PHOSPHATASES INVOLVED IN ISCHEMIA-INDUCED DEPHOSPHORYLATION OF THE GAP JUNCTION PROTEIN CONNEXIN43 IN CARDIOMYOCYTES

BY

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Phosphatases Involved in Ischemia-Induced Dephosphorylation of the Gap Junction Protein Connexin43 in Cardiomyocytes

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ABSTRACT

Connexin43 (Cx43) is a major constituent of cardiac intercellular communication channels termed gap junctions and is responsible for ensuring proper electrical and metabolic coupling of cardiomyocytes and maintaining cardiac rhythm. Abnormalities at the level of Cx43 distribution, accumulation, and phosphorylation exist in all cardiac pathologies and have been implicated in the induction of arrhythmias.

More specifically, ischemia of the intact heart is reported to cause rapid dephosphorylation of Cx43, an event correlating closely with the development of electrical abnormalities and tissue damage. Several categories of phosphatase inhibitors have been shown to confer a degree of cardioprotection during ischemia; their ability to prevent Cx43 dephosphorylation is not known. In fact, the phosphatase(s) mediating Cx43 dephosphorylation during ischemia have not been identified as yet. Thus the overall objective of our studies was to identify the phosphatase group(s) mediating Cx43 dephosphorylation during ischemia. An additional objective was to establish whether simulated ischemia would cause Cx43 dephosphorylation in an isolated cardiomyocyte model.

In the present study we have examined the following hypotheses: Hypothesis 1: Simulated ischemia of isolated rat cardiomyocytes will cause Cx43 dephosphorylation in a manner comparable to the adult heart. Hypothesis 2: Cx43 dephosphorylation is mediated by protein phosphatases belonging to the PP1/2A and/or the PP2B group.

Hypothesis 3: Prevention of cardiomyocyte Cx43 dephosphorylation during ischemia correlates with decreased myocardial injury.

We have used two in vitro systems to test our first hypothesis: In the first, cell pellets from isolated calcium tolerant adult rat cardiomyocytes were subjected to simulated ischemia by an oil overlay technique; In the second, confluent differentiated neonatal rat myocyte cultures were incubated in ischemic medium and a hypoxia chamber. In both models, simulated ischemia caused increases in dephosphorylated Cx43 accompanied by decreases in the phosphorylated 43-46 kDa Cx43 species, in a manner similar to the intact heart. This enabled us to use these models to address the hypotheses 2 and 3.

Adult myocyte pellets were subjected to ischemia in the presence or absence of the following inhibitors of:

* PP1/PP2A phosphatases, okadaic acid (OA) (1 μM), calyculin A (1 μM)
* PP1 phosphatase, tautomycin (10 nM)
* PP2A phosphatase, fostriecin (1 μM)
* PP2B phosphatases, cyclosporine A (CsA) (0.2 μM), FK-506 (150 nM)
* Combined inhibitors for PP1/PP2A and PP2B phosphatases.

All inhibitors were used at non-toxic concentrations. Cx43 content, composition and dephosphorylation were analyzed by western blotting of cell lysates and antibodies specific for dephosphorylated or total Cx43, followed by densitometry and statistical analysis. Calyculin-A and OA, used separately induced a partial prevention, by 28% and 31% respectively, of Cx43 dephosphorylation. Tautomycin also decreased

Cx43 dephosphorylation while fostriecin had no effect. CsA and FK-506, used separately, also decreased Cx43 dephosphorylation, by 33% and 28%, respectively.

Combined treatment with calyculin-A and FK-506 prevented Cx43 dephosphorylation by 94%. Confluent neonatal myocyte cultures were incubated in a hypoxia chamber for 4 hrs in the presence or absence of low concentrations of calyculin A (10 nM). Under these conditions, the inhibitor caused a reduction in dephosphorylated Cx43 as well as reduction in cellular injury, assessed by lactate dehydrogenase release. We conclude that phosphatases of both the PP1 and PP2B type, but not the PP2A type, mediate cardiomyocyte Cx43 dephosphorylation in ischemia. Prevention of Cx43 dephosphorylation by PP1 inhibition is linked to decreased ischemic injury of cardiomyocytes. The reported cardioprotection by PP1 and PP2B inhibitors therefore may be mediated, at least in part, by the preservation of Cx43 phosphorylation and cell coupling.

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ABBREVIATIONS

ANOVA.....Analysis of Variance

ATP.....Adenosine Tri Phosphate

BSA.....Bovine Serum Albumin

BCA.....Bicinchonic Acid

^{0}C.....Degree centigrade

CalA.....Calyculin A

CK-MB.....Creatine Kinase-MB

Cn.....Calcineurin

CsA.....Cyclosporine A

Cx.....Connexin

Da.....Dalton

dpc.....days post-coitus

ECL.....Enhanced Chemiluminescence

EGF.....Epidermal Growth Factor

FGF-2.....Fibroblast Growth Factor-2

FKBP.....FK Binding Protein

GFP.....Green Fluorescent Protein

GJ.....Gap Junction

GJIC.....Gap Junctional Intracellular Communication

HRP.....Horse Radish Peroxidase

hsp.....heat shock protein

I.....Inhibitor

ICD.....Intercalated Disc

IPC.....Ischemic Preconditioning

KDa.....Kilo Dalton

LDH-I.....Lactate Dehydrogenase-I

mM.....Milli molar

MCIP1.....Myocyte-enriched Calcineurin Interacting Protein 1

min.....minutes

mRNA.....messenger Ribo Nucleic Acid

MTP.....Membrane Transition Pore

NFAT.....Nuclear Factor of Activated T-cells

NFκB.....Nuclear Factor κB

OA.....Okadaic Acid

PKC.....Protein Kinase C

PLB.....Phospholamban

PMA.....Phorbol 12 Myristate 13-acetate

PMSF.....Phenylmethylsulfonylfluoride

PP.....Protein Phosphatase

PPI.....Protein Phosphatase Inhibitor

PTP.....Protein Tyrosine Phosphatases

PVDF.....Polyvinylidene Difluoride

SDS.....Sodium Dodecyl Sulfate

SEM.....Standard Error of the Mean

SMP.....Skim Milk Powder

TBST.....Tris Buffered Saline with TWEEN

TG.....Transgenic

TPA.....12-0-Tetradecanoyl-Phorbol-13-Acetate

µM.....Micro molar

CHAPTER 1

INTRODUCTION: LITERATURE REVIEW

A. GAP JUNCTIONS

A1: General Review

Gap Junctions (GJs) are communicating plasma membrane channels which connect the cytoplasm of adjacent cells and ensure electrical and metabolic coupling between cardiomyocytes {Beyer et al., 1990}. GJ channels are composed of connexons or hemi-channels, formed by proteins called connexins, which are members of a multigene family {Beyer et al., 1990; Willecke et al., 1991}. Connexins in the hemi-channel are arranged in a hexameric fashion {Manjunath and Page 1985}. The end-end alignment of two connexons contributed by two adjacent cells form intercellular channels in places where the plasma membranes of the adjacent cells come within 2-3 nm {Severs 1994}. Connexons have a central pore, which at its widest point measures less than 2.5 nm and permits the passage of small molecules less than 1000 Da {Goodenough et al., 1996}.

Based on the information from the human genome, 20 members of connexin family have been identified {Willecke et al., 2002}. The naming of connexins can be divided into two major systems: the molecular weight nomenclature that includes the species of origin and the molecular weight of the protein, (for example, rat Cx43) {Beyer et al., 1987}, and the α and β

nomenclature, that depends on genetic and primary sequence relationships {Kumar and Gilula 1992}.

Connexins contain two extracellular domains that are highly conserved, four transmembrane domains and one carboxy terminal, amino terminal and an intracellular loop {Yancey et al., 1989}. The extracellular domains are responsible for docking of the hemi-channels of the adjacent myocytes to form the GJ channel; they are linked by intramolecular disulfide bonds and have three conserved cysteine residues; the third transmembrane domain is responsible for pore formation {Hoh et al., 1991}. The intracellular cytoplasmic C-terminal acts as a gating particle and is considered responsible for the closure and opening of the channel by a ball and chain mechanism {Anumonwo et al., 2001}. The structure of the Cx43 with various phosphorylation sites is shown in Figure 1.

Gap junctions in mammals exist in nearly all cells and organs; notable exceptions are blood cells, spermatocytes and mature skeletal muscle {Dermietzel et al., 1997}. The gap junction channels are important for a variety of functions in different cells. Molecules less than 1000 Da, ions, aminoacids, sugars, second messengers like cAMP, IP_3 and ATP pass through the gap junctions from one cell to another, thus contributing to the metabolic coupling between the cells {Gilula et al., 1972}. They also play an important role in homeostasis {Goodenough 1992}, growth {Loewenstein 1979} and development {Lo 1996}.

STRUCTURE OF Cx43

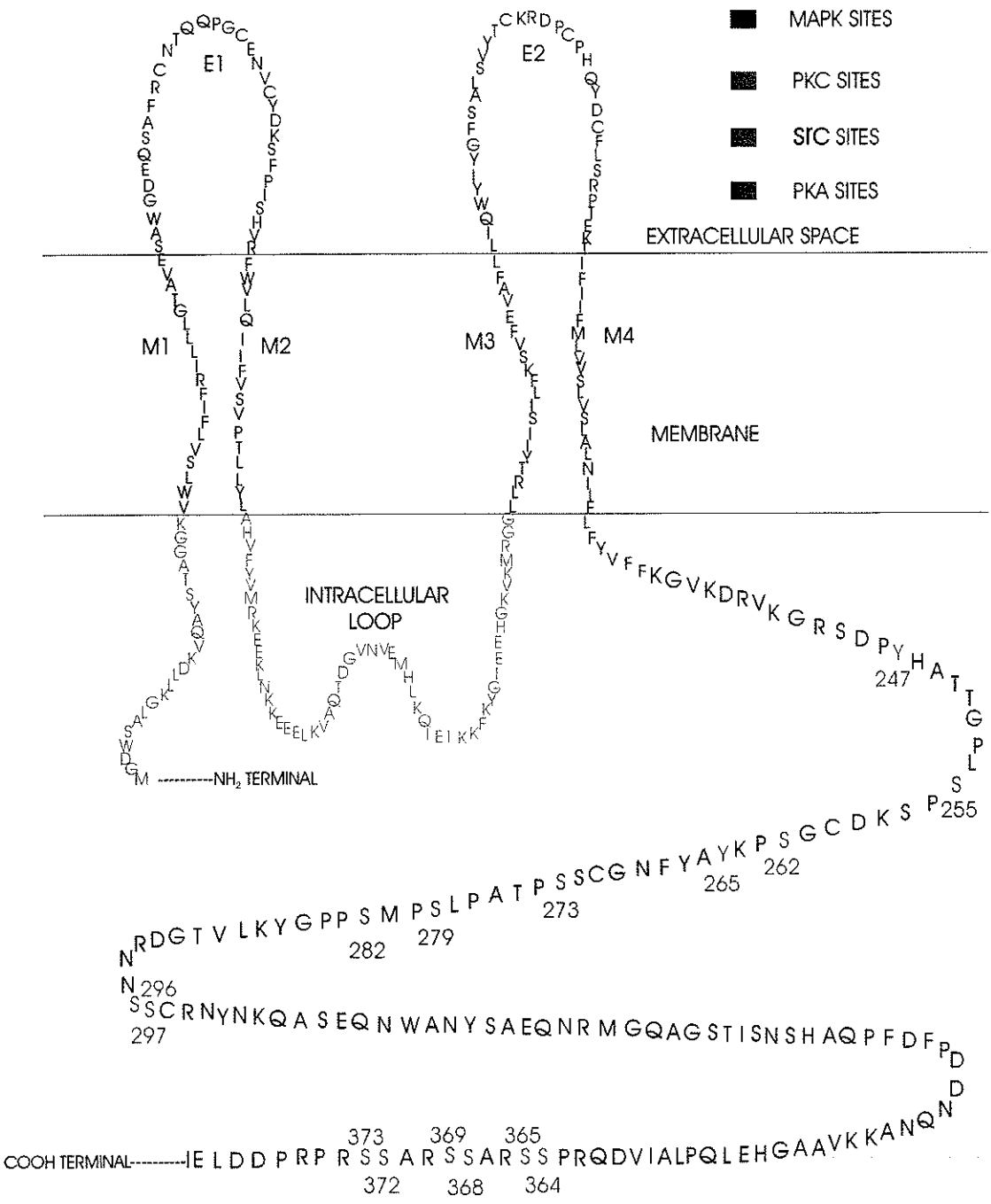


Figure 1

Selectivity and permeability of GJs depend on the type of connexin forming the channel {Veenstra 1996}. Permeability can be modified by a variety of factors such as, intracellular Ca^{2+} {Wang and Rose 1995}, pH {Ek-Vitorin et al., 1996}, voltage {Bennett and Verselis 1992}, and phosphorylation by various kinases {Lampe and Lau 2000}.

Phosphorylation of Connexins: Phosphorylation is addition of a phosphate group to serine/threonine or tyrosine residues thus altering the properties of the protein. The majority of connexins have been shown as phosphoproteins except Cx26 {Traub et al., 1989}. Phosphorylation of connexins, which occurs mainly at the C-terminal tail plays a key role in a variety of processes such as, trafficking {Puranam et al., 1993}, assembly/disassembly {Musil and Goodenough 1991}, and gating {Goodenough et al., 1996}. Since Cx43 is the subject of my project as well as the most abundant connexin, which is expressed widely in various tissues in the body, its phosphorylation is discussed in detail.

Multiple phosphorylation sites exist on Cx43 {Laird et al., 1991}, concerning mostly serine but also tyrosine residues {Lampe and Lau 2000} (see Figure 1 and Table 1). Increasing phosphorylation of Cx43 correlates strongly with its incorporation into the GJ plaque {Musil et al., 1990}. Non-phosphorylated Cx43 results in correspondingly faster electrophoretic mobility and apparently smaller molecular weight at 41 kDa {Lampe and Lau 2000}.

Table 1: Cx43 carboxy terminal residues demonstrated to be phosphorylated by various kinases

Cx43 Residues demonstrated to be phosphorylated	
Serine	255
	262
	279
	282
	368
	325
	328
	330
Tyrosine	247
	265

Various kinases such as those belonging to the protein kinase C (PKC) family {Lampe et al., 2000}, ERK1/2 {Warn-Cramer et al., 1996}, the tyrosine kinase src {Kanemitsu et al., 1997}, and casein kinase 1 {Cooper and Lampe 2002} have been implicated in the phosphorylation of Cx43. Growth factors generally inhibit GJ communication. Epidermal growth factor (EGF) inhibits junctional communication in some cell types {Maldonado et al., 1988} and stimulates serine phosphorylation of Cx43, which is reported to be mediated by ERK1/2 {Kanemitsu and Lau 1993}. Lau and coworkers have shown that Cx43 is phosphorylated by ERK1/2 on seryl residues 255, 279, and 282 and that the EGF-induced cell uncoupling is mediated by ERK1/2-dependant phosphorylation of Cx43 {Warn-Cramer et al., 1996}. Recently it has been demonstrated that ERK5 is responsible for the EGF-induced GJ uncoupling by specifically phosphorylating the Serine 255 residue of Cx43 alone, unlike the ERK1/2, which phosphorylates the Serine residues 279 and 282, in addition to 255 {Cameron et al., 2003}.

Additional Serine residues on Cx43 have been identified as targets of PKC phosphorylation. These include Serine 368 {Lampe and Lau 2000} and Serine 372 in vitro {Saez et al., 1997}. Src kinase has been shown to phosphorylate Cx43 on Tyr265 {Lin et al., 2001} and Tyr247 {Lau et al., 1996} and reduce the GJ coupling. Activated c-Src phosphorylates Cx43 on residue Tyr (265), resulting in a stable interaction leading to inhibition of gap junctional communication {Giepmans et al., 2001}. cGMP

dependant phosphorylation in rat Cx43 results in a decreased GJ conduction {Kwak et al., 1995}. Casein kinase 1 has been recently shown to phosphorylate Cx43 on Serine 325, 328, or 330; the cells treated with casein kinase specific inhibitor studied by immuno fluorescence show a reduction in the gap junctional Cx43, phosphorylated Cx43 and an increase in the non-junctional Cx43 {Cooper and Lampe 2002}. Our lab has evidence for the phosphorylation of Serine 262 in the physiological environment of the cardiomyocyte {Doble 2000 (abstract)}. Phosphorylation sites of Cx43 are summarized in Table 1.

A2: Gap Junctions in the Heart

In the normal adult heart, GJs are predominantly located in the intercalated disk region of the myocytes {Jongsma and Wilders 2000}. The periphery of the intercalated disk region contain larger GJ clusters while smaller ones are located in the central intermediate regions as shown by confocal microscopy {Gourdie et al., 1991}. Hoyt and others demonstrated that typically each ventricular myocyte in the heart is connected to approximately 11 other cells in side-to-side or end-to-end orientations in variable degrees and hence the electrical impulse propagates in both longitudinal as well as in transverse directions {Hoyt et al., 1989}. In the canine ventricular myocytes the GJs were shown to be located in an end-end as well as in a side-side fashion in equal proportion; in the atrial myocytes at the crista terminalis (part of the atrial tissue responsible for the conduction of impulses from sinus node to the atrioventricular tissue),

close to 80% of the connections were shown to occur in an end-to-end fashion as confirmed by ultrastructural analysis {Saffitz et al., 1994}. The impulse propagation in the normal heart is three fold faster along the long axis of the myocytes compared to the transverse axis {Delmar et al., 1987; Dillon et al., 1988}.

In the heart, the sequential propagation of impulse from the atria to the ventricle has been suggested to be due to the slow conduction in the AV node (due to the presence of small and sparse GJs) and the fast conduction in the Purkinje fibers and the working cardiomyocytes (due to the abundance of GJs) {Davis et al., 1995}. The Purkinje fibers were shown to be connected by extensive lateral and end-to-end connections and they form a huge network suggesting a rapid impulse propagation {Saffitz et al., 1995}. The size, number and the distribution of the GJs in the myocytes in 3D space have been attributed as the main determinants of the conduction properties of the myocardial tissue {Delmar et al., 1987; Dillon et al., 1988; Peters et al., 1993}. Together all these features contribute to the normal anisotropic conduction (the impulse propagation being different in different directions) of electrical impulses in the heart.

Cx43 is the major connexin of the working cardiomyocytes {Saffitz et al., 2000} and is one of the first connexins to be characterized by molecular cloning {Beyer et al., 1987; Kumar and Gilula 1986}. Four different types of connexins are expressed in the mammalian heart. They are Cx37 (predominantly expressed in endothelial cells), Cx40, Cx45

(predominantly expressed in cardiac conduction tissue), and Cx43 (predominantly expressed in vascular smooth muscle cells, fibroblasts and the working myocytes) {Doble and Kardami 1995; Gros and Jongsma 1996; Yeh et al., 1997}. Cx43, Cx40 and Cx45 are differentially expressed in certain regions of the heart in various combinations. There are differences in the composition of the atrial and ventricular myocytes. Davis and his colleagues reported that in the normal human heart, atrial GJs express Cx40, Cx43, and Cx45 in moderate amounts; sinus node GJs, express mainly Cx45 and smaller amounts of Cx40 but lack Cx43; AV nodal GJs express predominantly Cx45 and Cx40 and smaller amounts of Cx43; bundle branches express large amounts of Cx40, Cx43, and Cx45 {Davis et al., 1995}.

GJ channels perform several functions in the heart. They allow electrical coupling between myocytes, by conduction of impulses from one myocyte to another {Beyer 1993}. They also mediate metabolic coupling between myocytes, a process that may be linked to regulation of signal transduction, differentiation and growth {Doble et al., 2000}. GJ coordinate with other specialized junctions at the intercalated disk such as the adjacent fascia adherens and the desmosomes (responsible for connecting the contractile apparatus and the intermediate filaments of the adjacent cells respectively) to assist in the electromechanical coupling between adjacent cells {Severs 1994}.

Transgenic models have been developed to analyze the role of GJ proteins in the conduction properties of the heart. The Cx43 knock-out mouse was the first model to be developed and since homozygotes died immediately after birth (due to pulmonary outflow obstruction), it was not possible to measure the conduction properties in these mice {Reaume et al., 1995}. In contrast the heterozygous Cx43 mice ($Cx43^{+/-}$) survived till adulthood and bred normally; and no significant changes in the conduction parameters were noted compared to the wild type mice {Guerrero et al., 1997; Morley et al., 1999}, although earlier studies indicated that these mice had slowing of the activation of impulses {Guerrero et al., 1997}. Computer simulation studies by Jongsma and Wilders suggest that cell geometry and cytoplasmic resistivity but not necessarily gap junction content play a major role in the assessment of the conduction velocity, since 40% reduction in the GJ content has only a moderate effect on the cardiac conduction velocity {Jongsma and Wilders 2000}.

Cx40 knock-out mice models have also been generated. The survival of these mice is thought to be due to the compensatory increase in the connexins co-expressed with Cx40 in the atrium (Cx45, Cx43), conduction tissue (Cx45), and the endothelial tissue (Cx37); the electrocardiogram of these mice show decreased conduction velocities but some of these mice also show arrhythmias and atrioventricular block indicating the fact that Cx40 can play an important role in the arrhythmogenesis {Kirchhoff et al., 1998}.

Cx43/Cx40 double deficient mice were also developed and the electrocardiogram of the Cx43/Cx40 double heterozygous knock-out mice ($Cx43^{+/-}/Cx40^{+/-}$) show a slight increase in the QRS and QT_{max} interval suggesting slowed conduction in the ventricular myocytes (due to Cx43) and the conduction system (due to Cx40) compared to wild type mice ($Cx43^{+/+}/Cx40^{+/+}$); the Cx43 homozygous ($Cx43^{-/-}/Cx43^{+/+}$) mice were shown to have the same cardiac defects (conotruncus malformation) compared to the ($Cx40^{-/-}/Cx43^{+/-}$) mice in contrast to the Cx40 homozygous mice ($Cx40^{-/-}/Cx43^{+/+}$) in which the septation defects were aggravated by the presence of heterozygous Cx43 ($Cx40^{-/-}/Cx43^{+/-}$); from these findings the authors suggest that both these connexins have additive effects during cardiac septation {Kirchhoff et al., 2000; Reaume et al., 1995}.

A Cx45 knock out mouse model was also developed and they did not survive beyond the 10^{th} embryonic day. Even though contractions were initiated in these mice there was no coordination between the outflow tract and the ventricle and the atrioventricular tissue exhibited conduction block {Kumai et al., 2000}. These findings are consistent with the previous studies that show that Cx45 is expressed in the atrioventricular tissue and the outflow tract {Alcolea et al., 1999; Delorme et al., 1997}.

All the above germline knockout studies (in which the connexins were knocked out not only in the heart, but all tissues) discussed so far, were limited by the developmental phenotype associated with them and

the lethality of these mice around term. Gutstein and his colleagues developed a cardiac specific Cx43 conditional knock-out mouse (Cx43 CKO) model using the Cre-lox method {Baubonis and Sauer 1993} to address the role of the Cx43 GJs in arrhythmogenesis specifically in the heart. These transgenic mice were born alive without any defect seen in the Cx43 germline knock-out mice, but a few weeks after birth they died of sudden spontaneous lethal ventricular arrhythmias as shown by telemetry recordings {Gutstein et al., 2001}. Optical mapping studies also indicated the development of lethal arrhythmias in the Cx43 knock-out mice compared to normal rhythm in control mice {Gutstein et al., 2001}. The above study also indicated that Cx43 is not essential for cardiac development, because when Cx43 expression was inactivated in heart before embryonic day 12.5 there was no defect in the heart, in contrast to the findings from mice with germline deletion of Cx43; they also suggested that the cardiac defects in the previous studies with germline knockouts can be of neural crest origin {Gutstein et al., 2001}. From all the above transgenic studies it is clear that Cx43, while not essential for early cardiac development, plays an important role in the development of arrhythmias. Cx43 therefore can serve as a novel therapeutic target in various diseases that pose a risk factor for lethal arrhythmias.

