 10^6 cells.

Cells were rendered hypoxic in an environmental chamber containing 0.5% O_2, 5% CO_2 (balance N_2) with access via gas-tight sleeves as described previously.[23] Control cells were maintained in ambient (21%) O_2 and 5% CO_2. Reagents were added at defined times before or during hypoxia/ischemia, or included in reoxygenation media as required. Myocyte contractility was monitored using a dedicated Nikon IM microscope housed in the chamber.

Immunoblot Analysis

Cells were washed in ice-cold PBS, pH 7.4, and harvested in 1% SDS, 1 mmol/L dithiothreitol, 1 mmol/L sodium vanadate, protease inhibitors, and 1 mmol/L phenylmethylsulfonyl fluoride or phenylmethanesulfonyl fluoride, and disrupted by sonication. Protein concentrations were determined using a BioRad detergent-compatible assay. Western blots were prepared, probed, and imaged as described previously[21] using either a mouse monoclonal antibody against dephosphoCx43 or a rabbit polyclonal antibody recognizing phosphoCx43 and dephosphoCx43 (both from Zymed). Equal blot transfer was confirmed by Ponceau S staining. Primary monoclonal and polyclonal antibodies against phospho-extracellular- regulated kinase-1/2 were obtained from Cell Signaling and Promega, respectively. The sum of the densities of Cx43-specific bands in the control sample was arbitrarily set to 1.0, and the density of individual phosphorylated and nonphosphorylated bands was normalized to this value.

ATP Assay

Cell samples were washed in ice-cold PBS and harvested on ice in 5% trichloroacetic acid. ATP was measured in cell lysates using a luminescence-based assay as described.[24] Values were normalized to lysate protein concentrations.

Statistics

ANOVA with multiple column comparisons and unpaired Student *t* tests where appropriate were performed as indicated using InStat 2.00 software for Macintosh (GraphPad). Data are expressed as mean±SEM.

Results

Effects of Hypoxia, Ouabain, and Metabolic Inhibition on Synchronous Myocyte Contraction

Synchronous beating is a sensitive indicator of normal gap junction coupling in cardiac myocyte cultures. During hypoxia, myocytes in HG media (3.0 g/L) continued to beat rapidly and synchronously for 20 hours, consistent with previous findings from our laboratory that myocyte ATP levels and viability can be maintained by induction of glycolysis even under conditions of severe hypoxia.[25] In contrast, hypoxic cells in LG media (0.2 g/L) stopped beating within 2 hours (data not shown). As in our earlier reports, cessation of beating was preceded by a gradual loss of contractile amplitude rather than by deceleration.[25] Loss of contractility was reversible up to 8 hours after hypoxia onset, and synchronous beating resumed within 30 minutes of reoxygenation. Previous studies have shown that myocardial gap junction communication is reduced by ouabain, a positive inotropic agent and Na^+/K^+ ATPase inhibitor.[26] Consistent with this, normoxic cells stopped contracting within 1 hour in the presence of ouabain and resumed beating within 60 minutes of washout. Metabolic inhibition with iodoacetate alone or in combination with KCN, or with antimycin A (10 μmol/L) plus DOG caused loss of beating within 30 minutes that was irreversible by 90 minutes. A combination of DOG and KCN in LG medium caused complete but reversible cessation of beating between 4 and 8 hours after treatment (data not shown).

Glucose Depletion Leads to Dephosphorylation of Cx43

We wished to determine whether hypoxia itself, as opposed to energy depletion, was the stimulus for Cx43 dephosphorylation. Cells were therefore rendered hypoxic in HG media, with refeeding every 12 hours to prevent depletion of glycolytic substrates and accumulation of waste products. Under HG hypoxia, the relative predominance of phosphoCx43 was maintained, although the absolute amount of both phosphoCx43 and dephosphoCx43 increased significantly over time (Figure 1A; 1.65-fold and 1.89-fold at 8 and 20 hours, respectively; $P<0.01$ for both). Total Cx43 levels returned toward baseline levels within 4 hours of reoxygenation (1.12±0.15-fold of control; $P<0.01$).

