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Connexin 43 Hemichannels and Pharmacotherapy of Myocardial Ischemia Injury

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1. Introduction

Connexin (Cx) is the basic unit in the composition of gap junction channels but also exist in non-junctional unapposed hemichannels (Hc). The gap junction channels are formed by apposition of two hexameric CxHc from adjacent cells and play an essential role in cardiac function by allowing conduction of electrical impulse and exchange of biologically important molecules between myocytes. While most CxHc are engaged in gap junction formation, some unapposed Hc are present in association with various organelles (mitochondria, ER, etc.) but also in sarcolemma where they connect the intracellular and extracellular spaces. Recent evidence indicates that unapposed Hc in the plasma membrane perform functions different from those achieved by gap junction channels, mainly by providing pathways between the cytosol and the extracellular space allowing movement of ions and other small metabolites (Bennett et al., 2003; Goodenough & Paul, 2003), release of ATP and NAD^+ (Bruzzone et al., 2001; Cotrina et al., 1998), regulation of cell volume (Quist et al., 2000), and the activation of survival pathways (Plotkin et al., 2002). These Hc are therefore believed to play a prominent role in cellular ion homoeostasis and signalling.

In normal myocardium, most of the Cx43 are phosphorylated (van Veen et al., 2001). However, under ischemic stress, the amount of non-phosphorylated Cx43 increases (Beardslee et al., 2000). Phosphorylation causes the unapposed Cx43Hc to close whereas, conversely, Cx43 dephosphorylation increases Hc conductance and permeability (Bao et al., 2007; Contreras et al., 2002; John et al., 1999b; Kondo et al., 2000; Li et al., 2001), an effect that can result in abnormal elevation of intracellular sodium and calcium loads (Li et al., 2001), release of ATP (Braet et al., 2003a; Braet et al., 2003b; Contreras et al., 2002), osmotic imbalance (John et al., 1999b; Li et al., 2001; Quist et al., 2000), swelling of myocytes (Jennings et al., 1986; Steenbergen et al., 1985; Tranum-Jensen et al., 1981) and lead to irreversible tissue injury (Shintani-Ishida et al., 2007) and cell death. Several kinases are involved in Cx43 phosphorylation (Cottrell et al., 2003; Duncan & Fletcher, 2002; Shi et al., 2001; TenBroek et al., 2001; Warn-Cramer et al., 1998), including protein kinase C (PKC) (Lampe et al., 2000). In humans and many other mammalian species, PKC is the protein kinase for which Cx43 contains the largest number of phosphorylation sites. For instance, rat Cx43 contains 14 putative sites for PKC whereas only 3 have been reported for PKA, 4 for PKG, and 3 for MAPK (van Veen et al., 2001). A major physiological importance of the PKC resides in the existence of multiple PKC isoforms that can selectively affect particular targets in different conditions. The apparent preferential activation in response to different conditions and stimuli suggests that

various PKC isoforms have specific cellular and cardiovascular functions. For instance, data from several laboratories (Chen et al., 2001a; Dorn et al., 1999; Gray et al., 1997; Inagaki et al., 2003; Inagaki et al., 2005; Liu et al., 1999; Tanaka et al., 2004; Tanaka et al., 2005) shows that selective activation of εPKC isoform provides protection against ischemia damage, whereas activation of δPKC aggravates the injury. Indeed, PKC-selective modulator drugs are currently under development for the treatment of a variety of diseases involving PKC isoforms (Bar-Am et al., 2007; Casellini et al., 2007; Serova et al., 2006).