GJs play an important role in normal tissue development in embryogenesis, by allowing essential morphogenetic signaling molecules to diffuse through and thereby control differentiation and organ

development {Guthrie and Gilula 1989; Loewenstein and Rose 1992; Warner et al., 1984}. Cx43 is detectable from the earliest stages of the fetal development, from 10 dpc (days post-coitus) onward as shown by Gourdie and his colleagues {Gourdie et al., 1992} using confocal microscopy. They also showed that at very early stages of development, Cx43 GJs were present in high amounts in the trabeculae of the ventricles and low amounts in the outflow tract and interventricular septum {Gourdie et al., 1992}. In the conduction tissues, Cx43 GJs were absent at the early stage of development but later became detectable in low amounts in the AV node alone in the postnatal heart {Gourdie et al., 1992}. The mRNA and the protein levels of Cx43 increase progressively in the embryonic and early neonatal stages of the heart and later with increasing maturity the levels start decreasing {Fishman et al., 1991}. After birth, changes occur in the distribution pattern of the GJ in the ventricles. In humans, GJs are located all over the myocytes until 6 years of age (a distribution which facilitates the remodeling associated with growth and changing hemodynamics) but later they become restricted to the intercalated disk region, a distribution which correlates with the relatively stable adult myocardium {Peters et al., 1994; Spach 1994}.

GJs are dynamic structures and cardiac GJ plaques have been shown to have a rapid turn-over in vivo (half-life of <1.5 hours) {Beardslee et al., 1998}. Cx43 has also been shown to have a rapid turnover in vitro (half-life of 1.5 to 2 hours) {Laird et al., 1991} indicating the fact that these

proteins are replaced several times in a day. Beardslee et al. provided evidence that Cx43 is degraded both by the proteasomal and lysosomal pathways in both adult and neonatal myocytes {Laing and Beyer 1995; Laing et al., 1997}. One mechanism of Cx43 GJ internalization has been shown using time-lapse imaging of Cx43-GFP {Jordan et al., 2001}. The Cx43 GJs were shown to be internalized as structures called "annular GJs" in to one of the interacting cells {Jordan et al., 2001}. These annular junctions are present in cardiac myocytes especially in the recently isolated adult myocytes {Mazet et al., 1985}. Safftiz and others suggested that, since Cx43 turns over rapidly, degradation of Cx43 can serve as one of the mechanisms that control the levels of electrical coupling between cells in the heart by adjusting the number of channels between them {Saffitz et al., 2000} both in physiological as well as in various pathological conditions.

Cx43 exists predominantly in a phosphorylated state in the normal heart {Lau et al., 1991}. Various kinases are involved including PKC-ε, PKC-α, PKA, PKG, ERK1/2, and p38 {Darrow et al., 1996; Doble et al., 2000; Kwak et al., 1995; Polontchouk et al., 2002}. Cx43 phosphorylation plays a key role in the regulation of all aspects of GJ function including intercellular communication. Regulation of the conductance of human Cx43 by phosphorylation was shown clearly by Moreno and colleagues {Moreno et al., 1994}. In cardiomyocytes the activation of the PKA (Protein kinase A) has been shown to increase the GJ mediated intracellular

communication {Darrow et al., 1996}. Kwak and colleagues have shown that in neonatal cardiac myocytes single channel conductances increase by activation of PKC and decrease by the activation of PKG {Kwak et al., 1995}. While activation of PKC caused increased electrical coupling, it decreased metabolic coupling between cells, as assessed by fluorescent dye transfer {Kwak et al., 1995}. Doble and colleagues have shown that in the neonatal rat cardiomyocytes fibroblast growth factor-2 (FGF-2) reduces GJ dye coupling and also phosphorylates Cx43 on serine resulting in a slower electrophoretic migration of Cx43 species {Doble et al., 1996}. Using specific inhibitors for PKC, ERK1/2 and dominant negative forms of PKC-ε they further showed that it is PKC-ε and not ERK1/2 that mediates Cx43 phosphorylation by FGF-2 {Doble et al., 2000}. PMA (phorbol 12 myristate 13-acetate) stimulation of neonatal cardiomyocytes leads to increase in the phosphorylated species of Cx43 and this action has also been shown to be mediated by PKC-ε (using dominant negative forms of PKC-ε) {Doble et al., 2001}.

Findings by Doble et al. were subsequently confirmed by Bowling and colleagues who have shown that, in the failing and non-failing human hearts, PKC-ε directly interacts and phosphorylates Cx43 {Bowling et al., 2001}. The anti-arrhythmic peptide (AAP10) has been shown to increase the GJ conductance in adult cardiomyocytes from guinea pigs by phosphorylation via PKC-α {Weng et al., 2002}. Incubation of neonatal cardiac myocytes with angiotensin-II (AT-II) and endothelin-1 (ET-1) have

been shown to increase the expression and the phosphorylation of Cx43 where AT-II and ET-1 have been shown to likely act via ERK1/2 and ERK1/2 and p38 respectively {Polontchouk et al., 2002}. From all the above studies it is understood that phosphorylation of Cx43 GJ can play a vital role in maintaining the GJ mediated cell communication in the normal heart.

B. ISCHEMIC HEART DISEASE

B1: General Review

Cardiovascular disease is a leading cause of death worldwide. Myocardial ischemia, a major cause of cardiac pathology, is defined as severe reduction in the blood flow to the heart, such that the supply of oxygen does not match the demand. Reversible ischemia indicates the situation where ischemia if followed by reperfusion within a period of less than 20 minutes and leads to complete recovery of the cardiomyocytes from damage caused due to ischemia {Ip and Levin 1988}. Reversible ischemia leads to mild pathological changes and ultrastructural abnormalities such as leakage of enzymes {Wiggers et al., 1997} abnormalities in the ion channels {Shaw and Rudy 1997} and a degree of contractile dysfunction or 'stunning' {Opie 1996}.

Longer periods of ischemia cause activation of proteases and phosphatases causing degradation and dephosphorylation of cellular proteins respectively; progressive destruction of gap junctions,

sarcomeres and intermediate filaments can be observed; the cell cytoskeleton detaches from their cellular attachments; irreversible injury of heart cells and fatal complications such as arrhythmias occur {Ganote and Vander Heide 1987; Huang et al., 1999; Jennings et al., 1986}. When ischemia is prolonged for more than 20 minutes, the cytoskeletal protein vinculin is lost from the peripheral regions of the cardiomyocytes and serves as the early indicator of irreversible injury {Steenbergen et al., 1987}. Due to the reduction in the blood supply to the myocardial tissue during ischemia, there is a decrease in glucose and oxygen content and catabolites such as lactate, H^+, CO_2 and inorganic phosphates accumulate in the cells of the affected region {Ip and Levin 1988; Karmazyn 1991; Opie 1991}. A shift from aerobic to anaerobic glycolysis occurs and this leads to decrease in the amount of available ATP and also lactic acidosis {Opie 1976}. The decrease in the energy production leads to improper functioning of the ion channels, which leads to accumulation of intracellular Ca^{2+} and Na^+ ions and extracellular K^+ ions (due to the opening of the K_{ATP} channel) {Carmeliet 1999} and also Ca^{2+} accumulation in mitochondria {Piper 2000}. The action potential duration is shortened during ischemia and since the resting membrane potential is less negative, depolarization rate becomes slow and there is a early return to the resting level {Opie 1996}. The increase in hydrogen ion concentration causes decreased binding of the Ca^{2+} to the contractile filaments leading to reduced contractility of the myocardium {Blanchard and Solaro 1984}.

Ischemic damage of sarcolemma allows cytosolic enzymes such as CK-MB (creatine kinase-MB), LDH-I (lactate dehydrogenase-I) or myofibrillar proteins such as cardiac troponin-T or myosin to leak to the circulation; the concentration of cardiac proteins in the blood is used to assess ischemic damage {Lavie et al., 1991; Remppis et al., 1995}.

Reperfusion interventions are required to re-establish blood flow and thus replace depleted oxygen and energy supplies and remove metabolic wastes from the ischemic myocardium; nevertheless, the act of reperfusion itself can exacerbate of myocardial injury and further tissue loss, and is linked to contractile dysfunction; all of which is collectively termed "reperfusion injury" {Piper et al., 2003} and will be discussed in the next section.

B2: Ischemia and Gap Junctions

Since GJ mediate electrical, mechanical and metabolic coupling of the cardiac syncytium, abnormalities at the level of connexin concentration and three-dimensional distribution would be expected to have a major impact on several aspects of cardiac physiology, including cardiac rhythm, mechanical strength of contraction, and metabolic state. Indeed, all cardiac diseases examined to-date are associated with GJ abnormalities.

Arrhythmia: Arrhythmias are a feature of several cardiac pathologies. The mechanism underlying development of arrhythmias may be a unidirectional block of impulse propagation or a slowing of conduction leading to re-entrant electrical circuit {Hoffman and Dangman 1987}.

Increases in intracellular Ca^{2+} and H^{+} {Burt 1987}, accumulation of lipid metabolites {Massey et al., 1992} and abnormalities at the GJ level are believed to contribute to the development of arrhythmias during ischemia. GJ changes generally include decreases in Cx43 levels, and re-distribution of Cx43 from the intercalated disk area to the lateral surface of myocytes {Severs 1994}. The number of cells connected via intercalated disks to a single myocyte is reduced from 11 to 6 in the infarct border zone {Luke and Saffitz 1991}; these GJ changes are expected to impact on the propagation of electrical signals.

GJ abnormalities may play a critical role in arrhythmogenesis since resting membrane potential contributed by ions in the myocardium can be normal in ventricular arrhythmias {Dillon et al., 1988; Spach et al., 1988; Ursell et al., 1985}. Compelling evidence for the role of Cx43 in the development of arrhythmias and sudden death was provided by Gutstein et al; sudden death due to arrhythmia a few months after birth was observed in mice with cardiac-specific inactivation of Cx43 {Gutstein et al., 2001}. Similarly, Lerner and others reported that Cx43 deficient mice displayed increased incidence of ventricular arrhythmias when subjected to ischemia {Lerner et al., 2000}.

In addition to the formation of intercellular channels, Cx43 is capable of forming hemi-channels at non-junctional surfaces of the cell {Lal and Lin 2001}. Hemi-channels are closed under physiological conditions but can open during metabolic inhibition, and have thus been

proposed to be responsible for increases in intracellular Na+, Ca2+ and extracellular K+ ions during ischemia, contributing to ionic imbalance {John et al., 1999; Li et al., 2001}. Opening of hemi-channels during metabolic inhibition has been proposed to be the origin of arrhythmias during ischemia {Kondo et al., 2000}.

Ischemia: During acute ischemia, in the myocardium deprived of oxygen, increase in the intracellular Ca^{2+} and H^{+} and reduction in the ATP could lead to a decrease in the GJ conductance and slowing of conduction. Early studies indicated that following 30 minutes of ischemia there is alteration of the GJs and freeze-fracture studies showed a reduction in the center-center GJ particle distance {Ashraf and Halverson 1978}. Later, Hoyt and coworkers {Hoyt et al., 1990} suggested that uncoupling at 30 minutes of hypoxia was not associated with alteration of the GJ surface density. They also suggested that since the reduction in the GJ particle distance occur at 15 min of ischemia, when there is no uncoupling, it is possible that loss of coupling could be a result of a conformational change in the GJ channel {Hoyt et al., 1990}. More recent studies indicated that GJs undergo various alterations in levels, distribution and phosphorylation during ischemia {Beardslee et al., 2000; Huang et al., 1999; Lerner et al., 2000}. In adult canine myocardium a heterogenous (loss of Cx43 more in the middle layer of the heart) degradation of Cx43 (about 50% in ~4.8 hours) has been shown to occur after ischemia {Huang et al., 1999}. In a model of global ischemia of the

perfused heart it was shown that there is a decrease in the Cx43 staining at the ICD region suggesting that the ICD region is very sensitive to ischemic injury {Huang et al., 1999}. Cx43 dephosphorylation has also been detected approximately 1 hour after ischemia in coronary occlusion model and was suggested that this may precede Cx43 degradation that occurs after ischemia {Huang et al., 1999}.

Ischemic duration of more than 15 min is reported to cause electrical uncoupling detected as a sudden increase in GJ resistance and cessation of action potential propagation {Beardslee et al., 2000}. This electrical uncoupling has been proposed to be a consequence of Cx43 dephosphorylation {Beardslee et al., 2000}, although direct evidence is lacking. On the other hand, other studies show that dye coupling ('chemical' or metabolic coupling) persists in isolated myocytes and isolated perfused hearts even after 30 min ischemia {Ruiz-Meana et al., 2001}. Persistent metabolic coupling of ischemic myocytes is proposed to increase the range of ischemic injury. Spreading of death signals via GJs was observed in other cell types as well and likely represents a general GJ mediated mechanism {Cotrina et al., 1998}. For example, in cortical astrocytes in culture and in brain slices ischemia causes opening of GJ channels, which were proposed to contribute to the secondary expansion of ischemic lesions {Cotrina et al., 1998}. GJs, have also been shown to propagate signals leading to cell death. Ruiz-Meana and colleagues suggest that differential regulation of GJ-mediated electrical and metabolic

coupling may be responsible for the apparently different responses to ischemia, such as electrical but not chemical uncoupling {Ruiz-Meana et al., 2001}.

GJ changes have also been shown to occur in chronic ischemia. Chronic ischemia in a particular region of the heart could result in a mild disturbance in the electrical potential and an abnormal ventricular wall motion, due to improper coordination of contraction in the affected tissue {Peters 1995}. Some of the studies on the role of GJs in situations of chronic ischemia are summarized below. In healed canine infarct model a decrease in the surface density of the GJ has been shown to occur (using electron microscopy); GJs per unit length of ICD has been shown to be fewer and smaller {Luke and Saffitz 1991}. In the myocytes at the border of healed human infarcts, GJs are spread all over the surface of the myocytes instead of being aggregated into intercalated disks {Peters 1995}. A decrease in the GJs per unit cell volume occurs in the area distant to the infarct zone in the chronic ischemic human heart {Severs 1994}. In the ischemic heart samples a 40% reduction in surface area of the Cx43 GJ was noted {Gourdie et al., 1991}. All the above studies show that the decrease in GJ content may play a vital role in the generation of arrhythmias and electromechanical dysfunction in chronic ischemia.

Reperfusion injury includes further disruption of the ischemic sarcolemma and lethal calcium overload; energy depletion and calcium overload result in hypercontracture, a clear sign of irreversible injury {Piper

and Garcia-Dorado 1999; Piper et al., 2003}. Because hypercontracture seems to occur in a continuous zone of connected cardiomyocytes in necrotic regions, it is suggested that it is caused by a signal propagated via GJ {Piper and Garcia-Dorado 1999}. Passage of Na^+ through GJ is thought to result in calcium overload through Na^+/Ca^{2+} exchange {Ruiz-Meana et al., 1999}. In support of these claims, use of the GJ blocker heptanol during the reperfusion stage leads to cardioprotection {Garcia-Dorado et al., 1997}.

Additional ischemia-related phenomena have been described, including ischemic preconditioning and hibernation: Short periods of ischemia followed by reperfusion decrease the impact of a longer ischemic insult; this phenomenon is termed ischemic preconditioning (IPC) and is very well documented {Kosieradzki 2002}. Cardiac Cx43-composed GJs are essential for the preconditioning response; Schwanke and coworkers {Schwanke et al., 2002} demonstrated that hearts with reduced Cx43 content, from Cx43 (+/-) mice, do not manifest IPC. It is important to note that IPC is dependent on activation of protein kinase C-ε (PKC-ε) {Liu et al., 2001}. PKC-ε interacts with and phosphorylates Cx43, altering intercellular communication {Doble et al., 2000}. It is thus proposed that loss of IPC in Cx43 (+/-) mice may be caused by preventing the protective effects of PKC-ε to be properly applied at the GJ region {Schwanke et al., 2002}. In addition to the above, another group {Li et al., 2002} reported that use of the gap junction blocker heptanol prevented IPC; these authors

hypothesize that there may exist a 'survival' signal transmitted via GJ between cells during IPC. Recently using isolated perfused rat hearts, Jain and others have demonstrated (using confocal microscopy, immunoblotting and measurement of whole cell resistance) that electrical uncoupling, Cx43 dephosphorylation and the intracellular translocation of the Cx43 during ischemia are significantly decreased by IPC and suggest that these actions are mediated by K_{ATP} channels {Jain et al., 2003}. They also showed that, PKC-ε is responsible for the decrease in the translocation of the Cx43 but not the decrease in the Cx43 dephosphorylation during IPC {Jain et al., 2003}.

Hibernating myocardium: Hibernating myocardium is found in regions of hypoperfusion and is characterized by chronically impaired function {Frangogiannis 2003} that improves with revascularization; cardiac dysfunction correlates well with distinct qualitative and quantitative Cx43 changes at the intercalated disk level; reduced and disrupted GJ are therefore believed to contribute to the electromechanical dysfunction of the hibernating myocardium {Kaprielian et al., 1998}.

Hypertrophy: Cardiac hypertrophy is a compensatory response to pressure or volume overload and is also associated with remodeling and disorganization at the GJ level; generally Cx43 content decreases in chronically hypertrophied hearts {Peters et al., 1993}, although it may increase in the early stages of hypertrophy {Saffitz et al., 1994}. Cooklin et al. {Cooklin et al., 1997} reported increased gap junction resistance in the

hypertrophied myocardium, likely the result of decreased number of GJ channels. In a recent study, Emdad and coworkers showed that GJ redistribute over the whole surface of hypertrophic cardiomyocytes, and that GJ density at the intercalated disks decreases {Emdad et al., 2001}.

Heart Failure: Decompensated cardiac hypertrophy characterizes the transition to heart failure {Lips et al., 2003}, leading to further GJ changes. Significantly reduced Cx43 were reported in congestive heart failure in humans {Dupont et al., 2001; Severs 2002}. Dupont et al. reported that in idiopathic dilated cardiomyopathy there is significant reduction in the Cx43 content of the left ventricle, accompanied by an increase in Cx40 in the endocardial surface {Dupont et al., 2001}. Cardiomyopathies, in humans and animal models, are also associated with redistribution and disorganization of GJ {Chen and Jones 2000; Sepp et al., 1996}.

B3: In Vitro Models of Ischemic Heart Disease

Different in vitro and in vivo models of ischemic injury have been used to address a variety of issues related to heart disease. In vitro models consist of isolated perfused hearts and isolated cells. The cell-based models could be divided further in to three categories depending on the type of cells used namely, immature cardiac myocytes, mature cardiac myocytes, and non-myocytes {Marber 2000}.

In 1990, Vanderheide and colleagues showed that isolated cardiomyocytes could serve as a useful model to study the effects of

ischemia sharing characteristics similar to the in vivo systems {Vander Heide et al., 1990}. In 1994, Armstrong and his group described the first and most widely used model of in vitro ischemia of isolated adult cardiomyocytes, namely the mineral oil model of ischemia {Armstrong et al., 1994}. In this model, the freshly isolated cardiomyocytes are subjected to ischemia by pelleting them and overlaying mineral oil on top of the fluid layer containing the pellet. The damage due to ischemia can be assessed by taking samples during specific time periods of ischemia and analyzing them using various tests available. Another useful model of ischemia is the simulated ischemia of neonatal cardiomyocytes using a hypoxia chamber {Zhao et al., 1998}. In this model, confluent cultures of neonatal cardiomyocytes are subjected to hypoxia using a hypoxia chamber circulated with 95%N_2 and 5% CO_2 together with incubation in an ischemic solution. The advantages and the limitation of these in vitro models are described below.

Advantages:

(1) It allows for the precise control and manipulation of the external environment in the absence of blood borne factors

(2) The parameters obtained from a homogenous myocyte population in the in vitro model are free from influences from the other cell types and gives an indication of the role of that particular cell type under given circumstances

(3) Is easier to use for gene transfer studies, for example introduction of dominant-negative molecules, to delineate signal transduction {Zhao et al., 1998}

(4) The isolated model system is also cost effective and less time consuming, since more than one experiment can be done from one single isolation, which is not the case with the in vivo systems

(5) In vitro models make it possible for the valuable human cells to be used in experiments.

Limitations:

(1) After isolation the myocytes undergo shock due to sudden change in the environment

(2) The myocyte extracellular environment is not similar and so the results are influenced by these conditions

(3) They also lack the influences of the extracellular matrix, vascular tissue and neurohumoral substances as that of in vivo myocytes and so even if the myocytes are subjected to ischemia it is not similar to that which occurs in vivo.

In spite of the above-mentioned limitations, isolated myocytes provide a very valuable in vitro model of ischemia by which specific aspects of ischemia could be studied in detail. The isolated myocyte model also serves a useful model to study GJ channels in the absence

(isolated adult myocytes) or presence (neonatal myocytes) of intercellular communication. In the case of isolated adult cardiomyocytes, it has been shown that, upon cell seperation of the two contacting cells, one cell internalizes the GJ contribution from both the cells, contributing to the formation of annular junctions {Jordan et al., 2001}.

C. PHOSPHATASES

C1: Phosphatases/ Overview

Diverse biological processes (for example metabolism, growth, gene expression, muscle contraction) are regulated by phosphorylation (by kinases) and dephosphorylation (by phosphatases) of proteins in complex signal transduction pathways. Significant portions of cellular proteins (30%) are phosphoproteins, while 4% of the human genome codes for various kinases and phosphatases {Hunter 1995}. Levels and activity of these enzymes must be balanced and regulated to ensure appropriate cellular responses. Several pathologies (for example cancer, immunodeficiencies) are associated with abnormal levels of kinases/phosphatases {Jia 1997}. Proteins become phosphorylated on serine, threonine and tyrosine residues, although histidine phosphorylation has also been described {Swanson et al., 1994}. As a consequence protein phosphatases are classified into three categories depending on their substrate specificity: protein tyrosine phosphatases (PTPs) {Fischer et al., 1991} that dephosphorylate tyrosine residues; protein

serine/threonine phosphatases {Cohen 1989}, dephosphorylating serine or threonine residues; dual specificity phosphatases that dephosphorylate tyrosine as well as serine/threonine residues (they actually represent a subgroup of the PTP family) {Jia 1997}. The focus of this review will be predominantly on serine/threonine phosphatases. For information on the other categories the reader is directed to recent reviews {Theodosiou and Ashworth 2002; Zhang 2002}.

Serine/threonine phosphatases: The serine/threonine protein phosphatases are a unique class of enzymes, which are involved in the catalysis of serine and threonine residues of phosphoproteins. These enzymes have no sequence similarities with the protein tyrosine phosphatases. Two broad subgroups have been described: Type 1 (PP1) and type 2 (PP2) phosphatases; the type 2 phosphatases are further subdivided in three groups, PP2A, PP2B and PP2C {Herzig and Neumann 2000}.

PP1 (but not PP2A) are inhibited by endogenous protein inhibitors I-1 (Inhibitor-1) and I-2 (Inhibitor-2); PP2B (but not PP1, or PP2A) requires calcium and calmodulin for activity, while PP2C requires magnesium for activity {Cohen 1989; Herzig and Neumann 2000} (Table 2). With the exception of PP2C, the other PPs show stretches of conserved amino-acids characteristic of this family {Oliver and Shenolikar 1998}. PP1 and PP2A constitute more than 90% of serine/threonine phosphatase activity in mammalian cells. Several other serine/threonine phosphatases have

Table 2: Methods for distinguishing protein phosphatases

Phosphatases	PP1	PP2A	PP2B	PP2C
Inhibition by Inhibitor-1 and Inhibitor-2	Yes	No	No	No
Absolute requirement of divalent cations	No	No	Yes	Yes
Stimulation by calmodulin	No	No	Yes	No
Inhibition by okadaic acid	Yes	Yes	Yes (weak)	No

(Modified from Wera S et al, 1995)

been cloned (PP4, PP5, PP6, PP7); they represent a small fraction of phosphatase activity in the cell but have not been fully characterized as yet {Cohen 1997}.

C2: Protein phosphatase 1/2A

PP1: PP1 has a wide range of biological functions, including effects on glycogen metabolism, transition through the various stages of the cell cycle, and muscle contraction {Oliver and Shenolikar 1998}. They are composed of catalytic and regulatory (or accessory) subunits. Four mammalian isoforms for the catalytic subunit have been identified (α, β, γ_1 and γ_2), that display widespread tissue distribution (except γ_2, predominant in testes). The functional significance of the different isoforms is not yet clear.

Since the levels of catalytic subunits do not change in response to many physiological stimuli, hormonal/ growth factor-related regulation of activity is believed to be mediated by endogenous inhibitor proteins and the regulatory subunits {Oliver and Shenolikar 1998}. A large number of endogenous inhibitors targeting PP1 have been identified (Table 3). Of these, Inhibitor-1 (I-1) and Inhibitor-2 (I-2) have been the most widely studied. I-1 is a widely expressed cytosolic protein and it becomes an inhibitor of PP1 when it is phosphorylated by protein kinase A (PKA) on threonine 35. Many hormones that elevate cAMP also activate I-1. In general, it is believed that hormones/factors that can activate I-1 induce large and rapid changes in signal pathways that involve PP1 substrates

Table 3: Endogenous phosphatase inhibitors

Phosphatase inhibitors	IC_{50} (nM)
PP1 inhibitors	
Inhibitor-1 (I-1)	1.6
DARPP-32	1
Inhibitor-2 (I-2)	3.1
NIPP-1	0.01
RIPP-1	20
CP117	0.18
PP2A inhibitors	
Inhibitor-1(I1PP2A)	30
Inhibitor-2(I2PP2A)	25
PP2B inhibitors	
Cain	440

(Modified from Oliver CJ et al, 1998)

{Oliver and Shenolikar 1998}. I-2 binds and inhibits PP1 irrespectively of phosphorylation {Herzig and Neumann 2000}.