In contrast, cells in LG media exhibited loss of phosphoCx43×6 hours of hypoxia, with a corresponding increase in

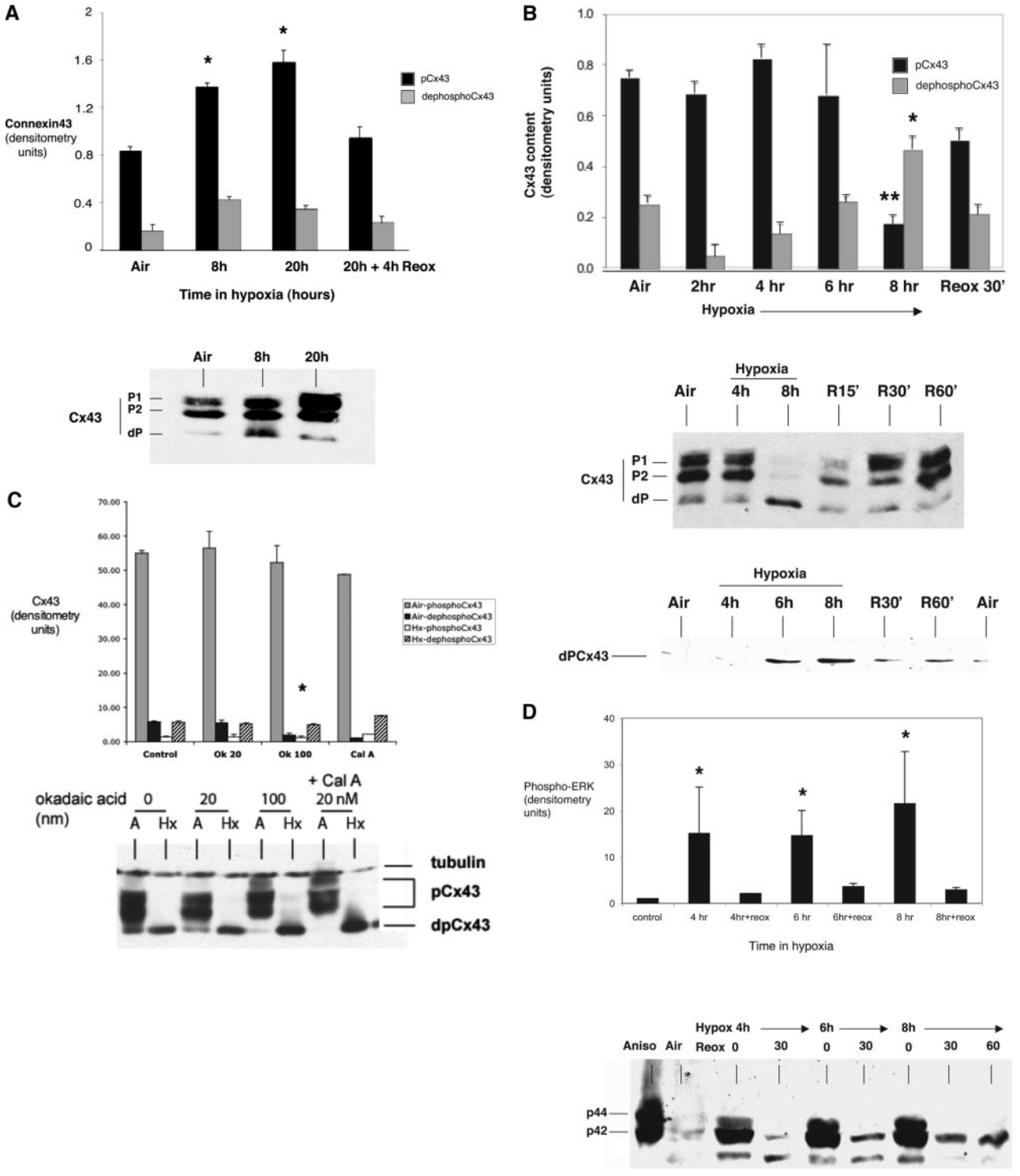

Figure 1. Cx43 phosphorylation during hypoxia, metabolic inhibition and simulated ischemia and simulated ischemia. A, Preservation of phosho-connexin43 (phosphoCx43) in hypoxia. Black bars indicate phosphoCx43; gray bars, dephosphoCx43; Air, aerobic cells; Reox, reoxygenation (n=5; *$P<0.01$ for comparison with Air and Reox samples). Cells were cultured in 0.5% oxygen as described in Methods, with regular glucose supplementation. B, Reversible dephosphorylation of phosphoCx43 during hypoxia with glucose depletion. Cells were cultured as above except that starting media glucose concentration was 0.2 g/L and was not replenished. Cell lysates were harvested and subjected to Western analysis using either a polyclonal Cx43 antibody recognizing all Cx43 species (center) or a monoclonal antibody specific for dephosphoCx43 (bottom; n=22; *$P<0.01$ for comparison with Air and Reox samples; **$P<0.0001$ vs Air). C, Lack of effect of phosphatase inhibitors. Okadaic acid (10, 20, and 100 nM) and calyculin A (20 nM) are shown in a representative Western blot (n=5; *$P<0.01$). Additional data are presented in the table. D, Maintenance of phospho-ERK levels in LG hypoxia. The same cell lysates shown in B were analyzed on Western blots probed with an antibody specific for phosphoERK (see Methods; n=6; *$P<0.05$ vs control). In all experiments, synchronously contracting monolayers of cardiac myocytes were cultured under hypoxia with or without glucose supplementation or in ambient oxygen as described in Methods. Cells were harvested at the indicated time points after treatment, after hypoxia, or after hypoxia and reoxygenation.