Until recently, unapposed CxHc were thought to remain closed and that their opening would induce metabolic stress and cell death. In fact, in the first study of Hc opening, which was conducted in Xenopus oocytes expressing Cx46, non-selective inward current, swelling, and cell death were observed (Paul et al., 1991). Subsequently, the opening of CxHc and consequent cell death following an ischemic insult were reported in different cell models including myocytes (Kondo et al., 2000; Li et al., 2001; Shintani-Ishida et al., 2007), astrocytes (Contreras et al., 2002; John et al., 1999a; John et al., 1999b), and renal tubule cells (Vergara et al., 2003). The involvement of Cx43Hc was also reported in cell lines, such HEK293 (John et al., 1999b) and HeLa (Contreras et al., 2002) cells, transfected with Cx43 that became more susceptible to death by simulated ischemia than non-transfected cells, a difference ascribable to Cx43Hc opening. Therefore, the delineation of PKC targets in the heart and the characterization of the functional role of the various PKC isoforms in the modulation of these targets will offer a mechanistic insight into the pathogenesis of ischemia/reperfusion injury and aid in the development of novel pharmacological interventions for cardioprotection.

The assessment of the functional role of PKC isoforms in the modulation of Cx43Hc and the role of Cx43Hc in cardiac protection against ischemia/reperfusion injury have largely been limited by the fact that selective modulators for PKC isoforms and selective inhibitors for Cx43Hc have not been available until recently. Therefore, two major objectives of the present study aimed respectively: 1) to determine the functional role of PKC isoforms - especially those involved in heart protection - in the regulation of Cx43Hc; and, 2) to reveal the importance of the inhibition of Cx43Hc in protection against ischemia/reperfusion injury. To this end, two unique sets of synthetic peptides were utilized in the course of the research: 1) a matrix of PKC isoform-selective activator and inhibitor peptides. In combination with the patch clamp technique, the peptides were individually delivered into the cytosol of the ion channels-deficient tsA201 cells exogenously expressing Cx43 to activate/inhibit each of the PKC isoforms and assess their specific roles in the modulation of Cx43Hc. 2) a pair of structural Cx43-mimetic peptides, Gap26 and Gap27. These peptides are presumed to have the ability to block Cx43Hc by binding to their extracellular loops and to have little or no immediate effects on gap junction channels (Evans et al., 2006; Leybaert et al., 2003; Martin et al., 2005; Pearson et al., 2005). The consequent effects of Cx43Hc inhibition following the administration of structural Cx43-mimetic peptides and their therapeutic potentials were assessed in isolated cardiomyocytes and in intact rat hearts under ischemic settings.

The use of Cx43-mimetic peptides to study unapposed CxHc encloses several benefits compared to other currently used techniques and represents an attractive pharmacological tool for *in vivo* studies. The specificity that Cx43-mimetic peptides have vis-à-vis Cx43Hc, and to a lesser extent gap junction channels, denotes a therapeutic advantage over PKC peptides and other compounds (e.g. heptanol) that are also involved in cardioprotection but concurrently affect a variety of other intracellular substrates. Furthermore, the accessibility to the binding sites on the extracellular loops of Cx43Hc eliminates the need to conjugate Cx peptides with cell penetrating carriers which may exert toxic effect on cells and delay the

delivery process (Zorko & Langel, 2005). Finally, the use of transgenic or knockout animal models has been widely utilized to study the roles of multiple cell proteins and ion channels; however, using such techniques to identify the role of specific Cx during ischemia/reperfusion is challenged by the fact that sustained loss of Cx43 severely affects gap junctional communication and alters cardiac development. Therefore, we believe that if our hypothesis is confirmed, these peptides will represent a promising pharmacological tool with which to counteract cell death in the cardiac ischemia/reperfusion pathology.

2. Knowledge to date

2.1 Structure and expression of connexins in the heart

There is at least twenty-one known types of Cx identified in human (Goodenough & Paul, 2003; Saez et al., 2003; Willecke et al., 2002) usually named according to their molecular weight (Beyer et al., 1987). Each Cx is composed of four transmembrane segments, two extracellular and one intracellular loop as well as one amino-terminal and one carboxy-terminal regions located in the cytosol (Fig. 1). Connexins differ in the length of their cytosolic carboxy terminus, which is characterized by the presence of several phosphorylation sites. A connexin-formed Hc consists of an oligomeric assembly of six connexins that delineate an aqueous pore. Several connexins are expressed in cardiac myocytes in different amounts and combinations depending on regions of the heart (Zipes & Jalife , 2004). The ventricular muscle fibres express abundant amounts of Cx43 but only trace levels of Cx45 and no detectable Cx40. Most studies of connexins in the heart have focused on their role in gap junctions, but a few studies have also characterized Cx43Hc properties in isolated cardiac myocytes (John et al., 1999b; Kondo et al., 2000).