The regulatory subunits are believed to affect PP1 by directing it to specific subcellular localizations and/or by altering its activity for specific substrates. For example, in the liver, a regulatory subunit termed G_L targets PP1 to glycogen particles, inhibits its activity towards glycogen phosphorylase and promotes glycogenolysis {Hubbard and Cohen 1993}. In muscle a different regulatory subunit, R_{GL} attaches PP1 to glycogen in rabbit skeletal muscle. Regulatory G_M, targets PP1 to the myofilaments {Johnson et al., 1996}. Other accessory subunits for PP1 are found in distinct subcellular compartments such as the nucleus or the ribosomes and are believed to affect PP1 activity at these sites {Herzig and Neumann 2000}. Although little sequence similarity can be found between the different PP1 regulatory subunits, they all seem to have in common a small "PP1-binding motif" containing the aminoacid sequence R/KVXF (where X is His or Arg) {Zhao and Lee 1997}. It has been speculated that the presence of this short motif in a protein may allow binding to the catalytic subunit of PP1, although additional binding sequences may also be required {Sim and Scott 1999}. PP1 activity may also be regulated by phosphorylation and methylation of their regulatory subunits {Herzig and Neumann 2000}.

PP2A: This ser/thr phosphatase is also involved in the regulation of many cellular functions similar to PP1. It is made up of one catalytic and

two regulatory subunits; the catalytic subunit (PP2Ac) is highly conserved, and has 50% sequence homology with the catalytic subunit of PP1. PP2Ac (catalytic subunit) is present as a heterodimer in cells; PP2Ac forms a dimer with a 65 kDa scaffold protein (A subunit) and the dimer then can recruit other regulatory subunits (B) to form a trimer {Sim and Scott 1999}. It is believed that several isoforms of B subunits bind to PP2Ac (suggesting the presence of several complexes of PP2A in the cell) and each complex in turn is responsible for a distinct function of PP2A in the cell although there is no evidence to this belief yet {Oliver and Shenolikar 1998}. PP2A is mainly cytosolic in its location even though it also is present in smaller extents in the nucleus {Turowski et al., 1995}. It has also been found to be localized in the membrane, cytoskeletal and mitochondrial compartments {Pitcher et al., 1995; Sontag et al., 1995}.

PP2A undergoes tyrosine phosphorylation {Chen et al., 1992} and methylation {Favre et al., 1994}, which results in alteration of its activity in vitro, but it is not known if it happens in vivo. It has also been shown that PP2A is inhibited by phosphorylation on a threonine residue {Herzig and Neumann 2000}. The PP2A is also regulated by the endogenous inhibitors, I_1^{PP2A} and I_2^{PP2A}, which are believed to inhibit PP2A by binding to the catalytic subunit {Janssens and Goris 2001} (Table 3). Many proteins and protein complexes have been identified as binding to the PP2A trimer. It is suggested that these elaborate complexes serve to provide specific regulation of signal pathways that involve the

phosphatase activity of PP2A; a 'classic' example of complex protein-protein interactions that include PP2A, center around the phosphorylation status (and stability) of beta-catenin in the wnt signaling pathway {Ratcliffe et al., 2000}.

Protein kinases are also substrates of PP2A. PP2A is required for the dephosphorylation of PKC leading to its down regulation, indicating that PP2A recruitment near PKC is likely to be required for the PKC signaling {Keranen et al., 1995; Ricciarelli and Azzi 1998}. PP2A also interacts with casein kinase 2alpha (CK2alpha) resulting in the regulation of MAPK; inhibition of CK2alpha on MAPK has been reported to be due to phosphorylation/activation of PP2A by CK2alpha resulting in the dephosphorylation/inhibition of the MAPK {Heriche et al., 1997; Sontag et al., 1993}. Thus, PP2A plays an important role in the various signaling pathways in the cell by the regulation of the activity of these kinases.

C2.1: Inhibitors of PP1/2A

Non-protein phosphatase Inhibitors:

Okadaic acid (OA: $C_{44}H_{67}O_{13}$) is a marine toxin first isolated from a black marine sponge of the species *Halichondria okadai* and it is also known to cause diarrhetic shellfish poisoning {Tachibana et al., 1981}. Though OA is an inhibitor of protein phosphatase 2A and protein phosphatase 1 (PP2A and PP1) {Ishihara et al., 1989}, it inhibits PP2A more potently (IC_{50} 0.2-1 nM) than PP1 (IC_{50} 3 nM) {McCluskey et al., 2002}. The IC_{50} values of OA for various phosphatases are listed in Table

4. It is suggested that OA inhibits PP1 activity by binding to aminoacid tyrosine 272 of PP1 {Zhang et al., 1996}. OA is a powerful tumour promoter {Suganuma et al., 1988} and tumor promotion by OA and related compounds such as calyculin A is suggested to be due to the inhibition of PP1/2A phosphatases {Fujiki and Suganuma 1993}. Use of OA increases phosphorylation of a variety of proteins that include vimentin, the 27 kDa heat shock protein, histone H3 and others {Herzig and Neumann 2000}. OA also can induce {Boe et al., 1991} or prevent {Chiang et al., 2001; Song et al., 1992} apoptotic death.

Calyculin-A ($C_{50}H_{81}N_4O_{15}P$) was first isolated from a marine sponge *Discodermia calyx* and seven other calyculins, B to H were additionally isolated from the same sponge {Ishihara et al., 1989}. PP1 and PP2A are equally inhibited by calyculin-A {Ishihara et al., 1989} having IC_{50} values at 0.3-0.7 nM and 0.2-1 nM respectively {McCluskey et al., 2002}. The IC_{50} values of calyculin-A for various phosphatases are listed in Table 4. It is suggested that calyculin-A binds to tyrosine 272 of PP1 {Zhang et al., 1996}. Calyculin A has been shown to have a good permeability through cell membranes {Herzig and Neumann 2000}.

Tautomycin ($C_{14}H_{66}O_{13}$) was first isolated from *Streptomyces spiroverticullatus* {Oliver and Shenolikar 1998}. Tautomycin exhibits antifungal property and is permeable to the cell membrane as well {Herzig and Neumann 2000}. The IC_{50} of tautomycin is 10 fold higher for PP2A than for PP1 {Oliver and Shenolikar 1998}. Tautomycin completely inhibits

Table 4: Protein phosphatase inhibitors

Inhibitor	IC_{50}		
	PP1	PP2A	PP2B
Okadaic acid	3 nM	0.2-1 nM	>10 mM
Tautomycin	0.7 nM	0.7 nM	~70 mM
Calyculin	0.3-0.7 nM	0.2-1 nM	>10 mM
Fostriecin	131 µM	3.4 nM	ND
FK-506	-	-	0.5 nM
Cyclosporine-A	-	-	5 nM

ND=not determined

(Taken and modified from McCluskey et al, Journal of Medicinal Chemistry, 2002)

PP1 at 3 nM and PP2A at 30 nM {Ubukata et al., 1997}. The IC_{50} values of tautomycin for various phosphatases are listed in Table 4. Similar to OA and calyculin-A, tautomycin has also been shown to interact with PP1 on Tyr-272 {Zhang et al., 1996}. Tautomycin is the first inhibitor, which has been shown as a specific inhibitor of PP1 both in vitro and in vivo {Favre et al., 1997; Yan and Mumby 1999} . A functional analogue of tautomycin called tautomycetin has been isolated in Japan from Streptomyces griseochromogenes and is shown to be a novel and more specific inhibitor of PP1 phosphatase {Mitsuhashi et al., 2001}. Synthesis of a hybrid called okadamycin containing okadaic acid and tautomycin has been reported and is claimed to be a potent inhibitor of PP2A {McCluskey et al., 2002}.

Tautomycin has been used as a specific PP1 inhibitor. For example, it has been used as a specific inhibitor of PP1, together with other PP1/2A inhibitors, to show PP2A but not PP1 that is involved in the dephosphorylation of proapoptotic BAD, which leads to apoptosis {Chiang et al., 2001}.

Fostriecin ($C_{19}H_{26}O_9P$) is a non-toxic water-soluble drug isolated from the fermentation beer of *Streptomyces pulveraceus* (fostreus subspecies) {Walsh et al., 1997}. It is a relatively weak inhibitor of PP1 but is a highly selective inhibitor of PP2A {Walsh et al., 1997}. The IC_{50} values for the PP2A and PP1 inhibition of fostriecin are 3.4 nM and 131 μM respectively {McCluskey et al., 2002}. The IC_{50} values of fostriecin for various phosphatases are listed in Table 4. Fostriecin inhibits type II DNA

topoisomerase and has antitumor activity, while the other phosphatase inhibitors are tumor promoters {Boritzki et al., 1988}. Fostriecin found to be active against breast, lung and colon cancer cells and leukemia and is proven so in clinical trials {Ho and Roberge 1996; Roberge et al., 1994}. There is evidence suggesting that phosphatase inhibition is at least in part responsible for the antitumor activity of fostriecin {Cheng et al., 1998}. It is a very polar molecule and is believed to be actively transported to the cells {Herzig and Neumann 2000}.

Other PP1/2A inhibitors also need mention in this review. They are microcystins (potently inhibits both PP1/2A similar to OA, but not permeable to the cell membrane {Armstrong and Ganote 1992}), cantharidin and its analogues (inhibits both PP1/PP2A), nodularin (inhibits both PP1/2A), heparin (inhibits PP1), and apomorphine (shown to inhibit PP2A only in the brain) {Herzig and Neumann 2000}.

C2.2: PP1/PP2A and Heart

PP1 and PP2A constitute the majority of the phosphatase activity in the heart {Cohen 1989; MacDougall et al., 1991}. Different isoforms of PP1/2A like PP1α, δ and γ, PP2Aα and β are expressed in the heart and the expression of these phosphatases is regionally regulated (the right ventricle has more PP1α, δ, and γ and PP2Aα compared to right atria and no difference in the expression of PP2Aβ) {Luss et al., 2000}. The accessory or the regulatory proteins of PP1/2A present in the heart are I-1, I-2, R_{GL} or G_M, PPP1R5, and NIPP1 {Herzig and Neumann 2000}. As in

the other tissues, the accessory proteins regulate the activity of the catalytic subunits of the phosphatases in the heart. For example, in the heart the dephosphorylation of myosin is carried out by the holoenzyme containing the catalytic subunit PP1δ in the presence of its 130 kDa myosin binding subunit which helps in specifically targeting the PP1δ to the myosin {Nishio et al., 1997}.

The cardiac contractile regulatory proteins activated by β-adrenergic stimulation are phosphoproteins {Bartel et al., 1993; Opie 1995}. Isoproterenol, a positive inotropic agent, increases phosphorylation of I-1 {Neumann et al., 1991}. The resulting decrease in PP1 activity prevents dephosphorylation of several proteins involved in excitation-contraction coupling (Na/K-ATPase, phospholamban, Troponin I, voltage sensitive calcium channels) and thus enhances the contractile response of the heart {Gupta et al., 1996; Neumann et al., 1991}. Among the contractile regulatory proteins phospholamban (PLB) is the key regulator of the β-adrenergic signaling of the heart and assists in both contraction and as well relaxation by regulating Sarco/Endoplasmic reticulum Ca^{2+} ATPase (SERCA) activity; the phosphorylation of PLB accelerates SERCA-mediated uptake of Ca^{2+} from the cytoplasm leading to relaxation of the cardiac muscle and its dephosphorylation (PP1 phosphatase has been implicated in the regulation of the phospholamban) leads to decreased uptake of Ca^{2+} {Neumann 2002}. PP1 has also been shown to bind to the cardiac ryanodine receptor (calcium release channel) that

plays an important role in the contraction {Zhao et al., 1998}. Thus PP1 and PP2A play an important role in the contraction and relaxation of the heart.

PP1 and PP2A have been implicated in various pathological conditions of the heart. Boknik and colleagues have shown an increase in the activity of PP1 and PP2A in the hypertrophied rat hearts following isoproterenol administration {Boknik et al., 2000}. They suggested that the chronic stimulation of β-adrenergic activity found in human cardiac hypertrophy and failure can contribute to altered phosphatase activity which might lead to altered contractile responses {Boknik et al., 2000}. Increased PP1 activity has been found in cardiac failure and this has been suggested to cause dephosphorylation of the regulatory protein phospholamban leading to a decrease in the uptake of Ca^{2+} from the cytosol / impairment of relaxation {Neumann 2002}.

Carr and coworkers {Carr et al., 2002} studied the role of PP1 in heart failure extensively by using TG (Transgenic) mice with cardiac-restricted PP1 over expression, I-1 knock-out mice, and failing human cardiomyocytes transfected with I-1 using adenoviral vectors. In TG mice with PP1 over expression, rates of contraction/relaxation and left ventricular developed pressure were significantly diminished compared to the wild type {Carr et al., 2002}. Echocardiographic studies on these mice at 6 months of age revealed changes similar to cardiac failure; the I-1 knock-out mice showed increased phosphatase activity and decreased

contractility {Carr et al., 2002}. In the cardiomyocytes taken from failing human hearts adenoviral-transfection of I-1 resulted in the restoration of the contractile parameters in response to β-agonists {Carr et al., 2002}. Thus, the above results suggested that PP1 serves as a vital regulator of cardiac function and it may be a potential target for the treatment of cardiac failure.

PP1 and PP2A inhibitors have been shown to be cardioprotective in various studies. OA has been shown to reduce infarct size in pig myocardium and protect it from ischemia-reperfusion injury in vivo, while stimulating the SAPKs/JNKs {Barancik et al., 1999}. It also causes a decrease in the rate of development of osmotic fragility (the susceptibility of the cardiomyocytes to lyse in the presence of hypotonic solution) in metabolically inhibited isolated rat cardiomyocytes {Armstrong and Ganote 1992}.

Several reports have shown calyculin-A is a cardioprotective agent. Armstrong and coworkers {Armstrong and Ganote 1992} also showed in the metabolically inhibited isolated rat cardiomyocytes that, similar to OA, calyculin-A also decreased the rate of development of osmotic fragility. In rabbit myocytes subjected to ischemia, calyculin-A delays osmotic fragility but has no effect on the sarcolemmal blebbing {Armstrong et al., 2001}. The protective effects of calyculin-A were suggested to be mediated by p38 MAPK and not by PKC {Armstrong et al., 1998}. In the in vitro model of ischemia, even when added 75 min after ischemia, calyculin-A was

found to be cardioprotective at a dose similar to that obtained during preconditioning {Armstrong et al., 1998}. The mechanism of this late protection was suggested to occur through an increase in the phosphorylation status of cardioprotective proteins by inhibition of dephosphorylation {Armstrong et al., 1998}. Heat shock proteins have been implicated in preconditioning and calyculin-A has been shown to block the ischemic dephosphorylation of cytoskeletal hsp27 in rabbit cardiomyocytes {Armstrong et al., 1999}. Highly phosphorylated forms of alpha B crystalline, a putative effector protein of ischemic preconditioning, translocates from cytosol to the cytoskeleton and calyculin-A has been shown to maintain the phosphorylated form of alpha B crystalline even during prolonged ischemia {Armstrong et al., 2000}.

Fostriecin, the selective PP2A inhibitor, has also been shown to be cardioprotective in different studies. In pig and rabbit cardiomyocytes subjected to in vitro ischemia, fostriecin was found to be cardioprotective and the effects mimicked preconditioning; the cardioprotective effects of fostriecin in pig but not rabbit cardiomyocytes have been suggested to be dependent on K_{ATP} channel {Armstrong et al., 1997}. Fostriecin was found to limit myocardial infarct size, as assessed by TTC (Triphenyl tetrazolium chloride), when administered before and after ischemia in rabbit hearts. It also protected ischemic rabbit cardiomyocytes from injury in a manner similar to that obtained with preconditioning {Weinbrenner et al., 1998}. Fostriecin continued to be protective to an extent similar to

preconditioning, when added during late ischemia (after 75 min) to rabbit cardiomyocytes subjected to simulated ischemia {Armstrong et al., 1998}.

C3: Protein phosphatase 2B/Calcineurin

PP2B is a calcium-calmodulin dependant protein phosphatase otherwise called calcineurin (Cn). It is present in almost all mammalian cell types but is particularly abundant in the brain {Rusnak and Mertz 2000} and it consists of a 60 kDa catalytic subunit (CnA) (related to PP2A) and 19 kDa calcium binding regulatory (CnB) (inhibitory) subunit {Herzig and Neumann 2000}. The catalytic subunit (CnA) consists of an N-terminal region and a C-terminus that has an auto-inhibitory domain (when Ca^{2+}/calmodulin is not bound, this domain normally folds such that it occludes the active site) {Rothermel et al., 2003}. The catalytic subunit (CnA) also has binding regions for both regulatory subunit (CnB) and Ca^{2+}/calmodulin. Any change in intracellular concentration of Ca^{2+} results in the binding of Ca^{2+} directly to CnB and to CnA as Ca^{2+}/CaM complex, displacing the auto-inhibitory domain, thus resulting in the activation of calcineurin {Rothermel et al., 2003}. The structure of calcineurin representing the active and the inactive form is shown in Figure 2.

Both calcineurin subunits exist in different isoforms. The different isoforms of A subunit are CnAα ($\alpha_1$and α_2 (splice variants)), CnAβ (β_1, β_2, and β_3 (splice variants)) and CnAγ, of which the CnAα and CnAβ are expressed in high levels in the brain whereas the CnAγ is expressed in the testis {Chang et al., 1994; Ueki et al., 1992}. The CnB subunit has 2

CALCINEURIN STRUCTURE

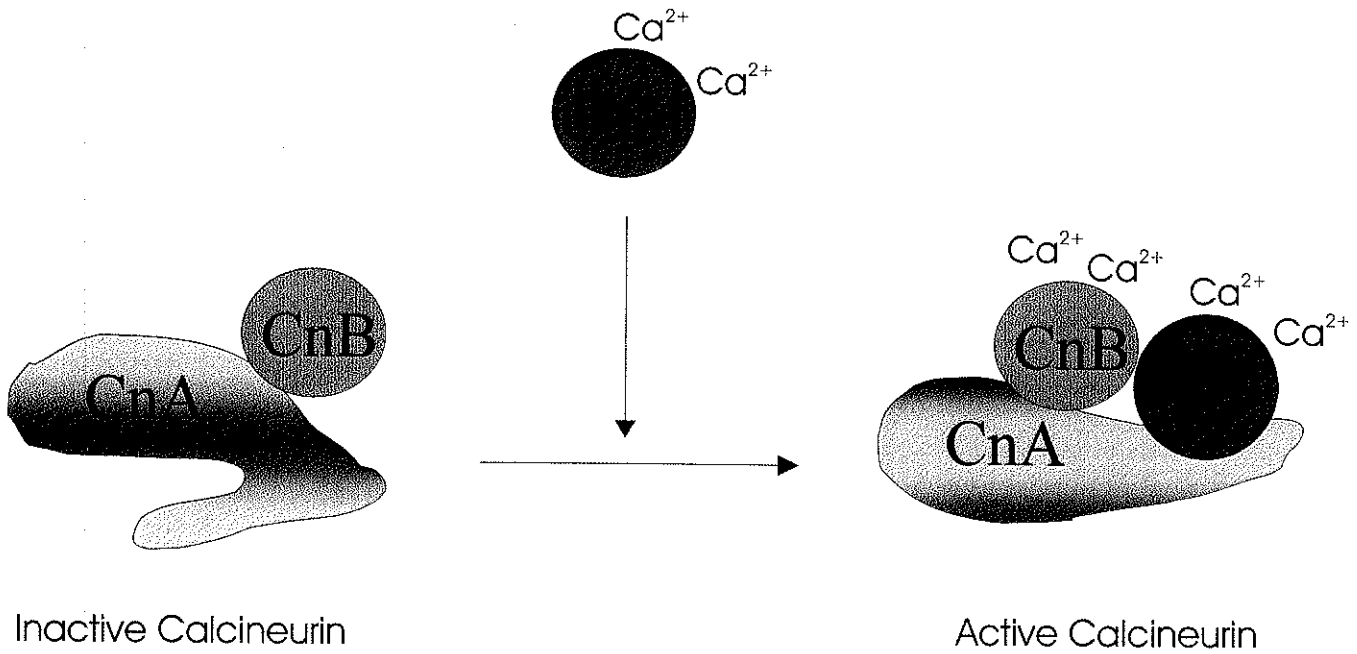


Figure 2

isoforms, CnBα (α_1, α_2 (splice variants)) and CnBβ; CnBα_2 and CnBβ are testis specific whereas CnBα_1 is present in many tissues {Herzig and Neumann 2000}. Two endogenous inhibitors of PP2B have been identified. The most potent is cabin1/cain, a 240 kDa protein {Herzig and Neumann 2000}. It binds to PP2B at a site other than that targeted by FK506. The other inhibitor is the PKA-binding protein AKAP79 {Coghlan et al., 1995}.

Crabtree and coworkers were the first to establish the mechanism of calcineurin signaling in T-lymphocytes {Flanagan et al., 1991}. These initial studies showed that the activation of T-cell receptor results in an increase in Ca^{2+} concentration and subsequent activation of calcineurin {Flanagan et al., 1991}. Calcineurin, in turn, binds to NFAT (nuclear factor of activated T-cells) and dephosphorylates it, resulting in the translocation of NFAT to the nucleus and activation of gene transcription {Flanagan et al., 1991}. Myocyte-enriched calcineurin interacting protein 1 (MCIP1) gene is a calcineurin responsive gene which is transcribed during calcineurin activation, resulting in the synthesis of MCIP1 that in turn inhibits calcineurin, establishing a negative feedback loop {Rothermel et al., 2003}. The general pathway of calcineurin signal transduction is shown in Figure 3. The calcineurin signal transduction pathway has been shown to be stimulated at an intracellular Ca^{2+} concentration of > 150 nM but not at the resting level of 70 nM in the cultures of rabbit skeletal myocytes {Kubis et al., 2003}. In cardiac muscle there is a frequent

CALCINEURIN SIGNAL TRANSDUCTION
(GENERAL)

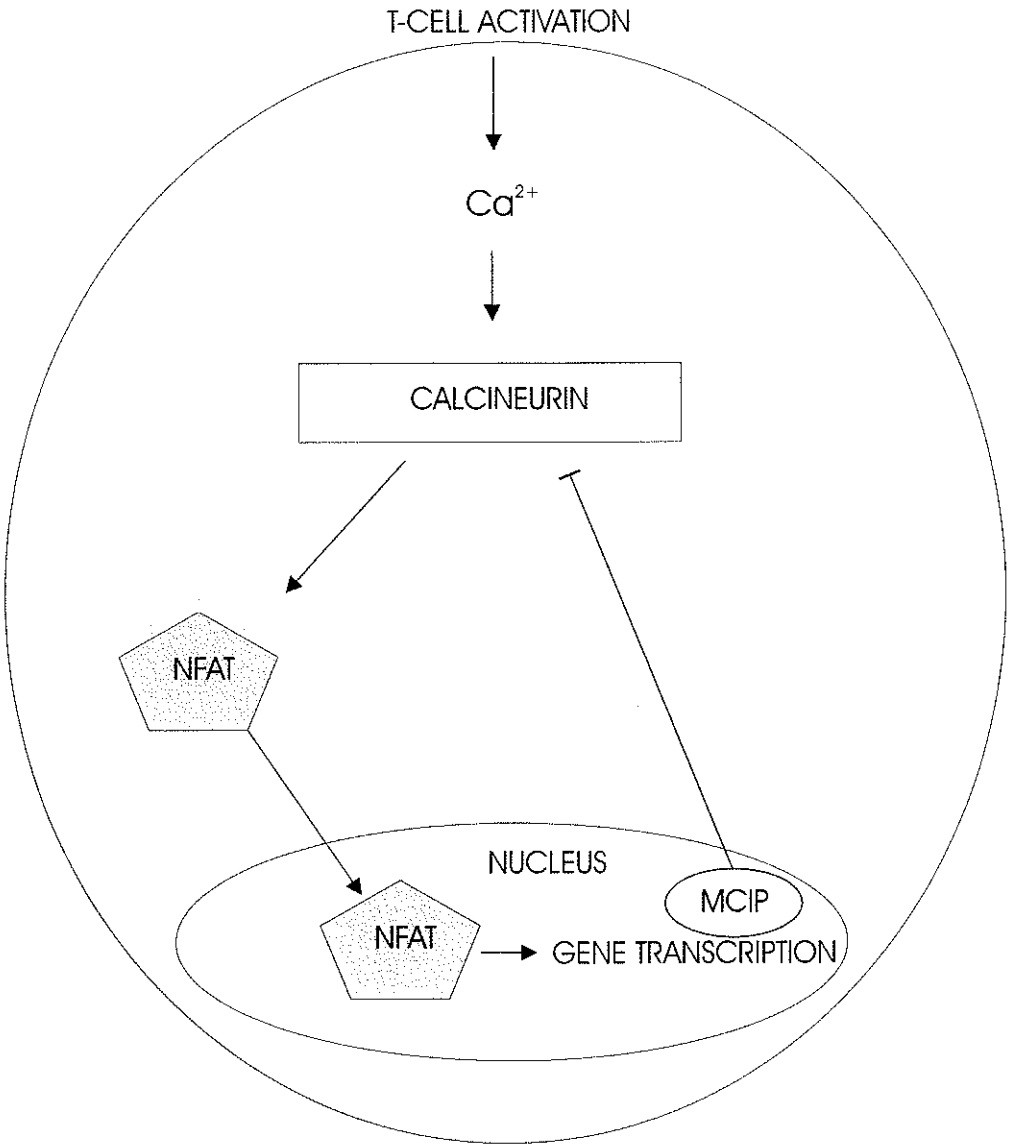


Figure 3

change in the intracellular concentration of Ca^{2+} during excitation and contraction cycles and CnA (which is dependant on Ca^{2+} for its action) has been shown to require a sustained low-level increase in intracellular Ca^{2+} in order to be activated {Shibasaki et al., 2002}.