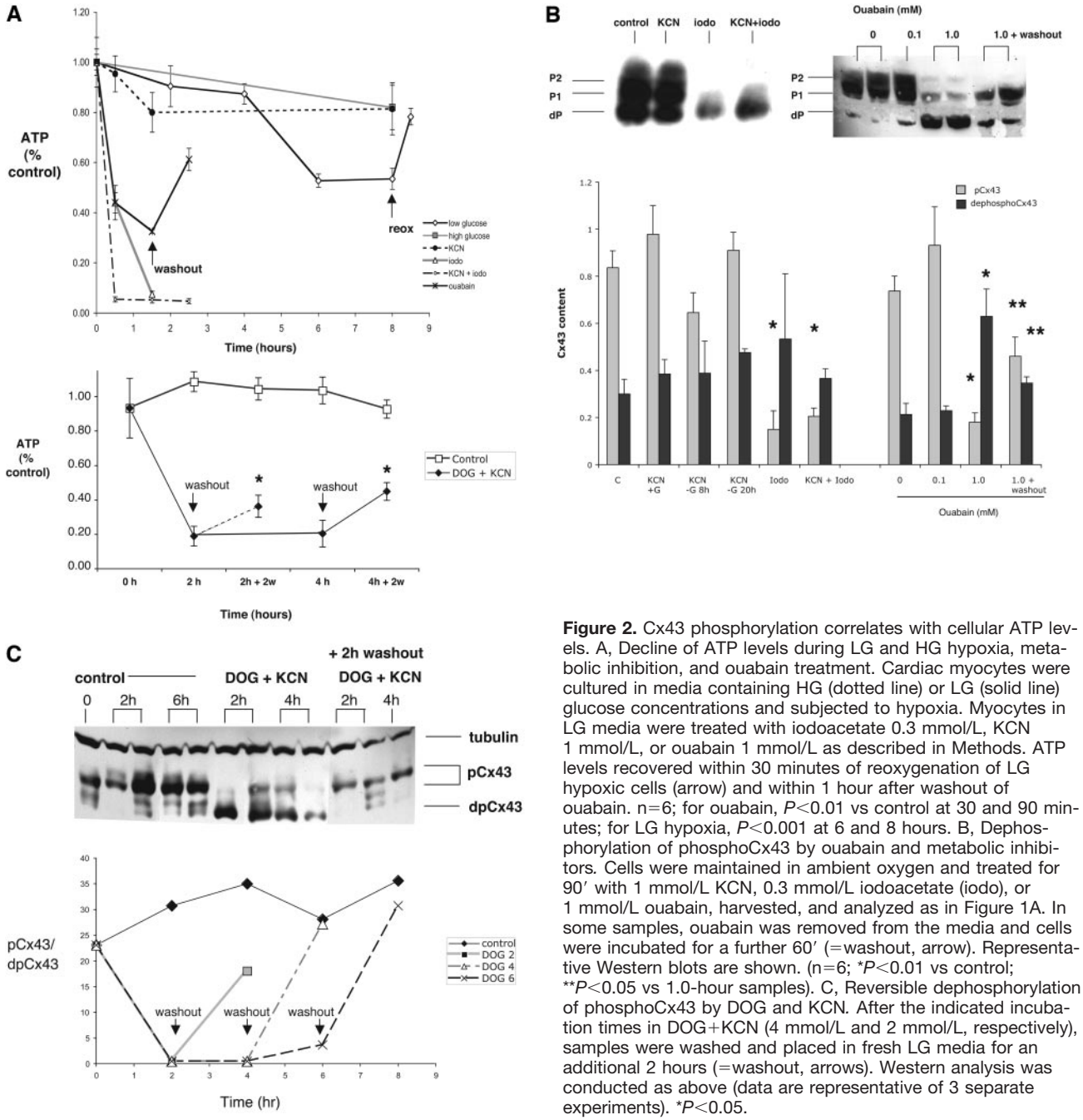

Figure 2. Cx43 phosphorylation correlates with cellular ATP levels. A, Decline of ATP levels during LG and HG hypoxia, metabolic inhibition, and ouabain treatment. Cardiac myocytes were cultured in media containing HG (dotted line) or LG (solid line) glucose concentrations and subjected to hypoxia. Myocytes in LG media were treated with iodoacetate 0.3 mmol/L, KCN 1 mmol/L, or ouabain 1 mmol/L as described in Methods. ATP levels recovered within 30 minutes of reoxygenation of LG hypoxic cells (arrow) and within 1 hour after washout of ouabain. n=6; for ouabain, $P<0.01$ vs control at 30 and 90 minutes; for LG hypoxia, $P<0.001$ at 6 and 8 hours. B, Dephosphorylation of phosphoCx43 by ouabain and metabolic inhibitors. Cells were maintained in ambient oxygen and treated for 90′ with 1 mmol/L KCN, 0.3 mmol/L iodoacetate (iodo), or 1 mmol/L ouabain, harvested, and analyzed as in Figure 1A. In some samples, ouabain was removed from the media and cells were incubated for a further 60′ (=washout, arrow). Representative Western blots are shown. (n=6; *$P<0.01$ vs control; **$P<0.05$ vs 1.0-hour samples). C, Reversible dephosphorylation of phosphoCx43 by DOG and KCN. After the indicated incubation times in DOG+KCN (4 mmol/L and 2 mmol/L, respectively), samples were washed and placed in fresh LG media for an additional 2 hours (=washout, arrows). Western analysis was conducted as above (data are representative of 3 separate experiments). *$P<0.05$.