2.2 Unapposed Cx43Hc opening in ischemia/reperfusion injury

The conductance and permeability of Hc, as well as gap junction channels, are regulated by intracellular protons changes (Ek-Vitorin et al., 1996; Morley et al., 1997; Spray & Burt, 1990) and calcium concentrations (Spray et al., 1985; Spray & Burt, 1990) but also via phosphorylation of specific serine, threonine and tyrosine residues by several kinases (Lampe & Lau, 2000) especially PKC (Bowling et al., 2001; Doble et al., 2000; Lampe et al., 2000). As indicated above, the conductance and permeability of the unapposed Cx43Hc are increased once Cx43 becomes dephosphorylated (Burt & Spray, 1988; Lau et al., 1991; Saez et al., 1986), while being reduced with increased Cx43 phosphorylation (Kwak & Jongsma, 1996). The dephosphorylation of Cx43 has been proposed as a key mechanism to open Hc during metabolic inhibition in cortical astrocytes in culture (Fig. 2) (Contreras et al., 2002). While Cx43 is phosphorylated under physiological conditions (Schulz et al., 2003; van Veen et al., 2001) and remains so in the first few minutes of ischemia (Schulz et al., 2003), subsequent dephosphorylation of Cx43 occurs with increasing duration of myocardial ischemia (Beardslee et al., 2000; Jain et al., 2003; Jeyaraman et al., 2003; Miura et al., 2004; Schulz et al., 2003) a process that has been associated with the opening of unapposed Cx43Hc (John et al., 1999b; Li et al., 2001) which results in metabolic stress and cell death. Based on single Hc conductance measurements, it was suggested that opening of only 50 Hc is sufficient to drown the cell with Na^+ (John et al., 1999b). As a result, Na^+ overload induces the activation of reverse Na^+-Ca^{++} exchange and hence promotes intracellular Ca^{++} accumulation (Barry & Bridge, 1993; Silverman & Stern, 1994), irreversible cell injury and arrhythmogenic transient inward currents (Tani & Neely, 1989). Altogether, these data

endorse the theory of the involvement of the unapposed Hc in ischemic-induced injury and support our hypothesis that inhibition of Hc during ischemia is an important determinant for cardioprotection against ischemia/reperfusion injury.