Calcineurin is inhibited by the immunosuppressive agents cyclosporine A (CsA) and FK-506; they form complexes with the cytoplasmic binding proteins cyclophilin, and FK-binding protein (FKBP12) respectively {Liu et al., 1992; Liu et al., 1991; Schreiber and Crabtree 1992}. Calcineurin is targeted to ryanodine receptor and IP_3 receptor by, FKBP12 and FKBP6 respectively {Rothermel et al., 2003}. In the heart FKBP 12.6 is associated with the cardiac specific isoform of the ryanodine receptor {Lam et al., 1995} and inhibits calcineurin dependant signaling in the presence of FK-506.

C3.1: PP2B inhibitors

Cyclosporine A ($C_{62}H_{111}N_{11}O_{12}$) was isolated first from fungi imperfecti of the species *Tolypocladium inflatum Gams* {Borel et al., 1976; Doutre 2002}. CsA is a potent inhibitor of calcium/calmodulin dependent protein phosphatase (PP2B) otherwise called calcineurin and through this action it can inhibit T cell activation {Clipstone and Crabtree 1992; Fruman et al., 1992}. The IC_{50} value for the inhibition of PP2B by CsA is 5 nM {McCluskey et al., 2002} (Table 4). It is widely used as an immunosuppressive agent, for example, in organ transplantation {Kahan

1989; Kahan 1989}. CsA is permeable to the cell membrane {Herzig and Neumann 2000}.

CsA is reported to be protective in liver {Broekemeier et al., 1992; Pastorino et al., 1993} (CsA protected the hepatocytes from anoxia and oxidative stress), brain {Shiga et al., 1992} (where CsA protected against the ischemia-reperfusion injury), lung {Krishnadasan et al., 2002} (preoperative administration of CsA protected against ischemia reperfusion injury and suggested a nuclear factor κB (NFκB)-dependent mechanism) and the heart {Weinbrenner et al., 1998} (described in detail later in the review).

CsA exhibits nephrotoxicity {Atkinson et al., 1983}, hepatotoxicity {Iacona et al., 1991}, neurotoxicity {Walker and Brochstein 1988} and cardiotoxicity {Owunwanne et al., 1993}. A high dose (50mg/kg) of CsA has been shown to be cardiotoxic in rats following long term exposure possibly due to cumulative effects of lysis and fibrosis of the myocytes {Owunwanne et al., 1993}.

Fk-506 ($C_{44}H_{69}NO_{12}$) an immunosuppressant and a macrolide antibiotic, also known as tacrolimus, is isolated from the fermentation broth of the fungus *Streptomyces tsukubaensis* {Kino et al., 1987}. It was discovered in 1984 in Japan during a search for new immunosuppressive agents. It is a specific inhibitor of PP2B (as a FK-506-FKBP complex) with a IC_{50} value of 0.5 nM {McCluskey et al., 2002}. It is more potent than CsA

producing similar in vitro effects at 100 times lower concentration than CsA.

FK-506 has been found to be neuro-protective {Sharkey and Butcher 1994} when administered 60 min post occlusion in an in vivo model of focal cerebral ischemia. FK-506 protected against the ischemia-reperfusion injury {Kubes et al., 1991} in the small intestine of cats. In the lungs {Krishnadasan et al., 2002} preoperative administration of FK-506 protected against the ischemia reperfusion injury involving NFκB). The cardioprotective effect is discussed in detail later in the review.

Fk-506 possesses toxic effects similar to CsA except that FK-506 causes lower clinically relevant hypertension {Kelly et al., 1995}.

Mechanism of action of FK-506 and cyclosporine: FK-506 and CsA bind to cytoplasmic proteins (immunophilins) called FKBP (FK binding protein 12.6 is the predominant species in the heart) and cyclophilin respectively and form a complex {Kelly et al., 1995; Lam et al., 1995}. The CsA/cyclophilin and FK-506/FKBP complexes in turn bind competitively with calcineurin to inhibit its activity {Liu et al., 1991}. These complexes have also been suggested to compete with MCIP1 for binding calcineurin and to disrupt the interaction of MCIP1 with calcineurin {Rothermel et al., 2003}. The diagrammatic representation of mechanism of inhibition of calcineurin by FK-506 and CsA is shown in Figure 4. A calcineurin independent mechanism of action via TGF-β1, has been hypothesized for CsA {Khanna et al., 1999}.

MECHANISM OF ACTION OF FK-506 AND CsA

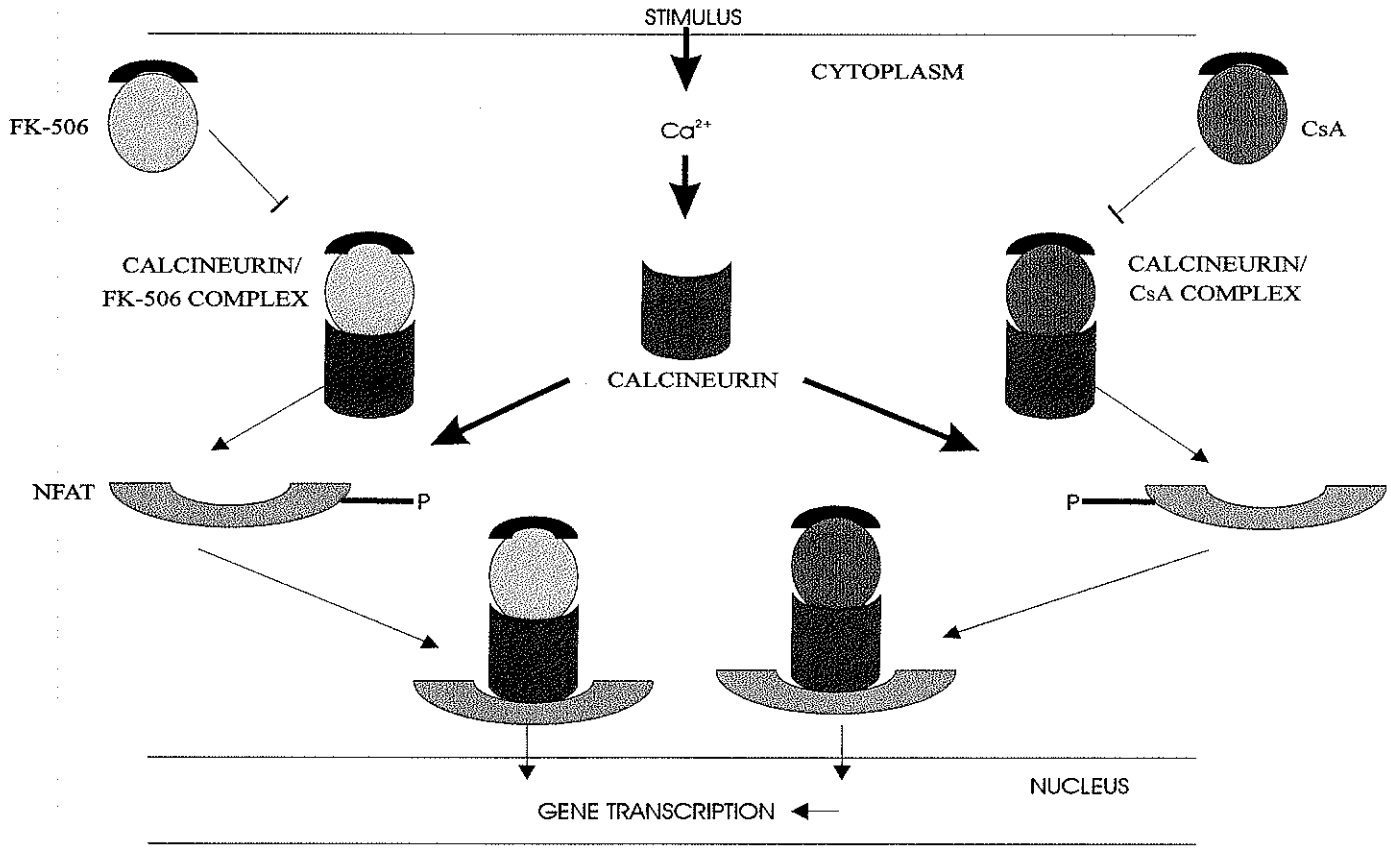
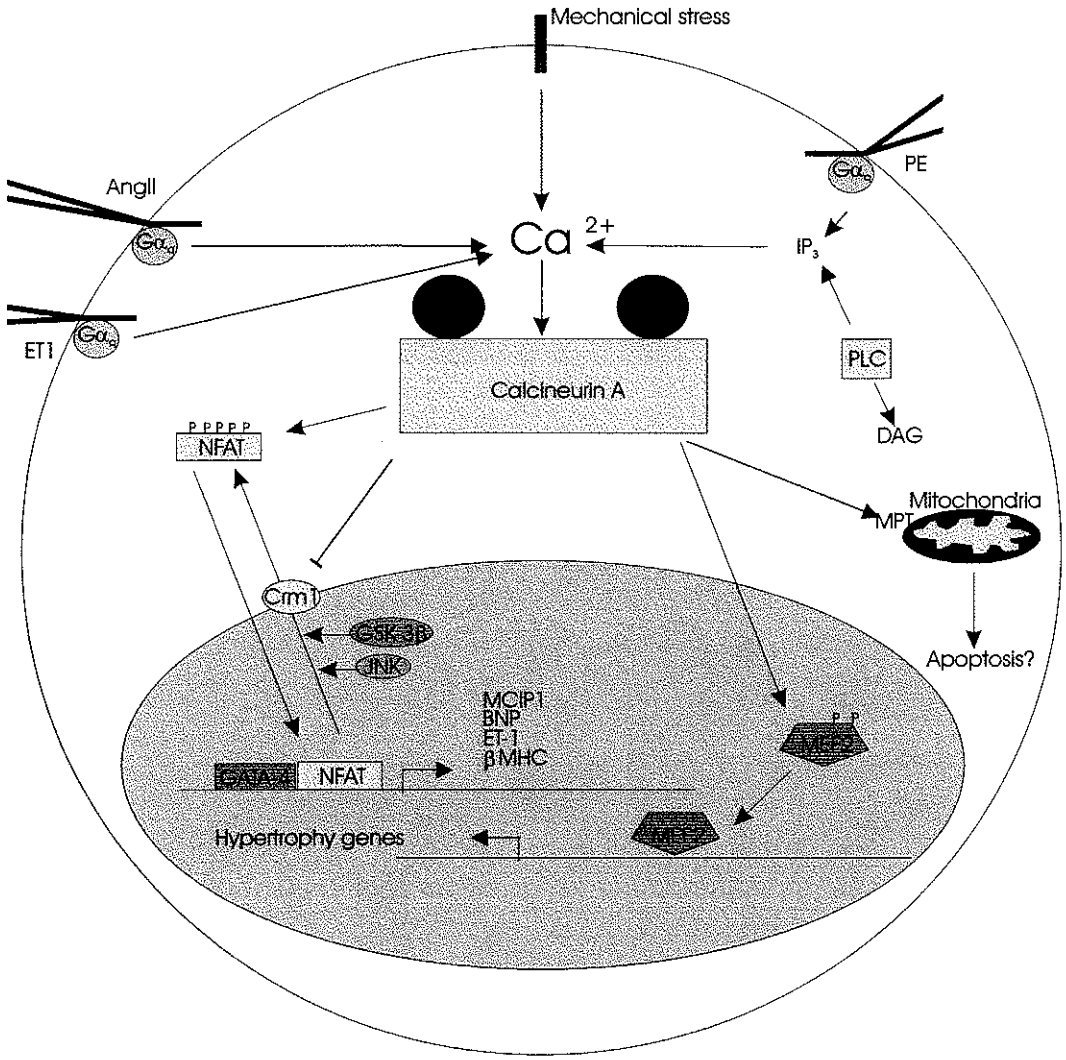


Figure 4

Figure 5

PATHWAYS OF CALCINEURIN IN CARDIAC MYOCYTES



(Taken and modified from Wilkins BJ et al, Journal of Physiology, 2002 & Bueno OF et al, Cardiovascular Research, 2002)

C3.2: PP2B and Heart

The heart consists of CnAα and CnAβ isoforms of calcineurin {Chang et al., 1994; Ueki et al., 1992}. In the cardiomyocyte, calcineurin has been reported to be localized to cytosol and nucleus, in association with anchoring proteins {Coghlan et al., 1995}, the SR or in association with calsarcin a docking protein which keeps the calcineurin with the sarcomeres {Frey et al., 2000}. The signaling pathway of calcineurin in cardiomyocytes is shown in Figure 5. In the cardiomyocytes activation of the $G\alpha_q$ and the mechanical stress receptors leads to increase in Ca^{2+} concentration which in turn stimulates calcineurin {Wilkins and Molkentin 2002}. Calcineurin causes nuclear accumulation of NFAT by directly dephosphorylating it and also by inhibiting crm1 which inhibits the export of NFAT from the nucleus {Wilkins and Molkentin 2002}. Calcineurin also acts on MEF2 factors and activates them; NFAT and MEF2 along with GATA-4 (a transcription factor) and other factors stimulates the transcription of the hypertrophy genes {Wilkins and Molkentin 2002}.

Cardiac hypertrophy occurs in response to both physiological as well as pathological stimuli; pathological hypertrophy gradually progresses to failure and it might also serve as a risk factor for arrhythmias. Calcineurin and its downstream effector NFAT have been shown by several studies as the main transducers of cardiac hypertrophy {McKinsey and Olson 1999}. In hypertrophied cardiomyocytes CnAβ mRNA and protein levels have been shown to be upregulated {Taigen et al., 2000}.

Using CsA and FK-506 several in vitro and in vivo studies have implicated the involvement of calcineurin in cardiac hypertrophy {Goldspink et al., 2001; Molkentin et al., 1998; Shimoyama et al., 2000}. Additional studies, however, indicate no correlation between calcineurin and hypertrophy {Ding et al., 1999; Fatkin et al., 2000; Luo et al., 1998} and this discrepancy is thought to be due to the neuro and nephrotoxicity of the CsA leading to secondary increase in the workload of the heart {Klee et al., 1998; Wilkins and Molkentin 2002}.

In order to overcome the difficulties posed by the use of the calcineurin inhibitors, genetic models were used to inhibit the activity of calcineurin and identify the exact role of calcineurin in hypertrophy. One of the first approaches studied rat neonatal myocytes adenovirally-transfected with the calcineurin inhibitory domain (or calcineurin binding domain) of cain or AKAP79 in the presence of stimulators of hypertrophy like AngII {Taigen et al., 2000}. Under these conditions hypertrophy was blocked and this was confirmed in studies of cain/AKAP79 transgenic mice, showing similar attenuation of hypertrophy {De Windt et al., 2001}. In another model, transgenic mice overexpressing MCIP1, in a cardiac specific manner, showed attenuation of hypertrophy in response to isoproterenol infusion and pressure overload {Hill et al., 2002; Rothermel et al., 2001}. Also, transgenic mice expressing dominant negative form of calcineurin showed a decreased hypertrophic response after banding of the abdominal aorta {Zou et al., 2001}. The CnAβ deficient mice, also

showed a reduction in the hypertrophic response in the presence of AngII or isoproterenol infusion/ aortic banding {Bueno et al., 2002}. Thus all these transgenic mouse models of calcineurin inhibition show attenuated hypertrophy in response to variety of stimuli in vivo indicating that indeed calcineurin is a critical mediator of hypertrophy. Various studies also show that calcineurin mediates physiological {Rothermel et al., 2001} and developmental hypertrophy {de la Pompa et al., 1998}.

Calcineurin inhibitors CsA and FK-506 have cardioprotective roles based on various studies of ischemia/reperfusion. However, It is not known whether these cardioprotective drug actions are actually mediated by calcineurin. Several reports point to CsA as a cardioprotective agent but the exact mechanism by which this occurs remains obscure. CsA administered before ischemia prevented reperfusion induced arrhythmias {Arteaga et al., 1992}. CsA also prevented post ischemic left ventricular dysfunction in isolated rat hearts {Griffiths and Halestrap 1993; Griffiths and Halestrap 1995}. CsA is known to block the degranulation of mast cells {Schreiber and Crabtree 1992} but studies by Wang and coworkers {Wang et al., 1996} showed that the cardioprotective effects of preconditioning do not involve the mast cells or its inhibition of degranulation. CsA has been shown to protect the heart from ischemia/reperfusion injury by binding to a mitochondrial protein cyclophilin and thereby inhibiting the formation of Ca^{2+} dependent mitochondrial membrane transition pore (MTP) {Halestrap et al., 1997}. It

has also been reported that CsA induces the synthesis of endothelin {Bunchman and Brookshire 1991} and Massoudy and others {Massoudy et al., 1997} showed that the cardioprotective effect of CsA could be due to a nitric oxide dependent mechanism mediated by endothelin. A reduction in infarct size is obtained in the isolated rat hearts pretreated with cyclosporine and the protection has been shown to be comparable to that obtained with preconditioning {Cai et al., 1998}. CsA is also highly protective against infarction in a model of isolated rabbit heart even when given after the onset of ischemia {Weinbrenner et al., 1998}. The above protection of CsA is similar to that obtained by preconditioning and the mechanism of protection was thought to be by the inhibition of phosphatases and prolongation of phosphorylation status of the proteins in ischemic myocytes.

CsA administered after occlusion has also been shown to decrease myeloperoxide activity (indicator of cellular infiltratum) and necrosis in the area at risk and in necrotic zones in rats subjected to total occlusion, leading to protection against ischemia reperfusion injury {Squadrito et al., 1999}. In another isolated rat heart model CsA administered prior to ischemia induced preconditioning-like protection {Minners et al., 2000}. CsA markedly decreased calcineurin activity in the post myocardial infarction remodeled left ventricle and markedly reduced incidence of heart failure {Deng et al., 2001}.

FK-506 also has been shown to be cardioprotective by various studies. In the canine heart FK506 protected from ischemia induced myocardial damage by reducing mitochondrial dysfunction, superoxide radical formation and development of reperfusion arrhythmias {Nishinaka et al., 1993}. FK-506 also reduced the incidence of reperfusion-induced arrhythmias (ventricular tachycardia and ventricular fibrillation) and improved post ischemic myocardial function in isolated rat hearts used in combination with Egb761, a free radical scavenger {Haines et al., 2000}. FK-506 was also found to be protective when used in an isolated rat heart model, where the infarct size was reduced {Cai et al., 1998}. Here, the protection was found similar to that of preconditioning

Calcineurin has also been shown to play an important role in apoptosis. In various studies, conducted in many cell types, calcineurin has been shown to stimulate {Shibasaki and McKeon 1995; Toth et al., 1999} or prevent apoptosis {Lotem and Sachs 1998; Zhao et al., 1995}. In the heart, cardiomyocytes have been reported to be protected from apoptosis by calcineurin both in vivo and in vitro {De Windt et al., 2000}.

C4: Connexin 43 and Phosphatases

Although the status and pattern of Cx43 phosphorylation is recognized as regulating all the properties of the molecule, most studies have focused on the identification of the kinases involved in its phosphorylation, while very little is known at present as to the

phosphatase(s) involved in Cx43 dephosphorylation in various cells and tissues especially the heart.

Studies on phosphatases involved in Cx43 dephosphorylation have been conducted on non-muscle cells. The WB-F344 rat liver epithelial cells, when treated with the GJ inhibitor 18 beta-glycyrrhetinic acid, disruption of GJ communication and Cx43 dephosphorylation occurred and was blocked by OA and calyculin-A {Guan et al., 1996}. In rat liver epithelial cells, EGF treatment led to initial disruption of the GJ communication and an increase in the phosphorylation of Cx43 followed then by dephosphorylation and recovery of GJ communication {Lau et al., 1992}. These effects were retarded by OA. In V79 fibroblasts, cyclosporine and FK-506 delayed the loss of hyperphosphorylated forms of Cx43 and GJIC after the washout of the cells treated with TPA (12-0-tetradecanoyl-phorbol-13-acetate), an activator of PKC {Cruciani et al., 1999}. Nagy and colleagues using astrocytes subjected to chemical hypoxia showed that PP2B but not PP1/2A mediate Cx43 dephosphorylation and uncoupling of GJs {Li and Nagy 2000}. Dephosphorylation of Cx43 also occur in the astrocytes subjected to metabolic inhibition {Martinez and Saez 2000}.

Moreno and his colleagues {Moreno et al., 1994} showed that OA increases the Cx43 phosphorylation in cultured rat myocytes. In neonatal ventricular myocytes Duthe and colleagues showed that the endogenous PP1 is responsible for the decrease in the GJ channel activity {Duthe et

al., 2001}. The role of phosphatases on the phosphorylation status of Cx43 during ischemia in the heart has/had not been determined.

D. PROPOSED STUDIES

Cx43 is a phosphoprotein, being phosphorylated (in the normal heart) on serine/threonine residues. It is well established that Cx43 becomes dephosphorylated during ischemia of the isolated perfused heart, or in whole animal coronary occlusion models. The phosphatases involved are not known; it is also not clear whether Cx43 dephosphorylation plays a protective or detrimental role during ischemia. Blocking the activity of phosphatases belonging to the PP1, PP2A and PP2B groups (all of which have been implicated in Cx43 dephosphorylation in non muscle systems) confers cardioprotection from ischemia.

Our overall objective was to examine which group(s) of serine/threonine phosphatases mediates Cx43 dephosphorylation, using isolated cardiomyocytes and simulated ischemia. Because is was not known whether Cx43 in isolated cardiomyocytes responds to ischemia in a manner similar to that in whole hearts, our first hypothesis was:

Hypothesis 1: Simulated ischemia of isolated rat cardiomyocytes will cause Cx43 dephosphorylation in a manner comparable to the adult heart.

Two in vitro systems will be used: cell pellets from isolated adult rat cardiomyocytes subjected to simulated ischemia by the well established mineral oil overlay approach; and confluent differentiated neonatal myocyte cultures exposed to ischemic medium and a hypoxia chamber. Cx43 phosphorylation/dephosphorylation will be analyzed by western blotting and specific antibodies.

Additional hypotheses investigated in this project are:

Hypothesis 2: Cardiomyocyte Cx43 dephosphorylation during ischemia is mediated by phosphatases belonging to the PP1/2A and/or the PP2B group.

Specific inhibitors of the various categories of phosphatases will be employed, at concentrations linked to cardioprotection, and their effect on Cx43 phosphorylation will be evaluated. Combination of inhibitors will also be examined.

Hypothesis 3: Prevention of Cx43 dephosphorylation during ischemia correlates with decreased cardiac injury.

We will examine the effect of various phosphatase inhibitors on cardiac ischemic injury in vitro, assessed by lactic dehydrogenase release.

The following inhibitors will be tested:

*PP1/2A inhibitor okadaic acid (1 μM)

*PP1/PP2A inhibitor calyculin-A (1 μM)

*PP2A selective inhibitor fostriecin (1 μM)

*PP1 selective inhibitor tautomycin (1 nM)

*PP2B selective inhibitor FK-506 (150 nM/L)

*PP2B selective inhibitor cyclosporine (0.2 μM).