dephosphoCx43 (Figure 1B). By 8 hours of hypoxia (6 hours after beating ceased), phosphoCx43 had fallen from 75.8±20.0% to 27.3±4.40% of total Cx43 ($P<0.0001$), with a corresponding rise in dephosphoCx43 from 25.3±3.3% to 72.7±6.6% of the total ($P<0.01$). Hypoxic dephosphorylation of Cx43 occurred despite inhibition of protein phosphatases (PPs) 1A and 2A using okadaic acid and calyculin A (Figure 1C), although both compounds increased the proportion of phosphoCx43 in aerobic samples. Inhibition of calcineurin also was without effect (data not shown). AICAR (0.5 mmol/L), a direct activator of AMP-activated protein kinase, had no effect on Cx43 phosphorylation in normoxic cells at time points between 30 minutes and 4 hours (data not shown). Neither Cx43 phosphorylation nor contractile activity was affected by passive transfer of medium from LG hypoxia cells (data not shown).

Within 30 minutes of reoxygenation, phosphoCx43 rose to 50.5±4.4% of total Cx43, and dephosphoCx43 decreased to 21.2±3.3%, both $P<0.01$ compared with hypoxia (Figure 1B), associated with the return of synchronous beating. In contrast, phosphoERK1/2 increased dramatically during LG hypoxia in the same cells (Figure 1D), indicating that the 2 proteins are subject to distinct ischemia-associated regulatory signals.