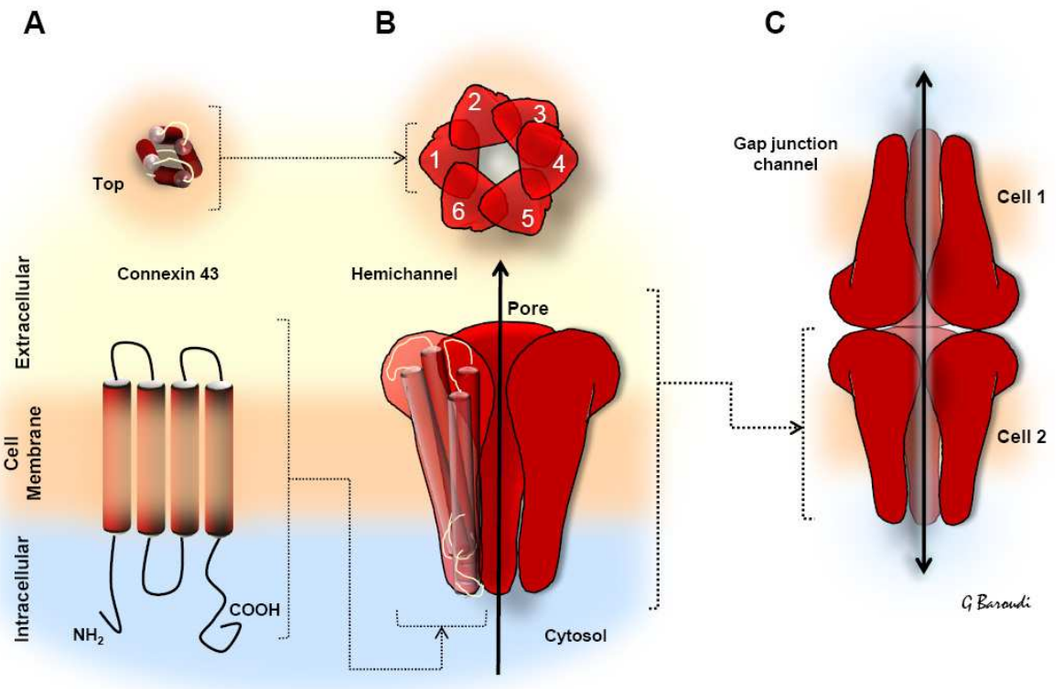

Fig. 1. Connexin 43 structures. A, secondary structure of Cx43 with insert showing a tridimensional view from the top; B, a single Hc is formed by the association of six connexins to form central permeable pore; C, a gap junction channel is formed when two Hc from adjacent cells appose and form a pathway between the cytosols of the neighbouring cells.

Ischemic preconditioning. The phosphorylation status of total Cx43 is affected by brief episodes of ischemia which reduce the adverse effects of subsequent myocardial ischemia (Cohen et al., 1991; Murry et al., 1986; Shiki & Hearse, 1987). In fact, ischemic preconditioned hearts from pigs (Schulz et al., 2003), rabbits (Miura et al., 2004), and rats (Jain et al., 2003) show preserved total Cx43 phosphorylation levels following prolonged ischemia. PKC is generally acknowledged as a key mediator of ischemic preconditioning (Schulz et al., 2003). One of the aims of this study is to establish that specific PKC isoforms are involved in the cascade of events leading to the preservation of the Cx43 phosphorylated state and cardioprotection. While cardioprotection is generally thought to involve phosphorylation of Cx43 gap-junction channels (Chen et al., 2005; Garcia-Dorado et al., 1997; Garcia-Dorado et al., 2004; Li et al., 2002; Rodriguez-Sinovas et al., 2006; Schwanke et al., 2002; Schwanke et al., 2003), experiments on isolated myocytes provided evidence that efficient ischemic preconditioning does not require the existence of gap junction channels (Li et al., 2004). Accordingly, we further hypothesize that unapposed Cx43 Hc (through their opening) are involved in ischemia-induced cell damage.

Other potential end-effectors of ischemic damage. Although this chapter focuses on the role of PKC isoform-dependent Cx43 HC phosphorylation in cardioprotection, one should

be aware that several other mechanisms have been implicated in ischemia/reperfusion injury and cardioprotection. These include, but are not limited to, Na/H exchanger, mitochondrial permeability transition pore (MPTP) and K_{ATP} channels. While inhibition of Na/H exchanger has been shown to be beneficial for protection against ischemia (Xiao & Allen, 2000), PKC activation during ischemic preconditioning induces enhancement rather than inhibition of the Na/H exchanger (Kandasamy et al., 1995). The involvement of Na/H exchanger in heart conditioning is therefore uncertain (Avkiran, 1999). Another potential end-effector in ischemia-related protection is the MPTP (Hausenloy et al., 2002). While it has been proposed that MPTP inhibition in ischemia/reperfusion underlies the protection against injury in isolated rat hearts (Hausenloy et al., 2002), only a single study, thus far, has provided direct evidence for attenuated MPTP opening in preconditioned heart (Javadov et al., 2003). A significant number of studies support the involvement of the mitochondrial K_{ATP} channels in cardiac protection against ischemia (Gross & Fryer, 1999; Liu & O'Rourke, 2001; Sato & Marban, 2000). It has been suggested that opening of mitochondrial K_{ATP}