CHAPTER 2

MATERIALS AND METHODS

A. MATERIALS

A1. Animals

Animals were obtained from the Central Animal Care Facility at the University of Manitoba. Hearts were obtained from male Sprague-Dawley rats (200-250g) after decapitation. Animal use was in accordance with Canadian Council on Animal Care regulations.

A2. Antibodies

Rabbit polyclonal antibodies, recognizing Cx43.res.367-382 (P.AB), have been described and characterized {Doble et al., 2000} and mouse monoclonal antibodies, #13-800, recognizing only the dephosphorylated Cx43 were characterized previously {Nagy et al., 1997} and purchased from Zymed Labs (South San Francisco, CA). The goat anti mouse and anti rabbit HRP (horse radish peroxidase) secondary antibodies, were obtained from Bio-Rad (Hercules, CA).

A3. Chemicals

Collagenase Type II was obtained from Worthington (Lakewood, New Jersey). Phosphatase inhibitors, calyculin-A, fostriecin, FK-506, and cyclosporine (CsA) were purchased from CalBiochem (La Jolla, CA), okadaic acid (OA) was purchased from Sigma (Saint Louis, Missouri) and tautomycin was purchased from BioMol (Pennsylvania, USA). The lactate

dehydrogenase (LDH) assay kit was obtained from Sigma (Saint Louis, Missouri).

B. METHODS

B1. Isolation Of Adult Rat Ventricular Myocytes

Calcium tolerant adult ventricular myocytes were isolated from Sprague Dawley (200-250 g) rats by collagenase perfusion as described previously {Padua et al., 1998}. The rats were euthanised and the thorax was opened and the heart exposed. The heart was excised and immediately placed in an ice-cold buffer. Within 60 seconds or less the heart was mounted on the Langendorff perfusion apparatus and it was perfused with Ca^{2+} free Krebs solution (110 mM NaCl, 2.6 mM KCl, 1.2 mM KH2PO4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 11 mM glucose, 15 mM HEPES) for 3 min to remove the blood cells from the heart. The perfusate was continuously gassed with 95% O_2 / 5% CO_2. The temperature of the perfusate was maintained at 35 °C. Then the heart was perfused with Krebs solution containing Ca^{2+} (25 µM) and collagenase (1 mg/ml) for approximately 25 min. The heart was taken out in a Petri dish containing Krebs solution with 25 µM Ca^{2+} and was teased apart very gently by using forceps and later by using a pipette. The isolated cell suspension was filtered with a 200 µm pore nylon mesh (Cefar) to remove tissue remnants. The filtered cell suspension was transferred to a 50 ml tube and centrifuged at 7 g for 2 min. Then gradually Ca^{2+} was raised from 25 µM

through 500 μM by adding solutions containing increasing concentrations of Ca^{2+}. The yield of the isolation was checked, by using a haemocytometer and a Nikon Diaphot epifluorescent microscope. The viability of myocytes ranged between 80-90%. Viability was assessed by finding the percentage of the rod shaped myocytes (impermeable to trypan blue) to the sum of the rod shaped myocytes impermeable to trypan blue and the rod shaped myocytes that were permeable to trypan blue.

B2. Experimental Protocol for Simulated Ischemia in Adult Myocytes

Isolated myocytes were subjected to a 30 min stabilization period, in which the cells were suspended in an oxygenated buffer (115 mM NaCl, 5 mM KCl, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgCl_2$, 0.1% BSA, 11.1 mM Dextrose, 1.25 mM $CaCl_2$). The cells were oxygenated continuously with 95% O_2 and 5% CO_2, in a way that the gas just touches the fluid layer, so that the cells in the pellet are not disturbed. The cell suspension from each preparation (one heart) was equally divided into 4 experimental groups (no ischemia/no inhibitor, no ischemia/+inhibitor, ischemia/no inhibitor, ischemia/+inhibitor). The myocyte pellets in different groups were obtained by making the cells settle down by gravity rather than by centrifugation because centrifugation itself caused some damage to the cells. After the stabilization period, myocytes in the different experimental groups were incubated for 10 min at 37 °C in the absence or

Experimental Design:

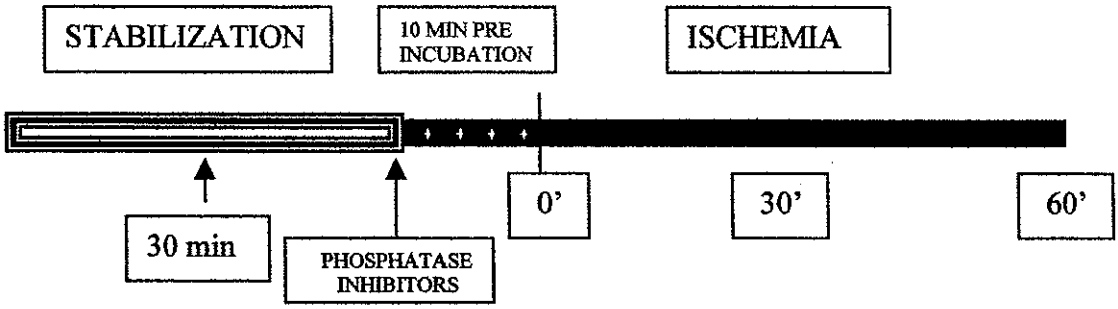


Figure 6: Graphic representation of the experimental design used for the identification of the effect of phosphatase inhibitors on Cx43 during ischemia. The leftmost rectangle represents the 30 min stabilization period. The phosphatase inhibitors were added to the pellets after the stabilization period and pre-incubated for 10 min at 37 °C. This is shown by the middle checked rectangle. Then the mineral oil was added subjecting the pellets to ischemia. Samples were taken for analysis at 0 min, 30 min and 60 min of ischemia. This is shown by the solid rectangle on the right hand side.

Ischemia Model:

Following the pre-incubation period each group of cells were transferred to a 1.5 ml microcentrifuge tube and the cells were allowed to settle down without centrifugation to avoid damage to the cells.

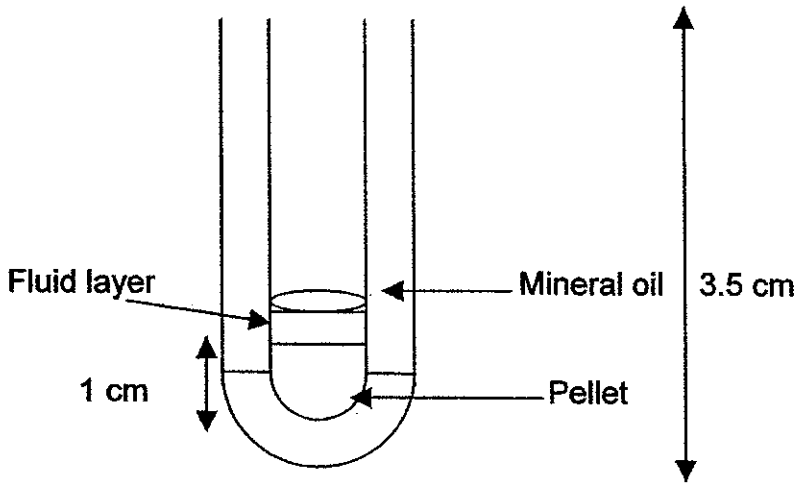


Figure 7: Graphic representation of the ischemia model. The procedure followed for mineral overlay-induced simulated ischemia on myocyte pellets was as described {Armstrong and Ganote 1992}. Each pellet occupied a volume of about 0.1 ml and measured 0.8-1 cm in depth. The fluid layer above the pellet occupied 1/3rd the volume of the pellet. The top of the fluid layer was layered with mineral oil, the depth of which was same as that of the fluid layer. The samples were taken out at 0 min, 30 min, and 60 min both from ischemic and the non-ischemic pellets.

presence of phosphatase inhibitors. This was followed by mineral oil overlay to simulate ischemia for 30 min, as indicated {Armstrong and Ganote 1992}. The pellets without the inhibitors served as controls.

B3. Drug Preparation

Okadaic acid (OA) was dissolved in distilled water to a stock solution of 100 µM. Calyculin-A and fostriecin were dissolved in 100% ethanol to a stock solution of 100 µM. FK-506 was dissolved in 100% methanol. CsA was dissolved in 100% ethanol. Tautomycin was dissolved in 100% ethanol to a stock solution of 100 µM. The stock solution was directly diluted into the cell suspensions to get the desired concentration of 1 µM for calyculin-A, fostriecin and OA, 10 nM for tautomycin, 150 nM/L for FK-506, and 0.2 µM for CsA.

B4. Western Blot Analysis

This procedure was performed exactly as described previously {Doble et al., 1996}. Ischemic samples were immediately frozen at –70°C. For analysis 1 ml of 1x SDS sample buffer (10% glycerol, 0.05 M Tris, and 1%SDS) containing PMSF (Phenylmethylsulfonylfluoride) (1 mM/L), sodium fluoride (10 mM/L), sodium orthovanadate (1 mM), β-glycerophosphate (60 mM), aprotinin (2 µg/mL), pepstatin (2 µg/mL), E64 (2 µg/ml), leupeptin (2 µg/ml) was added to the samples. The samples were sonicated and centrifuged (14,000 rpm for 10 min) to remove any undissolved residue and obtain the final sample. Protein concentration was determined by the BCA protein assay reagent (Pierce) followed by

spectrophotometry (Fischer Scientific). Lysates were analyzed on 10% polyacrylamide gels {Laemmli 1970} at 10 µg (10 µl) and 20 µg (20 µl) protein/lane using 60 mA constant current. To determine the molecular mass, broad range standards (Bio-Rad) were used. The proteins on the gel were electrophoretically transferred to PVDF (polyvinylidene difluoride) membranes. After transfer the membranes were incubated with a blocking solution containing 10% milk in Tris buffered saline with TWEEN-20 ((v/v)(SIGMA)(TBST)), for 1 hr at room temperature. Subsequently membranes were treated with primary antibody (1:500 for Zymed antibody and 1:10000 for the polyclonal P.AB) for 1 hr at room temperature. Antibodies were dissolved in 1% milk in TBST. The membranes were then rinsed with 1% milk in TBST (4 X 10 min) and incubated with goat anti mouse (1:10000) and anti rabbit (1:10000) HRP secondary antibodies for 1 hr at room temperature. Membranes were rinsed with plain TBST (4 X 5 min). Finally membranes were incubated in ECL+plus (enhanced chemiluminescence) Western blotting detection system obtained from Amersham Pharmacia, wrapped in the sheet protectors and exposed to the imaging film (Kodak X-omat LS) for 5-15 min to detect the chemiluminescent signal. Konica (SRX-101A) film processor was used to process the films.

B5. Lactate Dehydrogenase (LDH) Assay

Measuring the amount of LDH released during ischemia assessed the degree of myocyte injury. An LDH assay kit was used (Sigma

Diagnostics). One unit of LDH activity represented the amount of enzyme that catalyzes the formation of 1 μM/L of NAD per minute under the conditions in which the assay was done.

B6. Simulated Ischemia in Cultures of Neonatal Cardiomyocytes

Cardiomyocytes were isolated from the ventricles of 1 day old rat pups according to standard procedures {Doble et al., 1996; Doble et al., 2000} and were prepared by Robert R Fandrich in our laboratory. Freshly isolated myocytes were plated at a density of 800,000 cells per 35 mm collagen coated plate in the presence of 10 % bovine calf serum. They were then switched to low serum medium (0.5 % serum), replaced every 48 hrs, for 6 days. During this time myocytes formed a differentiated, synchronously contracting monolayer. To simulate ischemia, myocytes were placed in 'ischemic medium' (118 mM NaCl, 24 mM $NaHCO_3$, 1 mM $NaH_2PO_4 \cdot H_2O$, 2.5 mM $CaCl_2.2H_2O$, 1.2 mM $MgCl_2$, 0.5 mM sodium·EDTA·$2H_2O$, 20 mM sodium lactate, and 16 mM KCl, pH 6.2) {Zhao et al., 1998} and transferred to a hypoxia chamber maintained at 37 °C, in an atmosphere of 95 % N2 and 5 % CO2, for 2,4 or 6 hrs. Myocytes incubated in the presence of 'ischemic medium' but in the normal atmosphere of the culture incubator (not in hypoxia chamber) served as controls. The inhibitor calyculin-A was added to cultures (final concentration to 10 nM) together with the 'ischemic medium'. It was necessary to use the inhibitor at this low concentration because higher concentrations were highly toxic after 60 min of incubation.

Hypoxia chamber:

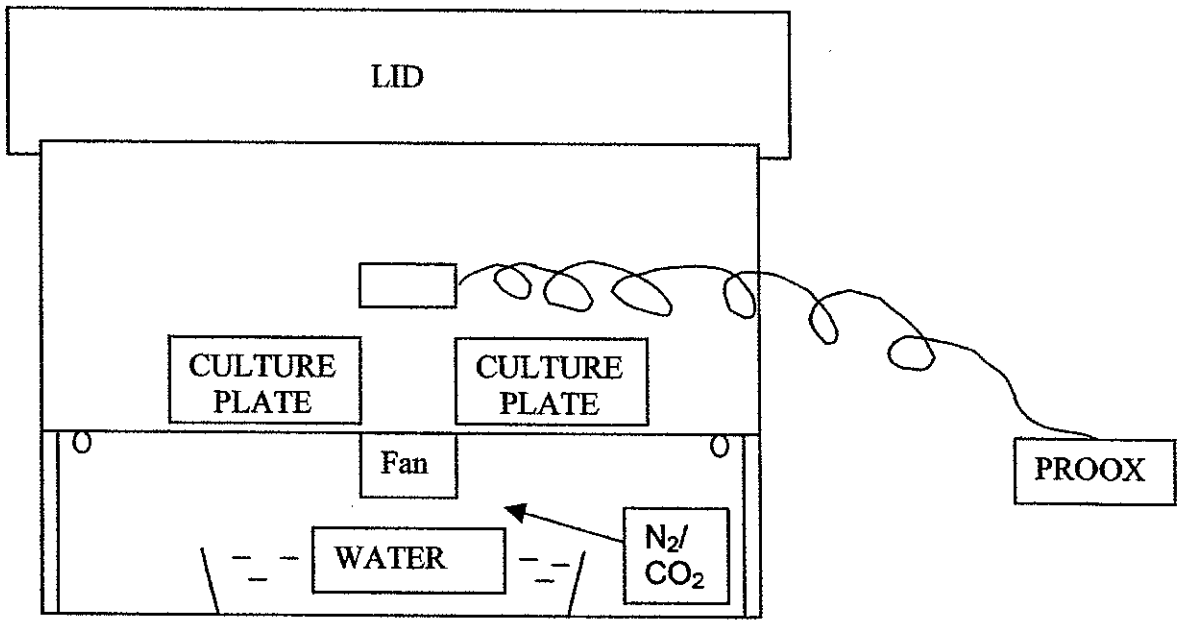


Figure 8: The hypoxia chamber is made of clear plexiglass and the parts consist of a lid and a square (size) chamber (40 cm x 40 cm x15 cm). The chamber sits inside the 37 ^{0}C incubator. The chamber is connected to the Nitrogen/CO_2 gas tank via the controller PROOX (Reming Bioinstruments Company). The gas tank was set to have a right concentration of CO_2, so that when the PROOX reaches the targeted set point of oxygen (0 %), by default the CO_2 reaches its required concentration (5 %).

B7. Densitometric Analysis of Phosphorylated and dephosphorylated Cx43 Species

Protein band density in Western blots was determined by using the volume analysis tools of the software program, Molecular Analyst (Biorad, Model-(GS-670)). The intensities of the phosphorylated and dephosphorylated Cx43 bands were determined individually. Intensities of both the bands combined together were also obtained. Percentage of Cx43 dephosphorylation was calculated as a function of the total (dephosphorylated and phosphorylated Cx43 species).

B8. Data Analysis

The Instat software program was used for the data analysis. Data are presented as means ± SEM (standard error of the mean). Statistical analysis was performed using the student's t test (paired, unpaired) to compare between two groups and ANOVA to compare more than two groups. $P<0.05$ was considered statistically significant. $P<0.01$ was considered statistically very significant.

CHAPTER 3

RESULTS

A. EFFECT OF ISCHEMIA ON THE PHOSPHORYLATION STATUS OF CX43

To examine the effect of simulated ischemia on Cx43 phosphorylation in the isolated adult myocyte model, we subjected myocyte pellets to increasing times of overlay with mineral oil. The phosphorylation status of Cx43 was analyzed by western blotting and probing with antibodies specific for all or only the dephosphorylated Cx43 species. Representative results are shown in Figure 9. Prior to ischemia, Cx43 was mostly phosphorylated, migrating at 43-45 kDa. Some dephosphorylated Cx43, migrating at 41 kDa, was also always present in these preparations. Baseline levels of dephosphorylated Cx43 varied between different preparations, between 5-45% of the total. Irrespectively of its baseline levels, ischemia induced an increase in the relative levels of dephosphorylated Cx43, accompanied by decreases in the phosphorylated Cx43. Increased Cx43 dephosphorylation was detectable as early as after 15 min of ischemia, reaching (in the experiment shown in Figure 9) 70% of total at 60 min. The percentage of dephosphorylated Cx43 was significantly increased at 30 min (*$P<0.05$, ANOVA, $n=3$) and 60 min (**$P<0.01$, ANOVA, $n=3$) of ischemia compared to 0 min time point.

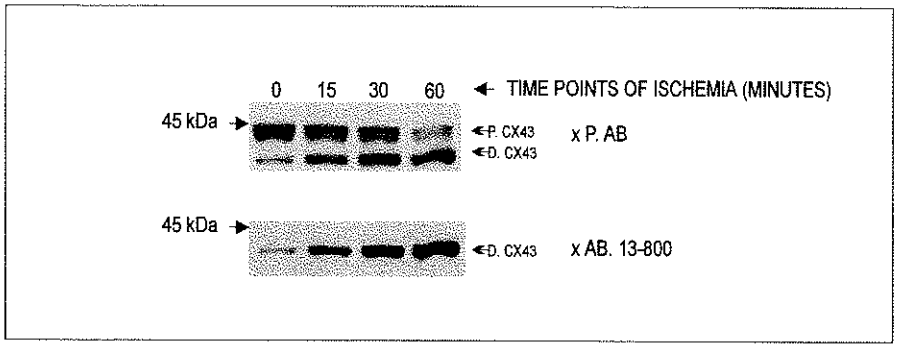
Figure 9. Effect of simulated ischemia on Cx43 dephosphorylation.

(a) Western blot of myocyte lysates probed for total (#P.AB) or dephosphorylated (#13-800) Cx43, as indicated. Myocytes were exposed to simulated ischemia for 0-60 min as indicated. Increased relative levels of dephosphorylated Cx43 (41 kDa) are observed as a function of time, in both blots.

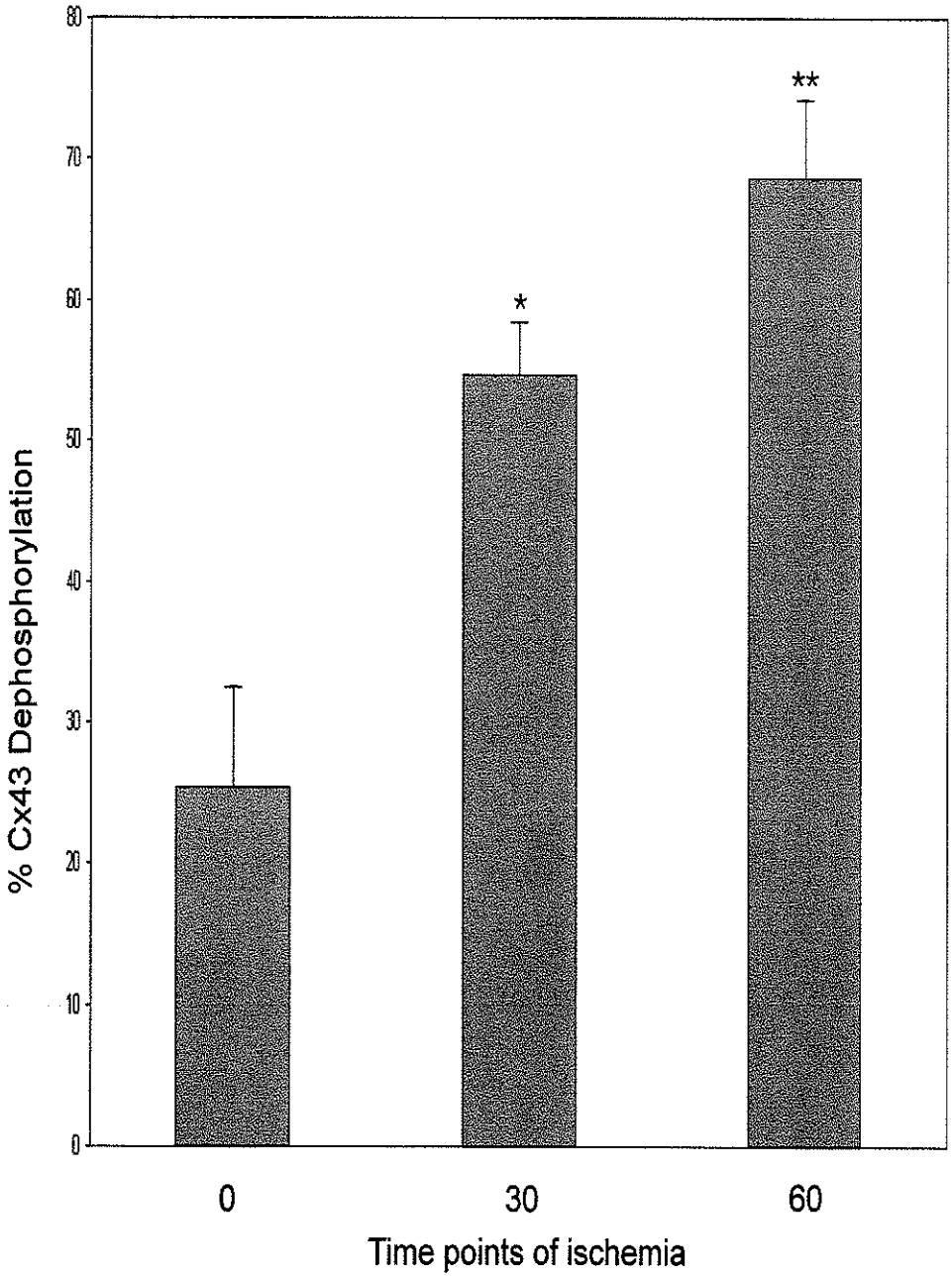
(b) Densitometric assessment of % of Cx43 dephosphorylation (y-axis) as a function of time of ischemia (x-axis). The percentage of dephosphorylation of Cx43 was significantly increased at 30 min (*$P<0.05$, ANOVA, n=3) and 60 min (**$P<0.01$, ANOVA, n=3) of ischemia compared to the 0 min. Data are mean$\pm$SEM.

Figure 9

(a)


0 15 30 60
TIME POINTS OF ISCHEMIA (MINUTES)
45 kDa
P. CX43
D. CX43
x P. AB
45 kDa
D. CX43
x AB. 13-800

(b)


% Cx43 Dephosphorylation
0
10
20
30
40
50
60
70
80
*
**
0
30
60
Time points of ischemia

B. EFFECT OF PHOSPHATASE INHIBITORS

B1: PP1/PP2A phosphatases

The PP2A/PP1 inhibitor OA {Hescheler et al., 1988} was used at different concentrations (10 nM, 100 nM, and 1 μM) to assess its effect on Cx43 dephosphorylation. Results are shown in Figure 10. Myocyte pellets incubated without the inhibitor served as control. OA did not appear to prevent Cx43 dephosphorylation at 10-100 nM. There was a significant reduction in Cx43 dephosphorylation at 1 μM OA (*$P<0.05$, ANOVA, n=3). This concentration was re-tested in subsequent studies, and representative results are shown in Figure 10b. Cells treated with 1 μM OA showed a significant 31% decrease in Cx43 dephosphorylation compared to the control (*$P<0.05$, student t test, n=3). A higher concentration of OA (10 μM) further prevented Cx43 dephosphorylation but was accompanied by high toxicity (cell shrinkage, incorporation of trypan blue-data not shown) and was not tested further.

Another PP1/PP2A inhibitor that was tested was calyculin-A {Armstrong and Ganote 1992}. In pilot dose-response studies, the group treated with 1 μM calyculin-A significantly decreased Cx43 dephosphorylation compared to the control group as shown in Figure 11(a) (*$P<0.05$, ANOVA, n=3). At higher concentrations calyculin-A was toxic to the cells (cell shrinkage, incorporation of trypan blue-data not shown) and was not tested further. Calyculin-A was thus used at a concentration of 1 μM in subsequent studies. One such study is shown in Figure 11(b). In the inhibitor treated cells the amount of dephosphorylation of Cx43 was significantly reduced by 28% at 30 min and 21% at 60 min of ischemia

Figure 10

(a). Dose response of the effects of okadaic acid on ischemia induced Cx43 dephosphorylation.