Cx43 Phosphorylation Correlates With Cellular ATP Levels

To explore the relationship between high-energy phosphate availability and Cx43 phosphorylation, we measured ATP

Loss and Recovery of Phospho-Cx43 During Ischemia and Reperfusion

		Control						Inhibitor					
		Air		HX		HX+Reox		Air		HX		HX+Reox	
Conditions	Target	P	NP	P	NP	P	NP	P	NP	P	NP	P	NP
Cycloheximide	Protein synthesis	66±6	34±6	28±3	50±8	40±4	29±3	55±4	29±7	24±4	41±7	43±10	26±3
Brefeldin 5μmol/L	Golgi processing	70±3	30±3	29±5	73±13	62±6	41±5	65±19	37±7	32±7	52±26	65±5	46±5
SB203850 5 μmol/L	p38MAPK	78±11	22±11	20±12	44±4	52±6	25±12	67±4.9	33±2.1	22±6.1	51±9.4	64±10	34±3.0
PD98059 50 μmol/L	ERK	94±1.4	6.3±1.4	16±20	35±18	68±12	9.5±5.2	113±2.0	11±0.8	10±10	59±21	95±18	39±13
Ad-dnJNK1	Jun kinase	67±1.5	33±1.5	8.7±4.1	27±3.6	44±19	25±4.3	67±2.8	27±3.0	5.4±2.6	33±2.7	48±11	22±2.8
KT5720 100 nmol/L	PKA	61±16	39±16	28±20	49±19	60±6.6	39±11	64±4.9	36±5.6	20±13	52±12	48±5.8	35±2.8
KT5823 40 μmol/L	PKG	70±3.2	30±3.2	22±12	43±5.0	50±10	35±2.1	72±6.1	32±0.1	20±8.4	46±6.7	53±17	35±3.6
Bisindomaleimide 5 μmol/L	PKC	77±2.3	23±2.3	17±2.7	60±21	43±10	23±9.5	64±16	20±20	15±4.8	53±22	25±9.8	27±11
Chelerythrine 5 μmol/L	PKC	70±8.3	30±8.3	16±9.1	42±2.9	45±5.8	23±5.9	69±4.8	21±8.0	16±9.3	40±2.8	18±7.8	19±9.3
Staurosporine 10 μmol/L	Multiple PKs	62±6.5	38±6.5	30±19	61±2.3	66±14	42±4.1	90±6.1	46±1.5	18±11	51±8.4	26±15	26±18

Effect of inhibitors of protein synthesis, processing, and protein kinases. NP indicates nonphosphorylated connexin; P, total phospho-Cx43.

Data were obtained by densitometry of 3–6 individual experiments. Data points from each experimental condition were normalized and expressed as a percentage of control P+NP Cx43 performed in parallel.

levels in cells under each condition. As predicted, ATP decreased only slightly during HG hypoxia (Figure 2A). In LG hypoxia, ATP remained stable for 4 hours (and at least 2 hours after cessation of beating) but fell to ≈50% of control between 6 and 8 hours and rose again with reoxygenation (Figure 2A), closely paralleling the time course of Cx43 dephosphorylation and recovery.

We then manipulated intracellular ATP levels with ouabain, which increases ATP consumption in contracting myocytes,[27] or with combinations of antimycin A, iodoacetate, KCN, and DOG, which inhibit ATP production. Ouabain induced a 70% fall in ATP levels and a significant dephosphorylation of Cx43 (compare Figure 2A and 2B, top right). Exposure to iodoacetate, antimycin A+DOG, or KCN+iodoacetate caused loss of phosphoCx43 within 90 minutes (Figure 2B; data not shown) together with loss of contractile activity. KCN by itself caused only a small fall in ATP, and no reduction in phosphoCx43 (compare Figure 2A and 2B, top left, lane 2), suggesting that loss of ATP and not the presence of specific chemical compounds was a key signal for Cx43 dephosphorylation. Although the effects of iodoacetate and antimycin A were irreversible (Figure 2A; data not shown), cells exposed to ouabain (Figure 2A and 2B) or DOG+KCN (Figure 2C) resumed beating after 1 to 2 hours of washout, accompanied by a partial recovery of ATP and near-complete recovery of phosphoCx43. These findings suggest that contractility and phosphoCx43 are supported above a threshold of ≈50% of basal ATP levels.