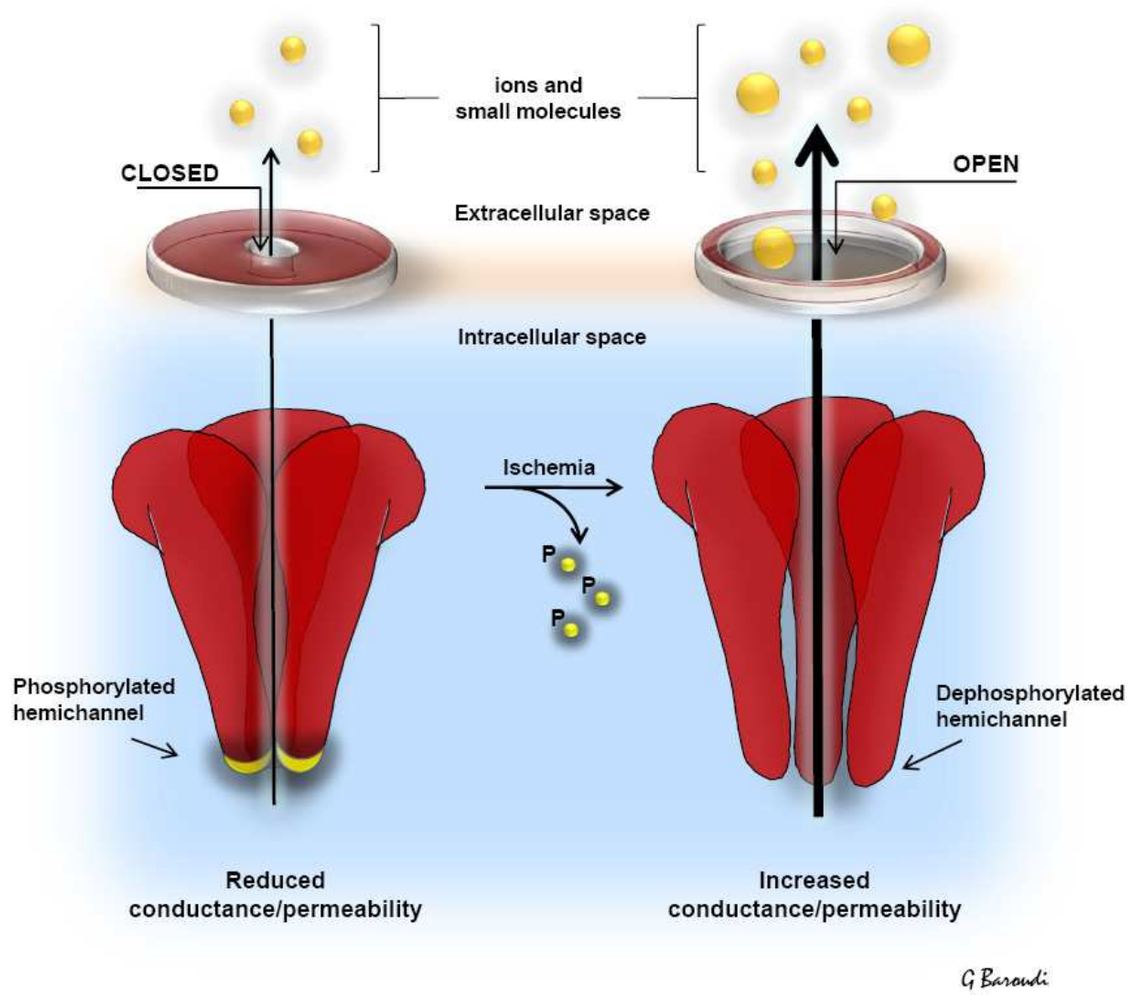

Fig. 2. Hemichannels activation. During ischemic stress, the decrease in PKC activity leads to increase in the dephosphorylated forms of Cx43, this causes the activation of Cx43Hc.