Myocytes were treated with 10 nM, 100 nM and 1 μM of okadaic acid (OA). Non-treated myocytes served as controls. The vertical axis indicates percentage of dephosphorylation and the x-axis indicates different treatments. There was a significant decrease in dephosphorylation with 1 μM OA (*P<0.05, ANOVA, (n=3)). Data are mean±SEM.

(b). Effect of okadaic acid on the dephosphorylation of Cx43 during Ischemia.

In a separate experiment, myocytes were subjected to 30 min ischemia in the presence or absence of 1 μM OA. Percentage of dephosphorylation (y-axis) is shown as a function of OA treatment, as indicated. Asterisk indicates statistically significant differences between OA treated samples and the control samples. OA decreased the Cx43 dephosphorylation by 31% when compared to the control. Compared to control group (*P<0.05, student's t test, (n=3)). Data are mean±SEM.

Figure 10

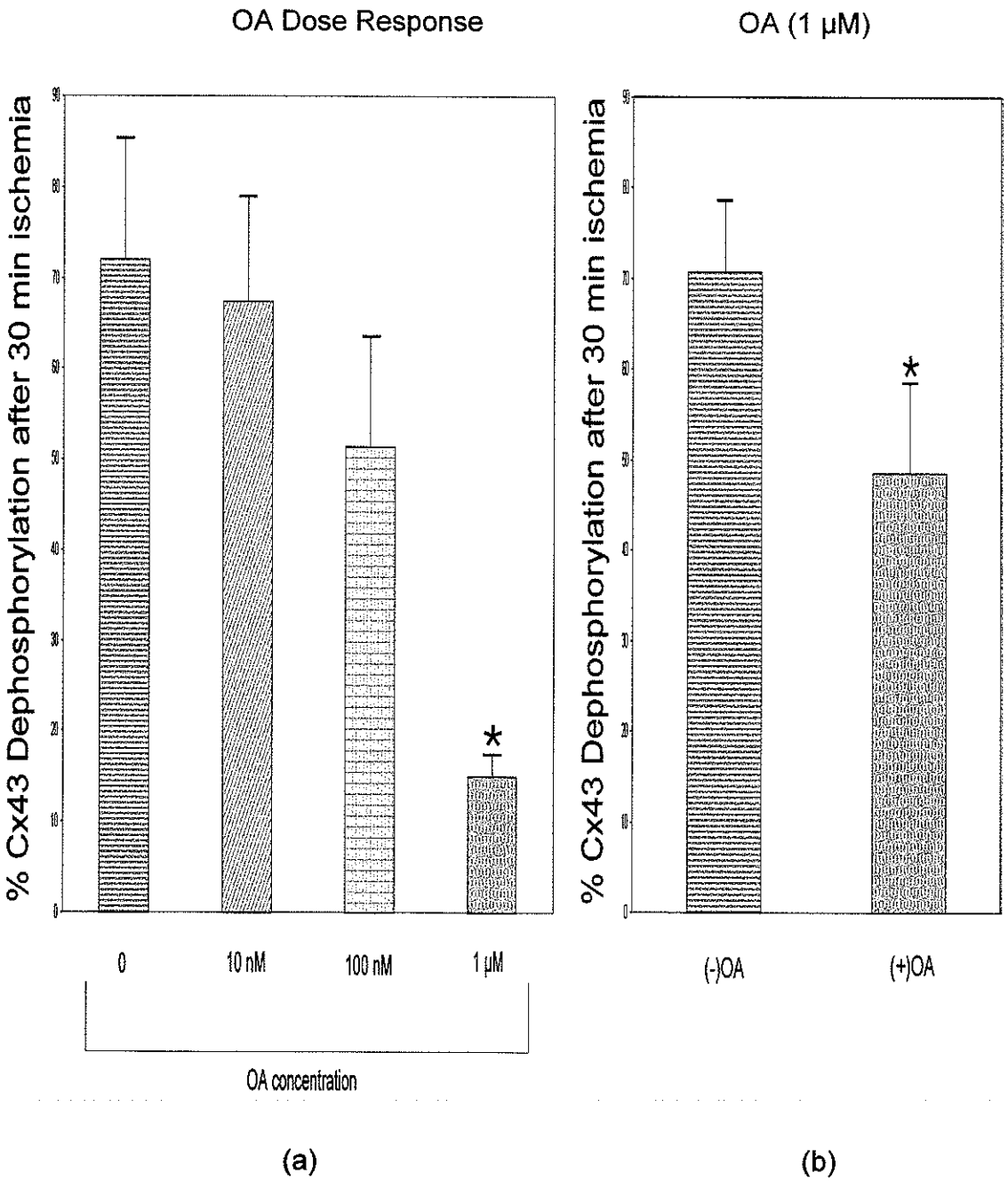

OA Dose Response
% Cx43 Dephosphorylation after 30 min ischemia
0
10 nM
100 nM
1 μM
OA concentration
(a)
OA (1 μM)
% Cx43 Dephosphorylation after 30 min ischemia
(-)OA
(+)OA
(b)

Figure 11

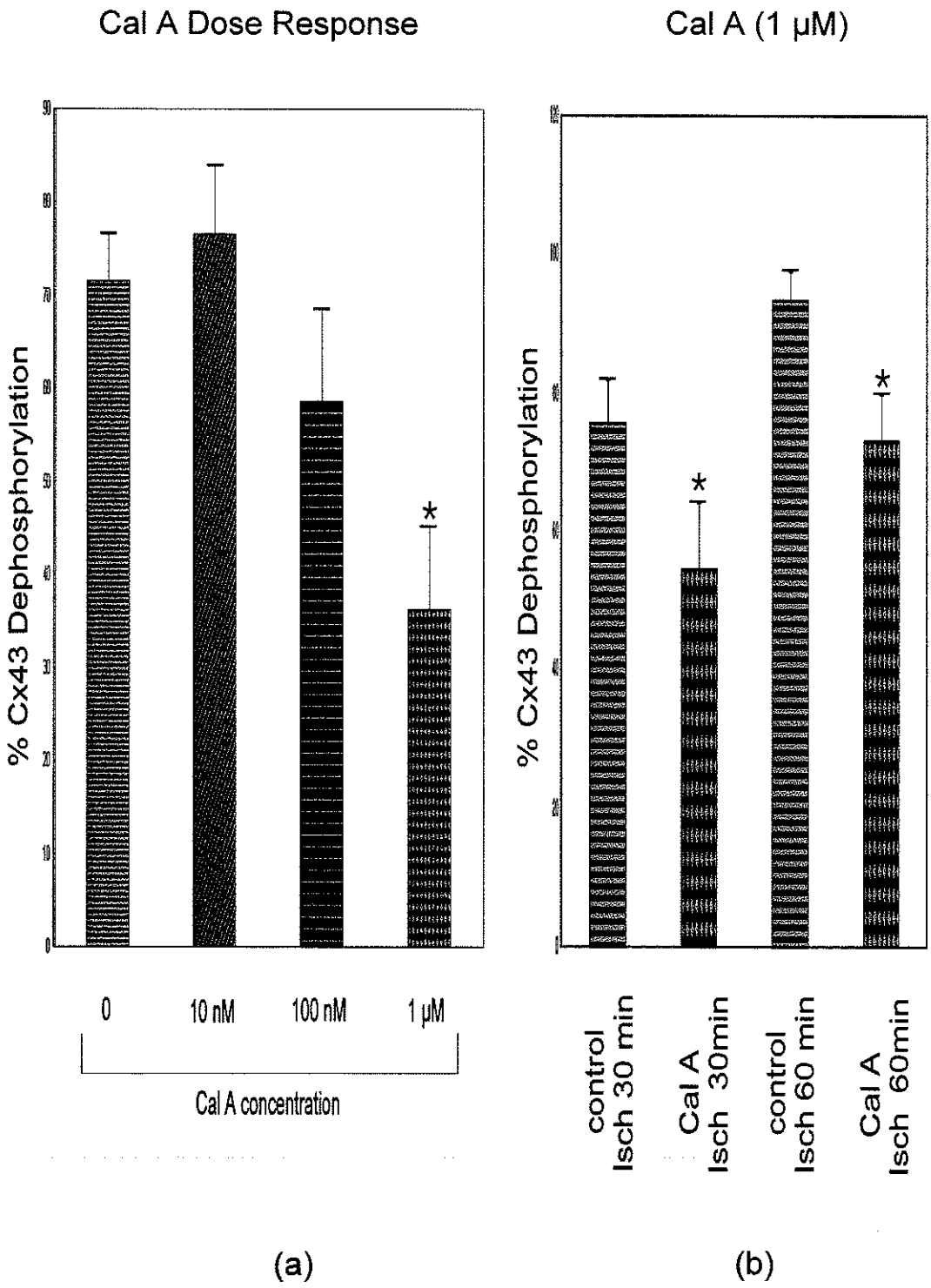
(a). Dose response studies for the effects of calyculin-A on ischemia induced Cx43 dephosphorylation.

Myocytes were treated with 0-1.0 μM of calyculin-A. Percentage of Cx43 dephosphorylation (y-axis) is shown as a function of treatment with different concentrations of Calyculin-A, as indicated. significant decrease in the dephosphorylation of Cx43 was obtained with 1 μM calyculin-A, compared to control (*P<0.05, ANOVA, (n=3)). Data are mean±SEM.

(b). Effect of calyculin-A on the dephosphorylation of Cx43 in the adult rat ventricular myocytes during ischemia.

In a different experiment, myocytes were subjected to 30 min and 60 min of ischemia with or without calyculin-A (1 μM). Percentage of Cx43 dephosphorylation (y-axis) is shown as a function of calyculin-A treatment at 30min and 60min of ischemia (x-axis). Calyculin-A reduced Cx43 dephosphorylation by 28% at 30 min and by 21% at 60min of ischemia. Asterisk indicates statistically significant differences between calyculin-A treated samples and the control samples both at 30min and 60min of ischemia. Compared to control group *P<0.05, student's t test (n=3). Data are mean±SEM.

Figure 11


Cal A Dose Response
% Cx43 Dephosphorylation
0
10 nM
100 nM
1 μM
Cal A concentration
(a)
Cal A (1 μM)
% Cx43 Dephosphorylation
control
Isch 30 min
Cal A
Isch 30min
control
Isch 60 min
Cal A
Isch 60min
(b)

when compared to the control ($P<0.05$, student's t test, n=3). Calyculin-A was dissolved in ethanol to a stock concentration of 100 μM. Ethanol used on its own and at similar concentrations used to dissolve calyculin-A did not prevent ischemia induced Cx43 dephosphorylation (data not shown).

The PP2A selective inhibitor fostriecin {Weinbrenner et al., 1998} was also tested for its effects on Cx43 dephosphorylation, at a concentration of 1 μM {Armstrong et al., 1997; Cheng et al., 1998; Weinbrenner et al., 1998}. Results are shown in Figure 12. Fostriecin did not prevent ischemia-induced Cx43 dephosphorylation. Two different lots of fostriecin gave similar results; increasing fostriecin dosage to 10 μM did not prevent dephosphorylation (data not shown). Representative western blots depicting the effect of the various inhibitors of PP1/2A phosphatases on Cx43 dephosphorylation during ischemia is shown in Figure 13.

We also tested the PP1 selective inhibitor tautomycin {Favre et al., 1997; Ubukata et al., 1997; Yan and Mumby 1999} at a concentration of I0 nM. As shown in Figure 14, while tautomycin had no effects on Cx43 in the absence of ischemia, it prevented Cx43 dephosphorylation during ischemia. This effect was especially evident in experiment 2 (Figure 14).

B2: PP2B phosphatases

FK-506, a PP2B inhibitor {Fruman et al., 1992} was tested at a concentration of 150 nM/L. The amount of dephosphorylated Cx43 was significantly reduced by 33% in the inhibitor-treated cells compared to controls

Figure 12. Effect of fostriecin on Cx43 dephosphorylation.

Percentage of Cx43 dephosphorylation after 30 min of ischemia (y-axis) is shown as a function of treatment with fostriecin (1μM), as indicated. Cx43 dephosphorylation in ischemia was not prevented by incubation with fostriecin. Data represented as mean±SEM (n=3).

Figure 12

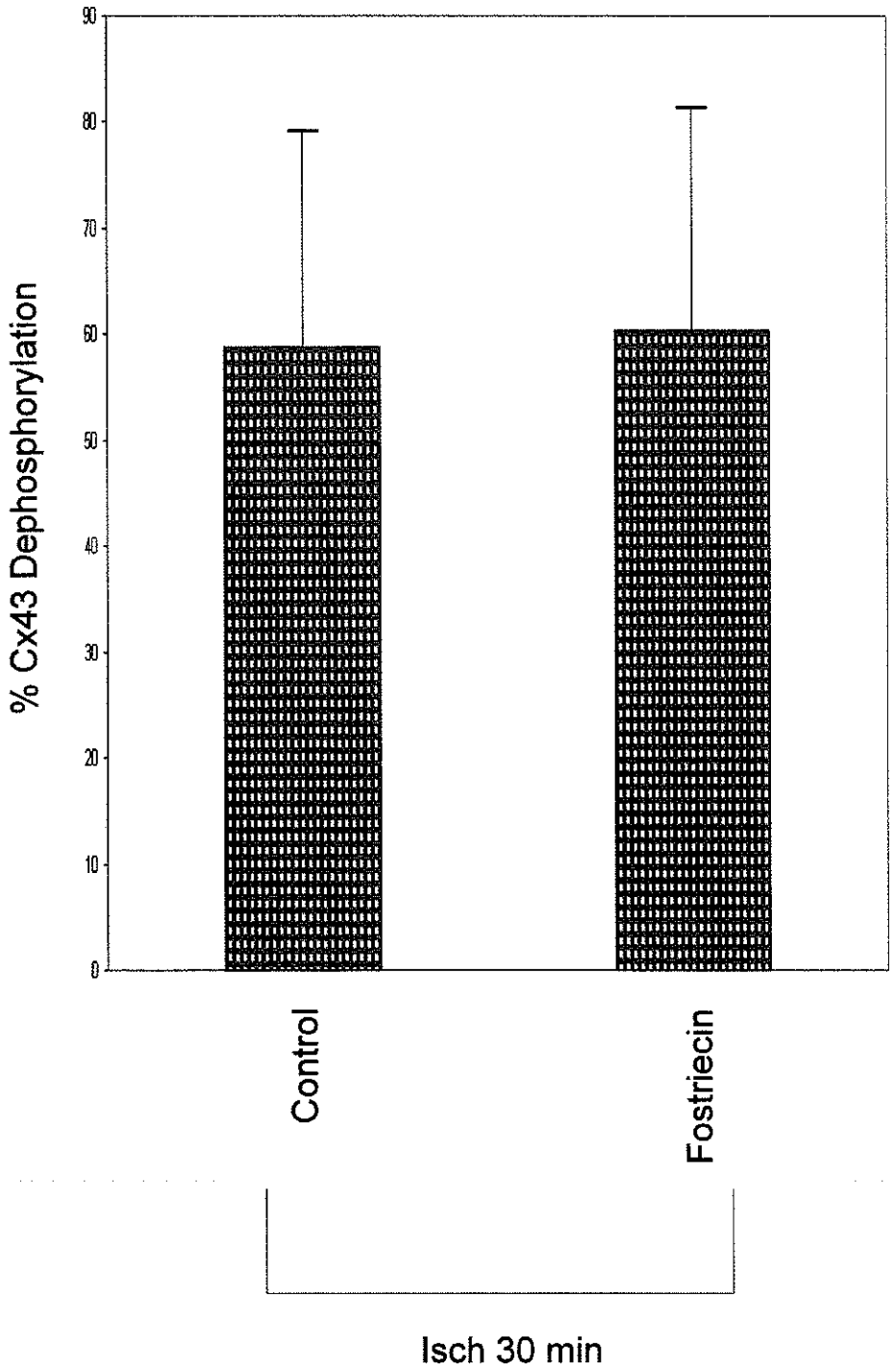

% Cx43 Dephosphorylation
90
80
70
60
50
40
30
20
10
0
Control
Fostriecin
Isch 30 min

Figure 13. Western blots showing the effects of PP1/2A inhibitors on ischemia-induced Cx43 dephosphorylation.

Representative western blots showing the effects of PP1/2A inhibitors (OA, calyculin-A, fostriecin) on ischemia-induced Cx43 dephosphorylation in isolated cardiac myocytes. Western blots were probed for total (#P.AB) or dephosphorylated (#13-800) Cx43, as indicated. (-) and (+) denote absence or presence of the inhibitor, respectively.

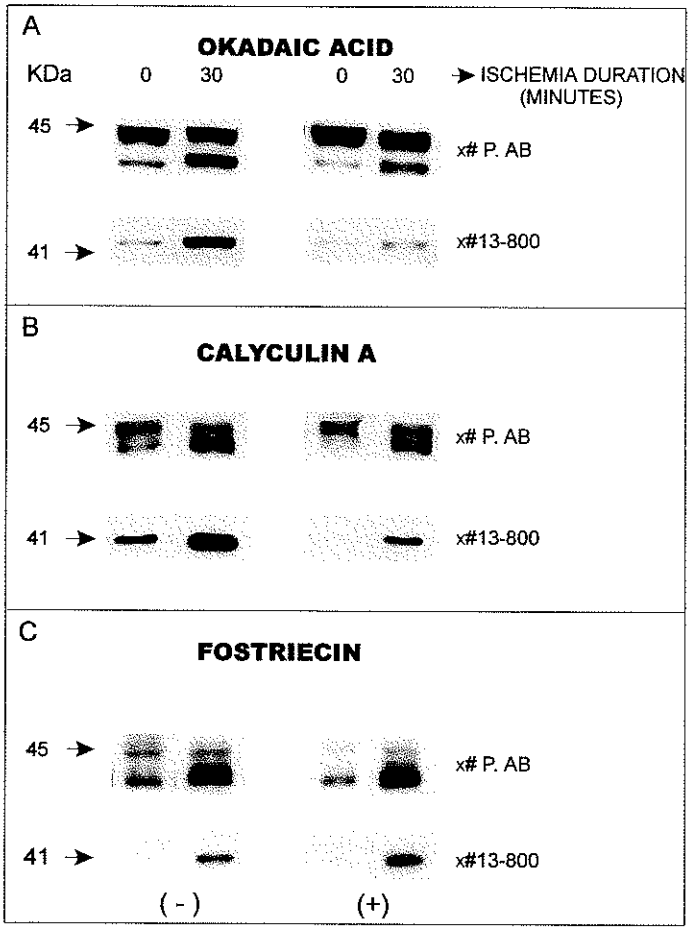

A
OKADAIC ACID
KDa
0
30
0
30
ISCHEMIA DURATION (MINUTES)
45
x# P. AB
41
x#13-800
B
CALYCULIN A
45
x# P. AB
41
x#13-800
C
FOSTRIECIN
45
x# P. AB
41
x#13-800
(-)
(+)

Figure 13

Figure 14. Effect of tautomycin on ischemia-induced Cx43 dephosphorylation.

Western blots from two different experiments, depicting the effect of the PP1 specific inhibitor tautomycin on ischemia-induced Cx43 dephosphorylation. Blots were probed for total (#P.AB) or dephosphorylated (#13-800) Cx43, as indicated. (-) and (+) denote absence or presence of tautomycin, respectively.

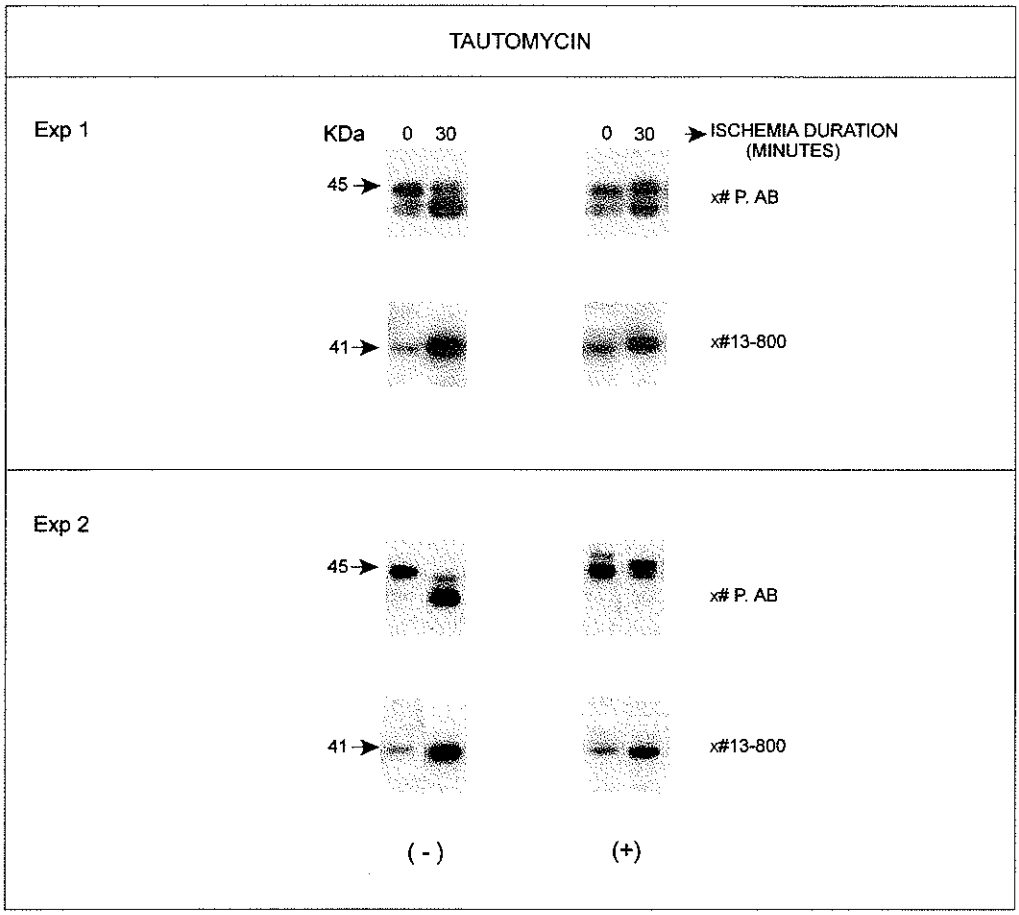

TAUTOMYCIN
Exp 1
KDa
0 30
0 30
ISCHEMIA DURATION (MINUTES)
45
x# P. AB
41
x#13-800
Exp 2
45
x# P. AB
41
x#13-800
(-)
(+)

Figure 14

($P<0.05$, student's t test, n=6, where n signifies independent experiment using a different myocyte preparation) (Figure 15). The corresponding western blot is shown in Figure 17.

Cyclosporine, another PP2B inhibitor {Fruman et al., 1992} was used at a concentration of 0.2 μM. As shown in Figure 16, the amount of dephosphorylated Cx43 was significantly reduced (by 28%) in cyclosporine-treated cells compared to the control cells ($P<0.05$, student's t test, n=3, where n signifies independent experiment using a different myocyte preparation). A representative western blot is shown in Figure 17.

C. COMBINED USE OF PP1/PP2A AND PP2B

In this experiment myocytes were divided in four groups. The first group received no treatment and served as control. The second and the third group were treated separately with FK-506 (150 nM/L) or calyculin-A (1 μM) respectively. The fourth group received combination treatment consisting of calyculin-A (1 μM) and FK-506 (150 nM/L). Results are shown in Figure 18. The control group displayed maximal dephosphorylation (as expected) at around 50-60% at 30 min of ischemia. The FK-506 treated group displayed a 53% reduction in dephosphorylated Cx43 ($^{*}P<0.05$, ANOVA, n=3). The calyculin-A treated group displayed a significant 80% decrease in the dephosphorylated Cx43 when compared to the control group ($^{\#}P<0.01$, ANOVA, n=3). In the group treated with both calyculin-A and FK-506, there was a marked and very significant decrease

Figure 15. Effect of FK-506 on the dephosphorylation of connexin-43 during ischemia.

Cx43 dephosphorylation (y-axis) is shown as a function of treatment with FK-506 (150 nM/L) (x-axis) as indicated. Asterisk indicates statistically significant differences between FK-506 treated samples and the control samples at 30 min of ischemia. FK-506 decreased the dephosphorylation of Cx43 by 33%, when compared to untreated cells. Data are represented as mean$\pm$SEM. Compared to control group *$P<0.05$, student's t test ($n=6$).