Recovery of PhosphoCx43 Is Not Attributable to De Novo Synthesis and Is Resistant to Inhibition of Mitogen-Activated Protein Kinases A and G

The recovery of phosphoCx43 after reoxygenation could arise from de novo synthesis and subsequent phosphorylation of Cx43 or from the rephosphorylation of existing protein. However, blocking new protein synthesis with 30′ exposure to cycloheximide had no effect on the recovery of phosphoCx43 during reoxygenation (Table). To identify the kinases responsible for the rephosphorylation of Cx43, we tested a series of selective inhibitors and dominant-negative mutants, the results of which are summarized in the table. The MAP kinases (p38, ERK, and c-Jun N-terminal kinase [JNK]) are activated by hypoxia and reoxygenation[20,28] (Figure 1D) and therefore were obvious candidates for the rephosphorylation of Cx43; moreover, JNK activation has been linked to loss of Cx43 during cell stress.[29] However, neither PD98059 and SB203850, selective inhibitors of ERK and p38 mitogen-activated protein kinase (MAPK), respectively, nor an adenovirus expressing a dominant-negative mutant of JNK shown previously to eliminate reoxygenation-mediated JNK activation,[20] had any effect on Cx43 abundance or phosphorylation whether in the presence or absence of hypoxia, despite essentially complete loss of kinase activity (Table; data not shown). Likewise, inhibition of protein kinases A and G with maximally effective concentrations of the selective inhibitors KT5720 and KT5823, respectively,[21,30] also failed to impair the reoxygenation-mediated recovery of phosphoCx43 (Table).

PhosphoCx43 Recovery Is Blocked by Broad-Spectrum PKC Inhibition

PKC has been implicated in phosphorylation of Cx43.[31] Inhibition of PKC with bisindolmaleimide or chelerythrine, and with the broad-spectrum protein kinase inhibitor staurosporine, prevented recovery of phosphoCx43 (Table; Figure 3A; data not shown). Interestingly, broad-spectrum PKC inhibition by either compound also caused strong enhancement of reoxygenation-mediated cell death, preventing the recovery of cellular ATP levels (data not shown), suggesting increased susceptibility to mitochondrial oxidative damage. In contrast, in normoxic cells, an equivalent 60′ exposure to chelerythrine had no effect on survival, ATP levels, or phosphoCx43 (Figure 3A, lane 4; data not shown). Because PKC isoforms δ and ϵ have been impli-