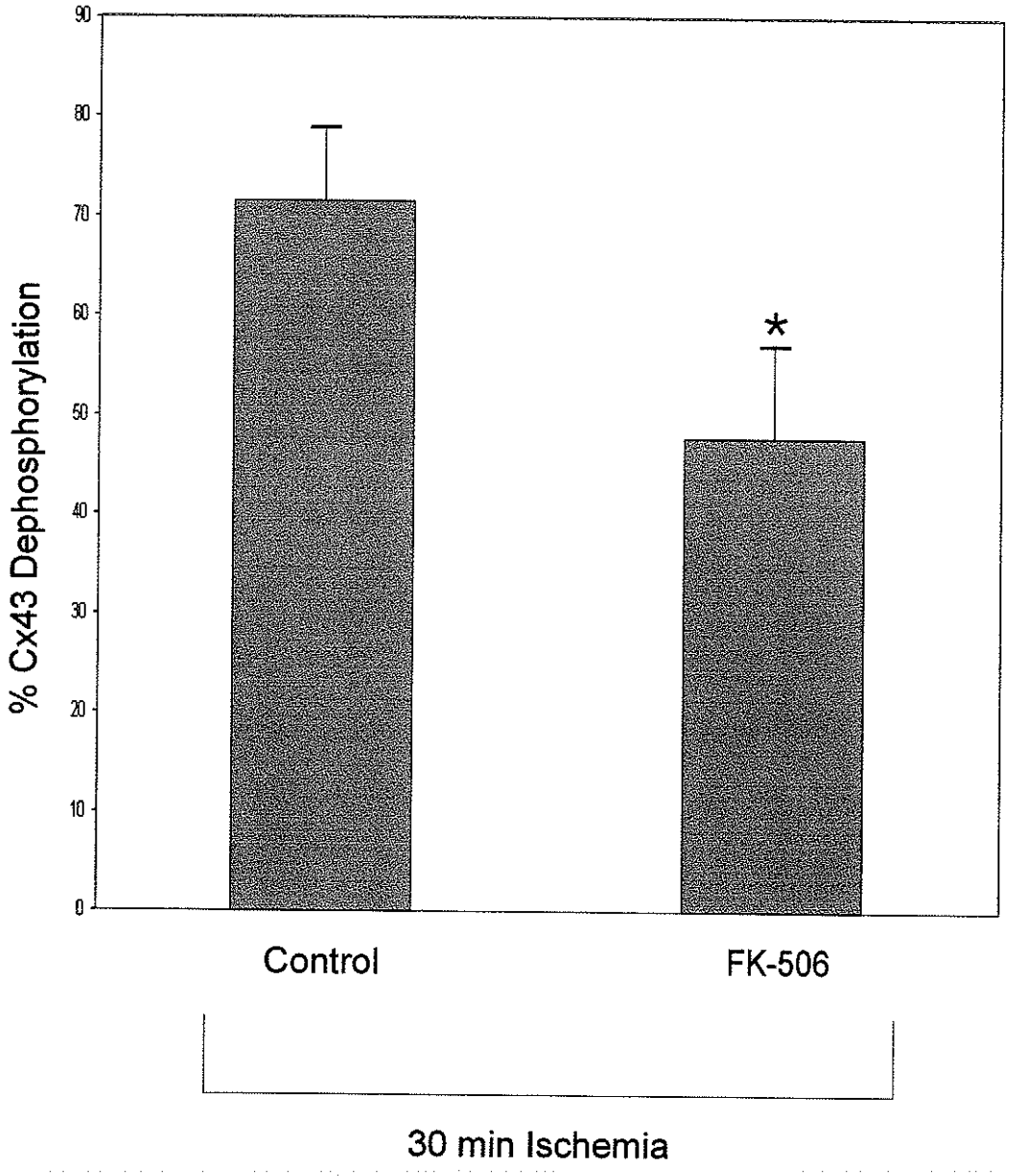

% Cx43 Dephosphorylation
90
80
70
60
50
40
30
20
10
0
*
Control
FK-506
30 min Ischemia

Figure 15

Figure 16. Effect of cyclosporine A on Cx43 dephosphorylation.

Percentage of Cx43 dephosphorylation during 30 min of ischemia (y-axis) is shown as a function of treatment with cyclosporine (CsA)(x-axis), as indicated. Asterisk indicates statistically significant differences between CsA treated group and the control group at 30 min of ischemia in terms of level of dephosphorylation of Cx43. Compared to the untreated samples CsA reduced Cx43 dephosphorylation by 28% (*$P<0.05$, student's t test (n=3)). Data is represented as mean$\pm$SEM.

Figure 16

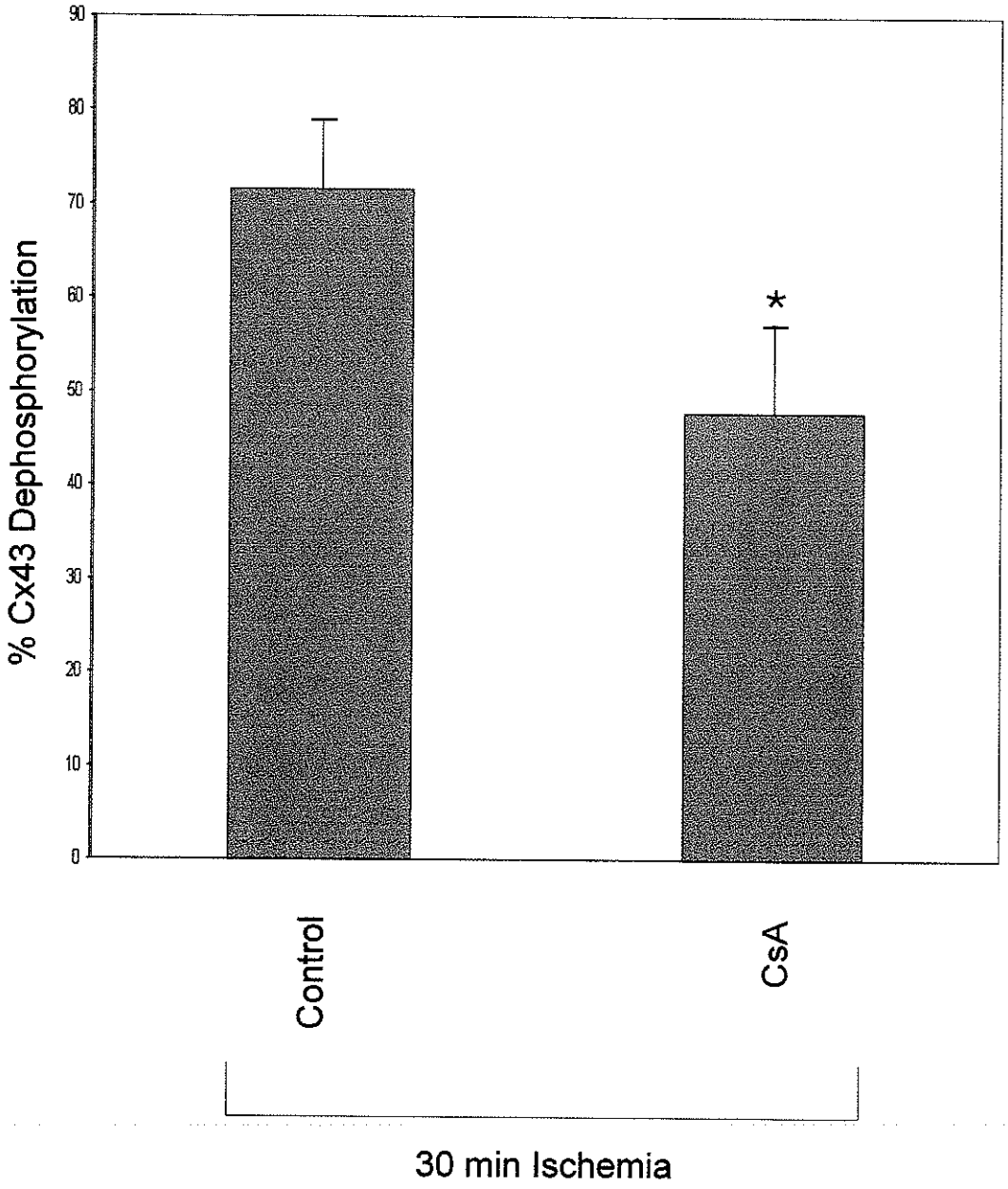

% Cx43 Dephosphorylation
90
80
70
60
50
40
30
20
10
0
*
Control
CsA
30 min Ischemia

Figure 17. Effect of PP2B inhibitors on Cx43 dephosphorylation.

Representative western blots depicting the effects of PP2B inhibitors on Cx43 dephosphorylation. Myocytes were treated with FK-506 (150 nM/L) or cyclosporine (0.2 μM) as indicated. Western blots were probed for total (#P.AB) or dephosphorylated (#13-800) Cx43, as indicated. Absence or presence of the inhibitor is denoted by (-) and (+) respectively. In the FK-506 treated group and in the cyclosporine treated group there is a partial decrease in Cx43 dephosphorylation. The top panel shows the effect of FK-506 and the bottom panel shows the effect of cyclosporine.

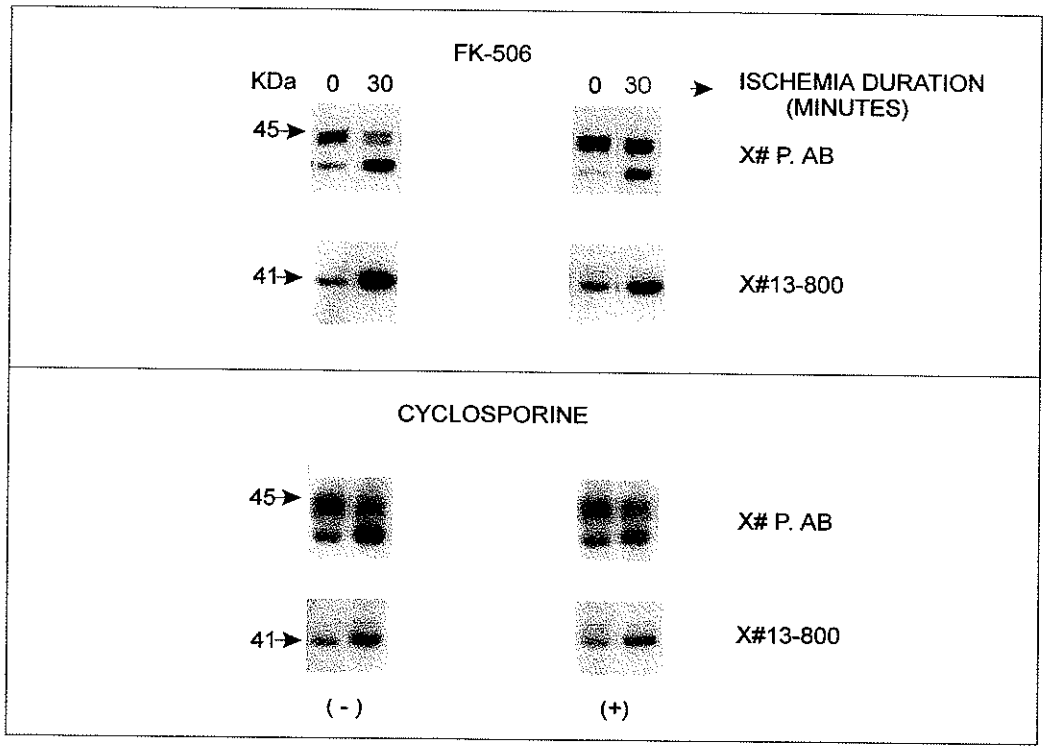

FK-506
KDa
0
30
0
30
ISCHEMIA DURATION
(MINUTES)
45
X# P. AB
41
X#13-800
CYCLOSPORINE
45
X# P. AB
41
X#13-800
(-)
(+)

Figure 17

Figure 18. Effect of combining PP1/2A and PP2B inhibitors on Cx43 dephosphorylation.

Fraction of dephosphorylated Cx43 is shown as a function of various treatments, as indicated. Myocytes were treated with 1 μM Calyculin-A, 150 nM/L FK-506, and 1 μM calyculin-A plus 150 nM/L FK-506 or left untreated as indicated. FK-506, Cal A, and (Cal A + FK-506) decreased Cx43 dephosphorylation by 53%, 80% and 94% respectively compared to the control group. Asterisk (*) indicates statistically significant differences from the untreated samples ($P<0.05$, ANOVA, n=3). The number sign (#) indicates statistically significant differences from the untreated samples ($P<0.01$, ANOVA, n=3, where n signifies independent experiment using a different myocyte preparation). The double number sign (# #) indicates very significant statistical difference from the control group ($P<0.001$, ANOVA, n=3). Data are represented as mean±SEM.

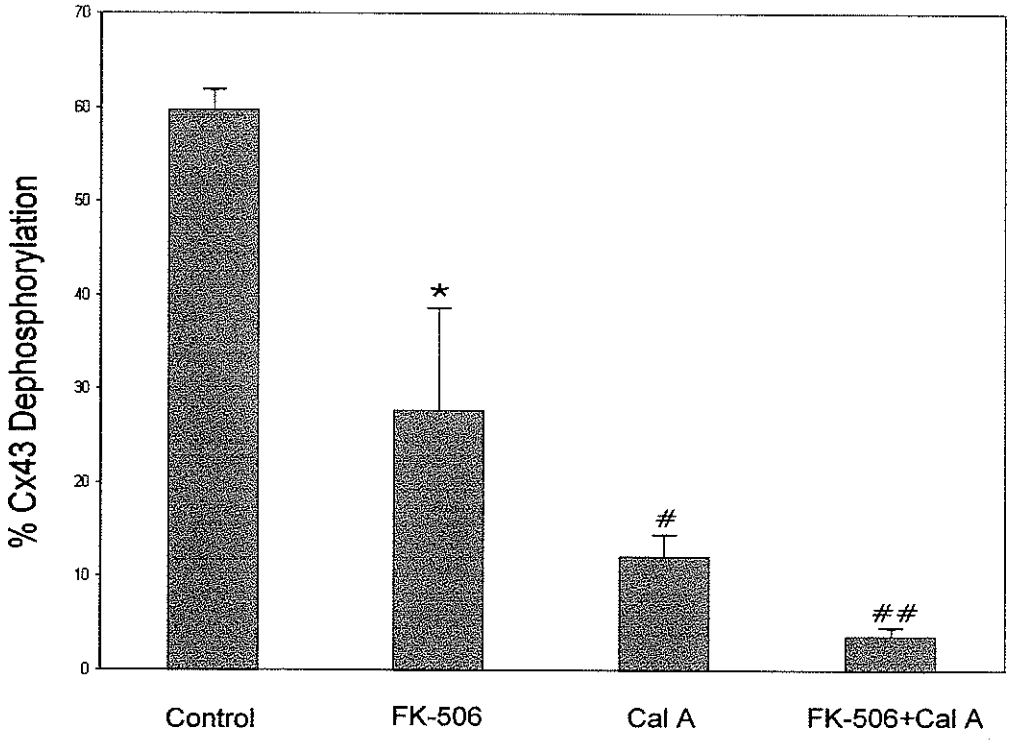

% Cx43 Dephosphorylation
70
60
50
40
30
20
10
0
*
#
##
Control
FK-506
Cal A
FK-506+Cal A

Figure 18

in Cx43 dephosphorylation (94%) when compared to the control group ($^{\#\#}P<0.001$, ANOVA, n=3 where n signifies independent experiment using a different myocyte preparation). A representative western blot is shown in Figure 19.

D. EFFECT OF PHOSPHATASE INHIBITION ON CARDIAC RESISTANCE TO INJURY

D1: Langendorff studies

In order to examine if the conclusions derived from the isolated myocytes were valid in the context of the whole heart, and to examine the relationship between prevention of Cx43 dephosphorylation and cardiomyocyte integrity, we collaborated with Dr. Stéphane Tanguy, using the isolated perfused rat heart model. Results from these studies have been published {Jeyaraman et al., 2003}. Briefly, perfused hearts were subjected to global ischemia in the presence or absence of pre-incubation with OA at 100 nM. Perfusion of whole hearts with 1 μM OA induced contracture and thus was not used further. Subsequently, relative content and distribution of dephosphorylated Cx43 was examined by western blotting and densitometry, as well as by immunofluorescence {Doble et al., 1996; Jeyaraman et al., 2003}. In the control hearts, global ischemia alone caused increases in the dephosphorylated Cx43 with increasing time points of ischemia. As shown in Figure 20 the amount of dephosphorylated Cx43 was significantly reduced by 38% in the OA-treated hearts compared to control hearts

Figure 19. Combined effect of calyculin-A and FK-506 on ischemia induced Cx43 dephosphorylation.

Representative western blots illustrating the effects of combined use of phosphatase inhibitors on Cx43 dephosphorylation. Blots were probed for total (#P.AB) or dephosphorylated (#13-800) Cx43, as indicated. Lanes 1, 2, 3 and 4 depict respectively, untreated control myocytes, and myocytes treated with FK-506, Cal-A, and combined FK-506 (150 nM/L) and Cal-A (1 μM). Combined treatment is most effective in preventing Cx43 dephosphorylation.

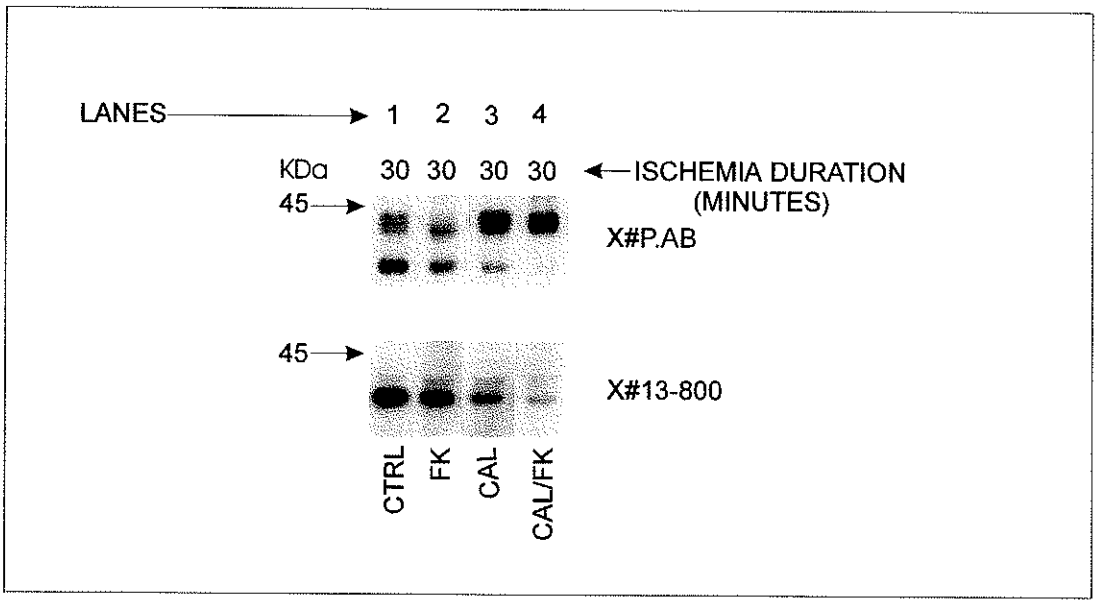

LANES
1 2 3 4
KDa
30 30 30 30
ISCHEMIA DURATION
(MINUTES)
45
X#P.AB
45
X#13-800
CTRL
FK
CAL
CAL/FK

Figure 19

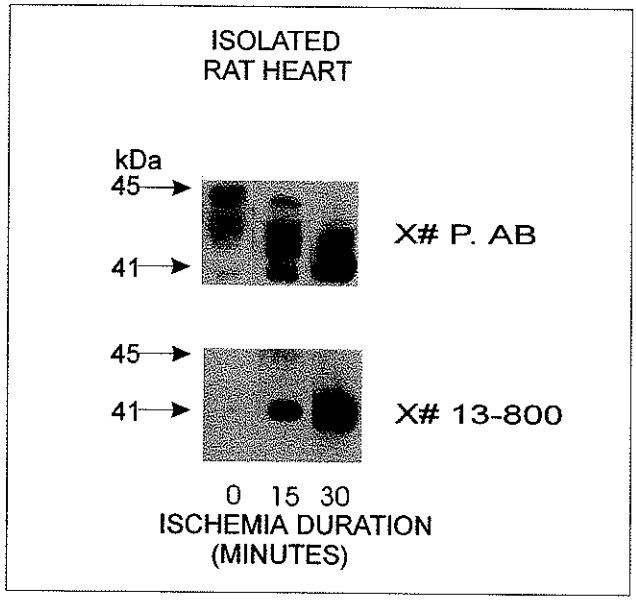
Figure 20(a): Effects of global ischemia on the phosphorylation status of Cx43 in the perfused intact heart.

Western blots were probed for total Cx43, using the polyclonal anti-Cx43 antibodies (#P.AB; upper blots) or for dephosphorylated Cx43 using the monoclonal #13-800 antibodies (lower blots), as indicated. Ischemia duration is indicated in minutes. Migration of molecular weight markers is indicated in kDa. Lysates were analyzed on large format 9% acrylamide gels. The blot shows global ischemia induced dephosphorylation of Cx43, in a time-dependant fashion.

Figure 20(b): Effect of okadaic acid on Cx43 dephosphorylation during global ischemia.

Percent dephosphorylation of whole heart Cx43 after 30 min of global ischemia, plotted as a function of absence (-) or presence (+) of okadaic acid, as indicated. Asterisk indicates statistically significant differences from untreated samples ($P<0.05$, unpaired student t-test, $n=4$, where n signifies individual hearts).

(a)


ISOLATED
RAT HEART
kDa
45
41
X# P. AB
45
41
X# 13-800
0 15 30
ISCHEMIA DURATION
(MINUTES)

(b)

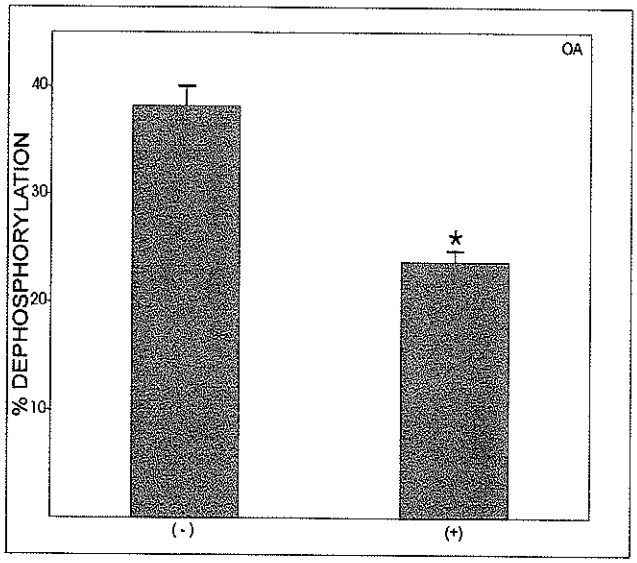

OA
% DEPHOSPHORYLATION
40
30
20
10
*
(-)
(+)

Figure 20

($P<0.05$, unpaired student t-test, n=4, where n signifies individual hearts) after 30 min of global ischemia. Ischemia caused increased distribution of dephosphorylated CX43 at, and a somewhat frayed appearance of, the intercalated disks between cardiomyocytes; OA treatment decreased dephosphorylated Cx43 at, and improved the apparent integrity of the intercalated disks {Jeyaraman et al., 2003}.

D2: Effect of calyculin-A on hypoxia-induced cardiomyocyte injury

Confluent, differentiated cultures of neonatal rat cardiomyocytes prepared by Robert R Fandrich in our laboratory were used for these studies. Ischemia was simulated in these cultures by incubation in the presence of ischemic medium (see methods), followed by incubation in a 'hypoxia-chamber', where oxygen concentration was maintained at very low levels, for different time points. Cultures were pre-treated with calyculin-A (10 nM) for 30 min before exposure to simulated ischemia. Higher concentrations of Calyculin-A were toxic after one hour of incubation in this model and were not studied further. A set of cultures was incubated in the presence of the 'ischemic' medium, and in normal oxygen levels (not in the hypoxia chamber). At the end of the incubation, myocyte injury was evaluated by determining LDH release in the supernatant. Results are shown in Figure 21.

At 2 hrs of incubation: minimal levels of LDH release were seen in cultures without inhibitor irrespective of hypoxia. At this time point therefore hypoxia did not induce detectable cell injury. Incubation with calyculin-A induced an increase

Figure 21: Effect of calyculin-A on LDH release from neonatal myocyte cultures in the presence or absence of hypoxia.

LDH release (y-axis) is shown as a function of incubation in ischemic medium in the absence (CTRL) or presence (Cal-A) of phosphatase inhibitor, and incubation (Hyp) or not (No Hyp) in hypoxia chamber, as indicated. Cal-A was used at 10 nM. Even at this concentration it caused some toxicity. However, it decreased the hypoxia-induced LDH release at 4 hrs of hypoxia (*$P<0.05$, ANOVA, $n=3$). Data are mean$\pm$SEM.

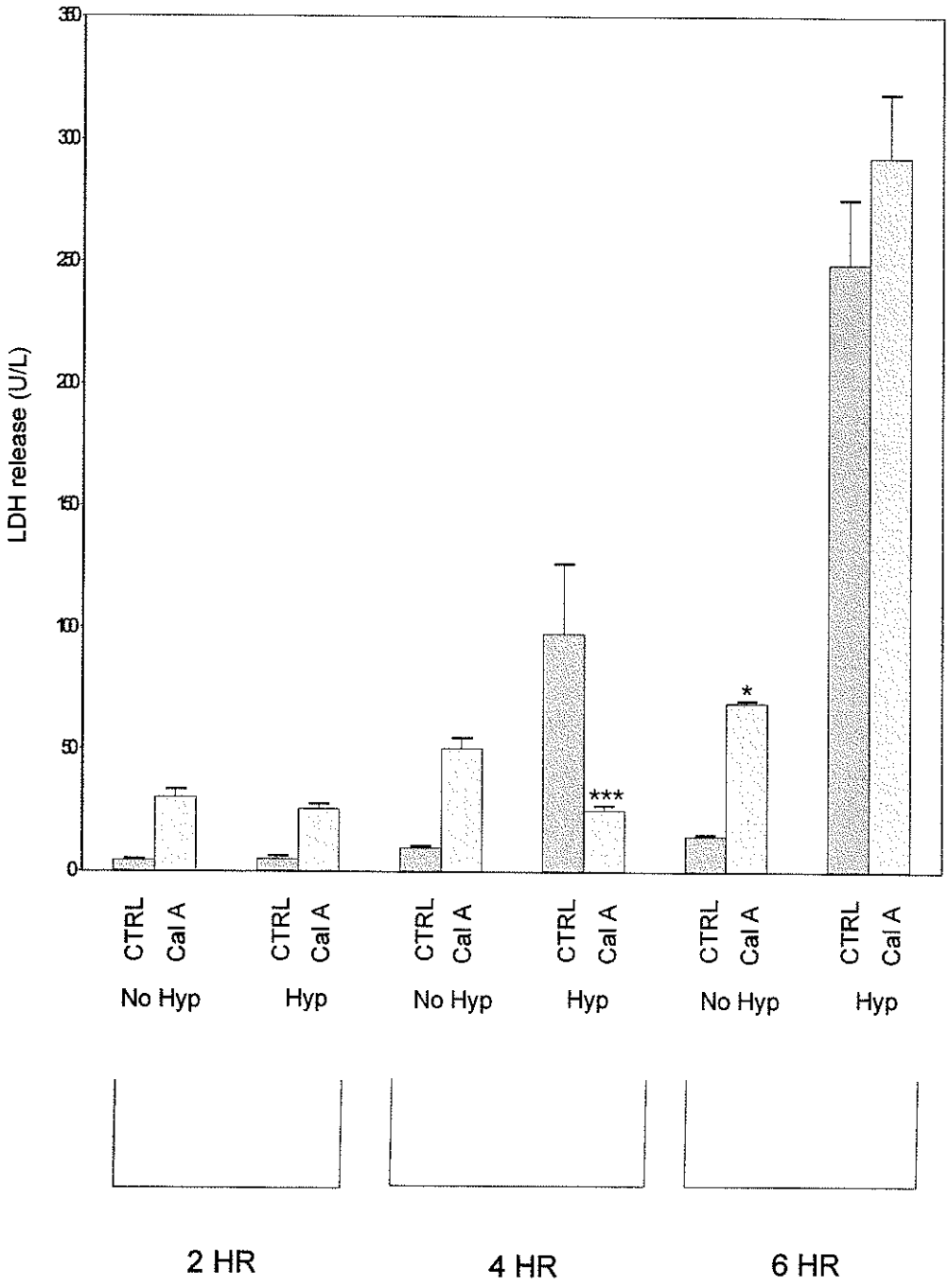

LDH release (U/L)
0
50
100
150
200
250
300
350
***
*
CTRL
Cal A
No Hyp
Hyp
2 HR
4 HR
6 HR

Figure 21

in LDH release (over corresponding controls); levels of LDH release in the presence of inhibitor were similar irrespective of hypoxia. The inhibitor itself therefore has some toxicity, even at this low concentration.

At 4 hrs of incubation: An increase in LDH release was detected in cells subjected to hypoxia in the absence of phosphatase inhibition compared to cells not subjected to hypoxia. Simulated ischemia (ischemic medium plus hypoxic chamber) therefore starts having clear effects in cell injury at this time point. Under these conditions presence of calyculin-A significantly reduced ischemia-induced LDH release (***$P<0.001$, ANOVA, n=3).

At 6 hrs of incubation, calyculin-A treatment continued to increase LDH release in the absence of hypoxia compared to untreated cells (*$P<0.05$, ANOVA, n=3). At this time point hypoxia induced a massive rise in LDH, irrespective of the presence of inhibitor.

We examined relative levels of dephosphorylated Cx43 at 4 hrs time point by western blotting. Results are shown in Figure 22. No dephosphorylated Cx43 was detectable for cells incubated under regular oxygen tension. Incubation in hypoxia chamber caused accumulation of dephosphorylated Cx43; calyculin-A decreased levels of dephosphorylated Cx43.

Figure 22: Effect of Calyculin-A and simulated ischemia on Cx43 dephosphorylation.

Neonatal cardiomyocyte cultures, subjected to 4 hrs of simulated ischemia, in the presence or absence of cal-A were lysed and analyzed by western blotting for accumulation of dephosphorylated Cx43 (AB 13-800). (+) and (-) signs indicate the presence or absence of cal-A respectively. Under hypoxic conditions, decreased relative levels of dephosphorylated Cx43 are observed in the presence of cal-A.

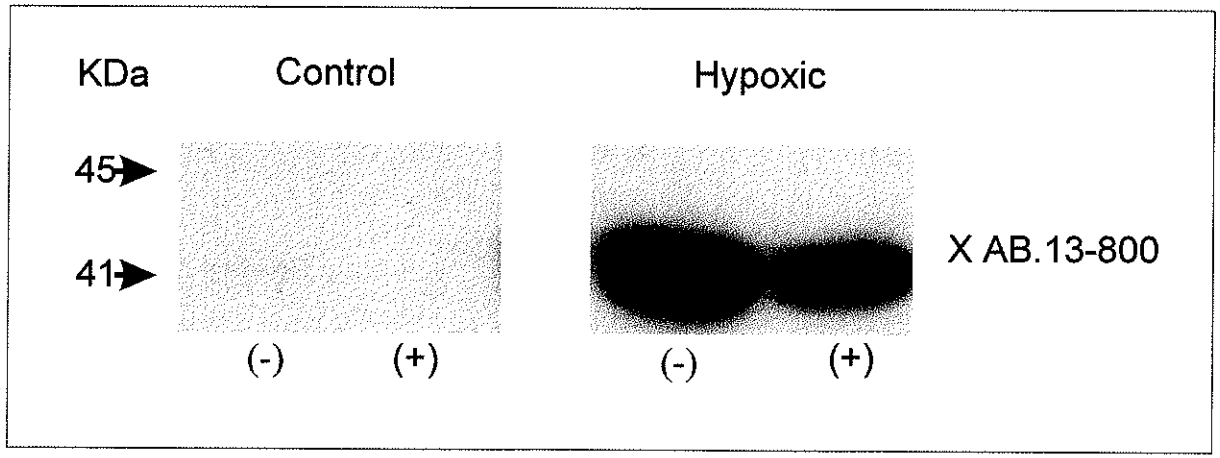


Figure 22

CHAPTER 4
DISCUSSION

A. RATIONALE

Ischemic heart disease is a major cause of mortality and morbidity in the world. In 1999 alone nearly 40,000 Canadians died of heart attack and related complications including malignant arrhythmias {www.heartandstroke.ca}. Since cardiomyocyte gap junction channels composed of Cx43 are important for the maintenance of proper cardiac function and rhythm {Saffitz and Yamada 2000} and changes at the level of GJ are implicated in arrhythmogenesis {Saffitz et al., 1999}, understanding and preventing ischemic changes at the level of Cx43 may provide a strategy to manage certain aspects of ischemia-induced cardiac function.

Cx43 is a phosphoprotein and ischemia induces its dephosphorylation in the heart {Beardslee et al., 2000; Huang et al., 1999}. Since phosphorylation has been demonstrated to affect coupling and/or membrane association in many systems, Cx43 dephosphorylation would be expected to impact on its properties in situ and affect cardiac function. Therefore, manipulations aimed at controlling Cx43 phosphorylation, or preventing its dephosphorylation, may also have therapeutic implications. One way to affect Cx43 dephosphorylation would be to identify the phosphatase(s) involved. This was the subject of our investigations.

These types of studies can be done using in vitro models, assuming they mimic the in vivo situation. In vitro models are highly advantageous since they allow tighter control of conditions as well as the testing of a large number of physiologically important molecules and 'drugs'.

B. OIL OVERLAY MODEL OF ISCHEMIA

In order to test our first hypothesis that isolated myocytes behaved in a manner similar to that of the intact hearts, we subjected isolated myocyte pellets to simulated ischemia by the mineral oil overlay technique, and then used Western blotting to analyze relative levels of Cx43 dephosphorylation, in comparison to equivalent studies conducted in the whole heart {Beardslee et al., 2000; Jeyaraman et al., 2003}. Our findings indicated that increasing periods of simulated ischemia induced Cx43 dephosphorylation to increase. Cx43 dephosphorylation seemed to occur within the same time window (15-60 min) as observed in intact hearts. Overall our data were consistent with the notion that cardiomyocytes in the in vitro model responded to simulated ischemia by Cx43 dephosphorylation in a manner qualitatively similar to that of intact hearts and that intact intercellular connections at the intercalated discs are not required for myocyte Cx43 dephosphorylation to occur. Our findings also allowed us to use this model for subsequent studies.

The in vitro method used here has been widely used and characterized {Armstrong and Ganote 1992; Vander Heide et al., 1990}. Vander Heide et al. provided evidence that isolated myocytes subjected to simulated ischemia share many aspects with their counterparts in intact hearts such as changes in morphology, decrease in ATP levels, increase in membrane fragility and response to hypothermia {Vander Heide et al., 1990}.

Our findings are not unique to the cardiac in vitro model. Li and Nagy have shown that cultured astrocytes subjected to hypoxia behave the same way as ischemic astrocytes in vivo with respect to Cx43 dephosphorylation {Li and Nagy 2000}.

It is important to note that although our results were qualitatively similar between experiments, there was variability between different myocytes preparations in the baseline levels of dephosphorylated Cx43. These differences likely reflect differences in cell quality, integrity and viability obtained after perfusion and collagenase digestion of the heart. In view of the sensitivity of Cx43 phosphorylation to ischemia, even minor changes in perfusion time, collagenase concentration and activity or handling of the isolated hearts would likely influence both cell viability and Cx43 phosphorylation. To address variability between preparations internal controls were included in every experiment, providing the baseline levels of Cx43 dephosphorylation before simulated ischemia.

C. CX43 DEPHOSPHORYLATION IS MEDIATED AT LEAST IN PART BY PP1-TYPE PHOSPHATASES

Different types of ser/thr phosphatases are present in the heart and may be involved in Cx43 dephosphorylation. We thus used different types of inhibitors to discern which type(s) of phosphatases mediate Cx43 dephosphorylation during ischemia.

To examine participation of PP1/2A phosphatases we used OA and calyculin A. More selective inhibitors within this category were also used such as fostriecin (PP2A selective inhibitor) and tautomycin (PP1 selective). These inhibitors were used at concentrations that would allow maximal prevention of dephosphorylation without exerting toxic effects, such as 1 μM for OA and calyculin A. These concentrations, shown previously to protect myocytes against ischemic injury {Armstrong and Ganote 1992}, would be expected to be effective against both PP1 as well as PP2A-type phosphatase(s). Fostriecin {Weinbrenner et al., 1998} was tested at concentrations previously shown to be effective in cardiac tissue {Armstrong et al., 1997; Weinbrenner et al., 1998}. Many PP1/2A inhibitors exist. Our criterion for selecting the inhibitors used in our studies is that they have been used successfully in various other studies to specifically inhibit PP1/2A in the heart {Armstrong and Ganote 1992; Armstrong et al., 1998; Armstrong et al., 1997}.

We found that while PP1/2A inhibitors OA and calyculin-A significantly reduced ischemia-induced Cx43 dephosphorylation, the PP2A

selective inhibitor fostriecin had no effect. Taken together our data pointed to PP1-type (but not PP2A) phosphatases as contributing to Cx43 dephosphorylation in ischemia. This was confirmed by the use of a specific PP1 inhibitor tautomycin. Previously, Duthe et al. reported that PP1 phosphatases are involved in the run-down of cardiomyocyte GJIC under conditions of ATP depletion {Duthe et al., 2001} implicating this group of phosphatases in the effects on Cx43 during ischemia. Our studies suggest that PP1-mediated Cx43 dephosphorylation may be responsible for the GJ effects reported by Duthe et al. {Duthe et al., 2001}.

In contrast to our findings in the cardiomyocyte model, Li and Nagy showed that PP1 phosphatases are not involved in astrocyte Cx43 dephosphorylation during hypoxia {Li and Nagy 2000}. The differences in the cell type may be responsible for this discrepancy. This raises possibility that different groups of phosphatases mediate Cx43 dephosphorylation in different cell types.

To evaluate the extent of Cx43 dephosphorylation we used SDS/PAGE, to separate the phosphorylated (43-46 kDa) from the dephosphorylated (41 kDa) Cx43, using two antibodies against Cx43. The polyclonal antibody recognizes both the phosphorylated and the dephosphorylated forms and served to assess total levels of Cx43 in all samples, while the monoclonal antibody recognizing only dephosphorylated Cx43 was used to evaluate differences in levels of dephosphorylated Cx43 between groups.

In the case of OA, studies on isolated hearts conducted in our laboratory revealed a similar response to the in vitro model, i.e., a decrease in Cx43 dephosphorylation during ischemia {Jeyaraman et al., 2003}. Prevention of dephosphorylation was observed at the level of intercalated discs between cells, where functional gap junction channels are located, and was associated with improved integrity of cardiomyocytes at the level of intercalated discs {Jeyaraman et al., 2003}.

None of the inhibitors used in these experiments was able to fully prevent ischemia-induced Cx43 dephosphorylation, although they were used within a concentration range expected to be effective. This suggested that additional phosphatase(s) may be involved in Cx43 dephosphorylation.

D. CX43 DEPHOSPHORYLATION IS MEDIATED, AT LEAST IN PART, BY PP2B-TYPE PHOSPHATASES

PP2B-type phosphatase, or calcineurin is a major phosphatase in the heart. We thus investigated the involvement of this type of phosphatase in Cx43 dephosphorylation, using the inhibitors CsA and FK-506. These inhibitors were used at concentrations previously shown to be cardioprotective, and effective in inhibiting PP2B {Weinbrenner et al., 1998}. Both inhibitors, having a different mechanism of action, were able to partially prevent Cx43 dephosphorylation during ischemia implicating PP2B in this process.

This is consistent with findings from other investigators reporting involvement of PP2B in the dephosphorylation of Cx43 in non-muscle cells {Cruciani et al., 1999; Li and Nagy 2000}. Both these inhibitors have also been shown to be cytoprotective by different studies both in vitro and in vivo in systems other than heart {Matsuda et al., 1998; Morioka et al., 1999}.

Although our findings imply that PP2B can have effects on Cx43 during ischemia, these may not affect GJIC (unlike the PP1 phosphatases). Duthe et al. have showed that PP2B phosphatases do not mediate the run down of GJIC during ATP depletion {Duthe et al., 2001}. Nevertheless, Chu and colleagues {Chu et al., 2002} have shown increased Cx43 dephosphorylation, in hearts overexpressing calcineurin, which is in agreement with our findings that PP2B plays a role in Cx43 dephosphorylation.

The PP2B inhibitors could not fully prevent Cx43 dephosphorylation, providing independent evidence that other types of phosphatases are also involved.

E. PREVENTION OF CX43 DEPHOSPHORYLATION BY THE COMBINED USE OF PP1 AND PP2B PHOSPHATASES

Having established that PP1 and PP2B are involved in ischemia-induced Cx43 dephosphorylation we proceeded to investigate if the combination of the PP1 and PP2B inhibitors will be able to completely

prevent Cx43 dephosphorylation. We used calyculin-A since it was generally well tolerated at the concentrations used, and FK-506, since it seems to have fewer non-specific effects compared to cyclosporine A. We found that indeed, combined use of calyculin-A and FK-506 (at the same concentrations as when used individually) achieved near complete prevention of Cx43 dephosphorylation (94%).

The question arises as to what is the significance of having two different types of phosphatases involved in Cx43 dephosphorylation. We speculate that since Cx43 is phosphorylated at multiple sites, and since its degree of dephosphorylation depends on ischemia duration, perhaps different types of phosphatases become activated during the time course of ischemia and possibly 'attack' different phosphorylation sites. It is likely that the drop in ATP occurring immediately upon ischemia {Duthe et al., 2001} would activate the PP1 phosphatases, as suggested by Duthe et al. During the initial seconds of ischemia when there is decreased supply of oxygen there is a shift from aerobic to anaerobic glycolysis {Opie 1978}. The resultant ATP depletion and accumulation of metabolites causes a decrease in pH followed by inhibition of the enzymes phosphofructokinase and glyceraldehyde-3-phosphate dehydrogenase, which leads to further decrease in ATP production {Das and Maulik 1996; Opie 1978}. Intracellular Na^+ increases to compensate for the decreased pH, but the Na^+-Ca^{2+}exchanger gets activated leading to intracellular accumulation of Ca^{2+} {Allen et al., 1993}. The accumulation of Ca^{2+} that occurs at later stages of

ischemia and would be expected to activate the PP2B phosphatase group. According to this scenario, Cx43 would be a substrate for PP1 during early ischemia, and for both PP1 and PP2B later on.

Cx43 has many phosphorylation sites, but the functional significance of phosphorylation at each site is not known as yet. Functional properties of Cx43 existing at different levels of phosphorylation are also far from understood. Another unknown aspect is whether the PP1 and PP2B phosphatases may have a preference for particular phosphorylation sites on Cx43. Another possibility is that ongoing changes in the microenvironment of Cx43 during ischemia might result in greater exposure of different phosphorylation sites. All these issues need further investigation.

The degree of inhibition of Cx43 dephosphorylation that we achieved with calyculin-A showed considerable variability. A 28% inhibition of dephosphorylation was obtained in the first series of experiments, while inhibition reached 80% in later experiments. This discrepancy may have been caused due to the fact that the first experiments were done initially when the quality of the cells obtained was not very good due to inexperience. In addition the variability in cardiomyocyte responses between different preparations and also the differences in calyculin-A activity may also have been the contributing factors. This difference raises the possibility however that under appropriate conditions PP1 inhibition may

be sufficient to fully prevent Cx43 dephosphorylation during ischemia, at least in the early stages.

F. IS THERE A LINK BETWEEN PREVENTION OF CX43 DEPHOSPHORYLATION AND PROTECTION FROM ISCHEMIC DAMAGE?

Even though the phosphatase groups involved in Cx43 dephosphorylation were identified, it is important to know if reduction in the dephosphorylation contributes to reduction in cell injury. Unfortunately, we were unable to answer this question conclusively. In the oil overlay model, myocyte viability assessed at various time points of ischemia, in the presence or absence of phosphatase inhibitors displayed extensive variability, precluding statistical significance. We also used another in vitro model consisting of confluent cultures of neonatal cardiomyocytes. These cells are well connected with each other and contract in a synchronous manner, indicative of a developed GJ network. They were incubated in the presence of 'ischemic medium' and a hypoxia-chamber for several hours. Unfortunately use of PP1 inhibitors such as OA and calyculin-A at the concentrations used for the oil overlay method elicited significant toxicity in this model.

Nevertheless, we tested calyculin-A, at very low concentrations, for its effects both on Cx43 dephosphorylation and cell viability. While even at

10 nM, calyculin-A had some residual toxicity (causing slightly elevated LDH release at 2 hrs of incubation) it did nevertheless result in reduced overall cellular injury at 4 hrs of incubation, when the effects of ischemia become detectable. Reduced cell injury at this time point correlated with reduced Cx43 dephosphorylation by calyculin-A, suggesting the possibility of a cause and effect relationship. Some correlative evidence for a beneficial effect of prevention of Cx43 dephosphorylation during ischemia was obtained in the isolated heart model {Jeyaraman et al., 2003}. In this model, OA treatment of ischemic hearts was associated with improved structural integrity of cardiomyocytes at the level of the intercalated disc. This would be in agreement with reports that okadaic acid (and calyculin A) prevents development of fragility of metabolically inhibited and/or ischemic cardiomyocytes {Armstrong and Ganote 1992}. The degree to which prevention of Cx43 dephosphorylation per se contributes to the okadaic acid-induced preservation of structural integrity of the ischemic cardiomyocytes remains to be established.

The functional consequences of Cx43 dephosphorylation during ischemia are a matter of some controversy. It is thus not clear whether cardiac Cx43 dephosphorylation would result in increased or decreased intercellular coupling. Channels composed of dephosphorylated Cx43, in oocytes {Moreno et al., 1994} or artificial membrane layers, have increased conductance properties {Kim et al., 1999}. A recent report indicated that gap junction permeable fluorescent dyes can still migrate between ischemic

myocytes of the ex vivo heart; gap junction channels furthermore were shown to be capable of allowing the spread of contracture that occurs after 45 min of ischemia {Ruiz-Meana et al., 2001}. These conditions are accompanied by substantial dephosphorylation of Cx43 {Beardslee et al., 2000}, and thus would imply that cardiac gap channels composed of dephosphorylated Cx43 are permeable to ions and small metabolites, and are thus are capable of conduction. This would also be in agreement with findings from ischemic astrocytes {Lin et al., 1998}. On the other hand, another group reported that Cx43 dephosphorylation seemed to coincide with loss of electrical coupling {Beardslee et al., 2000} and development of arrhythmias. Irreversible cardiomyocyte injury, brought about by calcium overload during ischemia would be expected to result in closure as well as loss of gap junctions {Beardslee et al., 2000}. ATP depletion (an event that would rapidly occur in ischemia, and thus be associated with Cx43 dephosphorylation) has been shown to decrease gap junctional conductance between myocyte pairs {Duthe et al., 2001}. In these studies, PP1 inhibition (a manipulation that would prevent Cx43 dephosphorylation) prevented the run down in gap junctional communication, again providing a link between Cx43 dephosphorylation and decreased conductance {Duthe et al., 2001}.

The reason for these apparent discrepancies is not yet known. It should be noted that Cx43 is phosphorylated at multiple sites and can have different levels/states of phosphorylation. As shown in Figure 9, early

ischemia is associated with conversion of Cx43 to less phosphorylated species, but not complete dephosphorylation; complete dephosphorylation is observed at later time points (30-60 min ischemia; data not shown). The different phosphorylation states of Cx43 may have different properties. In addition, conductance and metabolic coupling may be regulated in a different manner {Kwak et al., 1995}

G. CONCLUSIONS

We have shown that the isolated myocyte oil overlay model responds to simulated ischemia in a manner similar to whole hearts. Using this model and specific inhibitors, we also showed that PP1 and PP2B type phosphatases are involved in Cx43 dephosphorylation during ischemia. Finally we provide evidence for a correlation between prevention of Cx43 dephosphorylation and protection from ischemic injury of the cardiomyocyte. We suggest that manipulations that target the phosphorylation state of Cx43, by affecting the phosphatase(s) and kinase(s) involved deserve further consideration as potential cardioprotective treatments.

H. FUTURE DIRECTIONS

Our findings need to be confirmed by vivo studies. Limitations relating to inhibitor specificity and toxicity need to be addressed. Future studies could be directed to address the following aspects.

a. Since the PP1, PP2B phosphatases exist in different isoforms and are regulated regionally in the heart, specific targeting of these isoforms would be required. Genetic manipulations may allow selective inactivation or overactivation of specific phosphatases to examine their action.

b. Identification of the phosphorylated amino acids on Cx43 and of the sites that become dephosphorylated during progressing ischemia would be important steps in order to understand their function in the context of cardiac response to injury.

CHAPTER 5

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