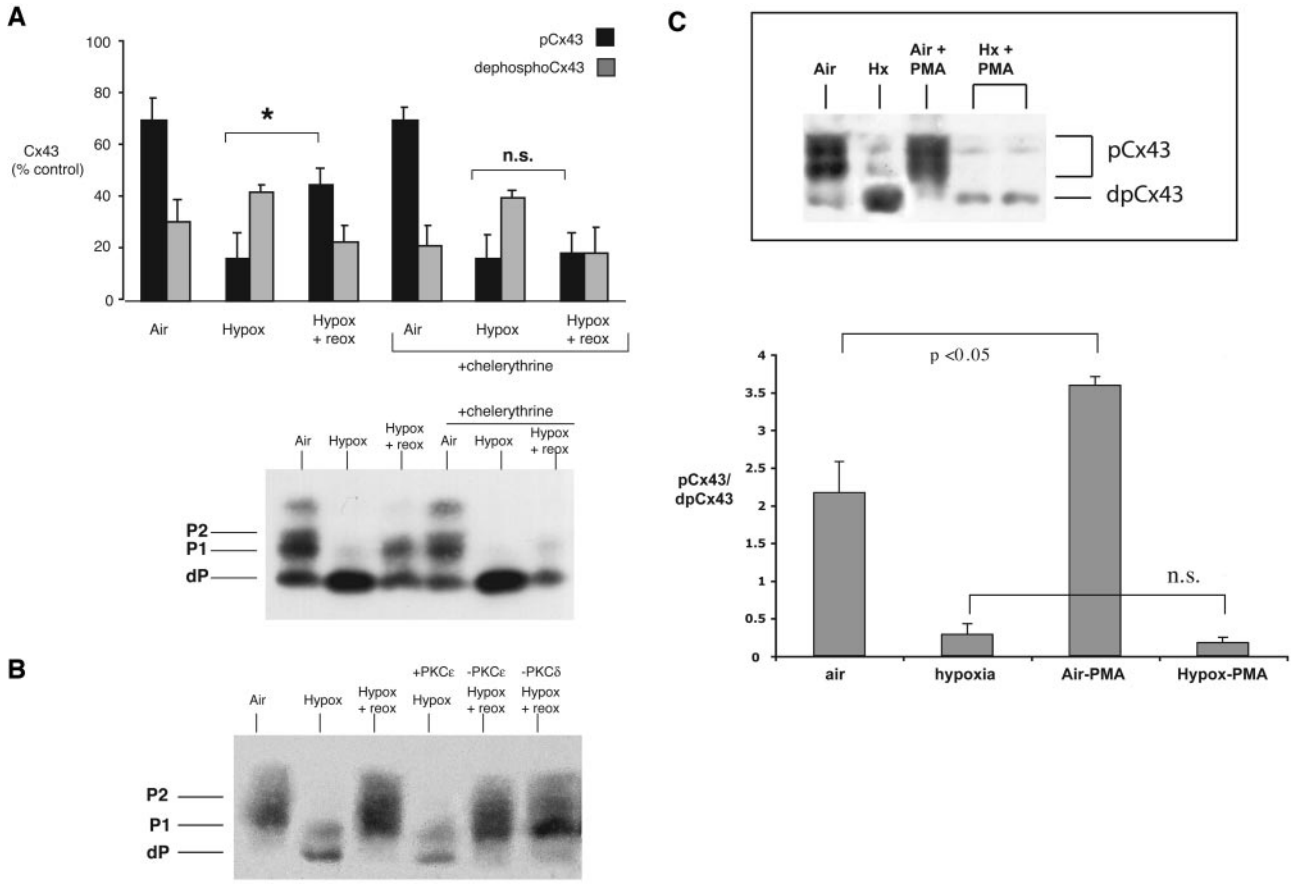

Figure 3. Effect of inhibition of PKC on rephosphorylation of Cx43 after reoxygenation. A, The broad spectrum PKC inhibitor chelerythrine blocks recovery of phosphoCx43 after reoxygenation. Chelerythrine (5 μmol/L) was added 1 hour before harvesting and 30′ before reoxygenation. n=4; *$P<0.01$ vs hypoxia 8-hour sample. Similar results were obtained with bisindolmaleimide and staurosporine (Table). B, Subtype-selective modulation of PKC-δ and -ϵ does not affect Cx43 dephosphorylation or recovery. Western blot shown is representative of 3 different experiments in which similar data were obtained. TAT-conjugated peptide activators and inhibitors of PKC isoforms were provided by Dr D. Mochly-Rosen and used as described in Dorn et al.[32] In brief, stock solutions of TAT-conjugated peptide activators and inhibitors of PKC-δ or -ϵ, or their vehicle (water; final concentration each 1 μmol/L) were added directly to the hypoxic cell cultures 30 minutes before reoxygenation and included in the normoxic media added at reoxygenation. C, PKC cannot prevent hypoxic dephosphorylation of Cx43. Cardiac myocytes were cultured at ambient O_2 tensions (air) or in hypoxia (H) for 8 hours. Either 10 nM PMA or its vehicle was added to cultures for 15′ before harvesting. Chart summarizes data from 3 experiments.

cated in the cell response to anoxia and reoxygenation in cardiac myocytes,[32] we also used Tat-conjugated peptide agonists and antagonists of these isoforms in the same system (Figure 3B, bottom). Inhibitory δ- or ϵ-specific PKC peptides did not block recovery of phosphoCx43 to control levels on reoxygenation (Figure 3B, lanes 5 and 6). Furthermore, a PKC ϵ-specific peptide agonist failed to reverse Cx43 dephosphorylation in hypoxic, glucose-depleted myocytes (Figure 3B, lane 4). None of the peptide reagents exhibited cytotoxicity under these experimental conditions. Finally, direct activation of PKC by PMA (10 nM, 15′) failed to phosphorylate Cx43 in hypoxic cells (Figure 3C, lanes and 5), although PMA did appear to eliminate basal dephosphoCx43 in aerobic cells (Figure 3C, lane 3), suggesting that additional factors are required to phosphorylate Cx43 when ATP levels are compromised.

Discussion

Ischemia is a complex insult, comprising hypoxia, acidosis, build-up of toxic metabolites, and ultimately ATP depletion. We show here that Cx43 dephosphorylation is not a consequence of irreversible myocardial damage but is a regulated and reversible process that is exquisitely sensitive to ATP levels. Neither hypoxia, per se, nor soluble metabolite accumulation accounted for Cx43 dephosphorylation, nor was it a passive consequence of high-energy phosphate depletion because ERK phosphorylation increased under the same conditions. Instead, the mechanisms regulating Cx43 phosphorylation appear to be selective and closely linked to the decline and recovery of cellular ATP levels below a critical threshold. Although such a relationship has been postulated,[13] the existence and significance of a specific correlation between connexin phosphorylation and ATP levels have not been established previously.

Gap junction activity is regulated at multiple levels, including Cx43 expression, degradation, and phosphorylation.[15,33,34] Basal phosphorylation–dephosphorylation cycles regulating phosphoCx43 content are largely dependent on PP1 activity,[35] and PP1 has also been reported to be involved in the dephosphorylation of Cx43 during ischemia.[36] The functional significance and specific molecular targets of phosphorylation in the gap junction are not clear, although electrical

conductance and dye permeability may be affected.[37–40] Surprisingly, metabolic dephosphorylation of Cx43 did not require PP1 because it was insensitive to calyculin A and okadaic acid in our system, although calyculin A and higher concentrations of okadaic acid reduced the relative amount of dephosphoCx43 in control cells. We also considered the possibility that AMP kinase, an ATP sensor, could be involved in this regulatory dephosphorylation indirectly via activation of unknown phosphatases; however, direct activation of AMP kinase by AICAR did not induce Cx43 dephosphorylation (data not shown).

The protein kinase involved in Cx43 rephosphorylation also remains to be identified. The lack of a requirement for new protein synthesis and the presence of significant amounts of dephosphoCx43 at areas of cell–cell contact[12] (data not shown) suggests that this protein kinase is likely to reside in the vicinity of myocyte plasma membrane gap junctions and to phosphorylate pre-existing dephosphoCx43. The Western analyses shown here do not shed light on specific residues regulated by ATP or provide information about which kinases may be mediating this regulation. Despite previous hints of ERK involvement[19] and the known activation of MAPKs during ischemia reperfusion,[41] our results indicate that no single one of the classical MAPKs is likely to be involved. Alternatively, Cx43 rephosphorylation may rely on multiple protein kinases.[18,42]

PKC has also been implicated in Cx43 phosphorylation,[17,31,43] although it is not clear whether this is a direct or indirect effect.[43] Our data exclude a direct role for either PKC-δ or PKC-ϵ isoforms in the rephosphorylation of Cx43 after hypoxia, suggesting that this represents a distinct physiologic signal pathway to that reported previously for fibroblast growth factor 2 stimulation.[17] However, our data are consistent with a role for the classical PKC pathway in phosphorylation of Cx43. In our system, the direct effects of PKC on Cx43 phosphorylation may be less important than its secondary effects on reoxygenation damage, mitochondrial ATP production, and cell survival; significantly, direct activation of PKC did not restore Cx43 phosphorylation under conditions of ATP depletion, and broad-spectrum PKC inhibitors prevented Cx43 rephosphorylation but also promoted reoxygenation-mediated cell death. We propose that PKC may be required for the resumption of mitochondrial ATP production after ischemic stress, and thus indirectly permissive for recovery of phosphoCx43. Further studies are required to identify the direct ATP sensor.

Dephosphorylation of phosphoCx43 could play a role in the cellular response to ischemia through effects on the transmission of cell fate signals. Cx43-deficient and heptanol-treated hearts are resistant to ischemia reperfusion damage, suggesting that gap junctions may communicate death signals between damaged and intact cells[10,11] or lead to loss of ATP and death through opening of membrane hemichannels.[44,45] On the other hand, loss of gap junction coupling also eliminates the protective effects of ischemic preconditioning.[46,47] Cx43 phosphorylation may affect cell survival and electrical activity differently;[48] dephosphorylation of Cx43 can impair transmission of chemical signals through gap junctions while maintaining electrical coupling.[49] Differential regulation of electrical and metabolic coupling also occurs in response to voltage gating[50] and may represent a protective adaptation.[51] Further work will be required to determine the influence of regulated dephosphorylation of Cx43 on the pathophysiology of myocardial ischemia.

Acknowledgments

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