channels causes mitochondrial depolarisation, resulting in attenuated Ca overload during ischemia (Weiss et al., 2003). However, the protective effect of this reduction has never been experimentally demonstrated. Nevertheless, it should be noted here, that K_{ATP} channels are also modulated by PKC (Wang & Ashraf, 1999). Indeed, other end-effectors were also proposed to be directly involved in protection against ischemia/reperfusion injury. These include cGMP/PKG, calpains, cytoskeleton, and the tumor necrosis factor; the definitive evidence for their role on cardioprotection remains however obscure.

2.3 Function and regulation of CxHc

Currents from Hc have been successfully characterized in various native cell types (Hofer & Dermietzel, 1998; Kondo et al., 2000; Stout et al., 2002; Vergara et al., 2003) as well as in Xenopus oocytes (Ebihara & Steiner, 1993; Gomez-Hernandez et al., 2003; Gupta et al., 1994; Pfahnl et al., 1997; Pfahnl & Dahl, 1999; White et al., 1999; Zoidl et al., 2004) and in mammalian expression systems (Bukauskas et al., 2006; Contreras et al., 2003; Valiunas, 2002). Basically, they behave like large conductance ion channels that share properties with gap junction channels in terms of permeability and gating (DeVries & Schwartz, 1992; Li et al., 1996; Trexler et al., 1996). Nevertheless, opening of these channels is significantly facilitated by exposure to calcium-free extracellular solutions (DeVries & Schwartz, 1992; Li et al., 1996), membrane depolarization (Hofer & Dermietzel, 1998), or metabolic inhibition (Contreras et al., 2002; John et al., 1999b). Cx43Hc have been implicated in diverse functions including volume regulation (Quist et al., 2000), efflux of NAD^+ and ATP (Bruzzone et al., 2001; Cotrina et al., 1998; Stout et al., 2002), influx of Na^+ and Ca^{2+} ions (Contreras et al., 2002; John et al., 1999b; Kondo et al., 2000) and activation of survival pathways (Plotkin et al., 2002).

Under resting conditions, Hc are predominantly in a closed state but their gating can be regulated by several factors, including by phosphorylation of Cx43 (Saez et al., 2005). Several functional states of single Cx43Hc have been described; closed Hc, fully open Hc, and an intermediate state between the fully open and the closed states (Contreras et al., 2003). On western blot, Cx43 exhibits three bands reflecting the presence of three functional states of Cx43: a highly phosphorylated, a partially phosphorylated and a non-phosphorylated state (Hertzberg et al., 2000; Musil et al., 1990; Saez et al., 1997; VanSlyke & Musil, 2000). Although Cx43 is a target protein for several kinases (Lampe & Lau, 2000), we are particularly interested in modulation by individual PKC isoforms. Because activation of εPKC and/or inhibition of δPKC prior to, during or even following an ischemic insult was shown to confer cardioprotection against injury (Chen et al., 2001a; Dorn et al., 1999; Gray et al., 1997; Inagaki et al., 2003; Inagaki et al., 2005; Liu et al., 1999; Tanaka et al., 2004; Tanaka et al., 2005); we believe that this protective effect is due, at least in part, to the inhibitory effect that εPKC exerts on Cx43Hc (Bao et al., 2004a; Bao et al., 2004b). Therefore, we investigated the functional role of the εPKC, as well as the other cardiac PKC isoforms, in the modulation of Cx43Hc.

2.4 PKC Isoform-selective activator and inhibitor peptides

PKC consists of a family of at least twelve closely related isoforms (McDonald et al., 1994; Nishizuka, 1988; Osada et al., 1992) which regulate biochemical processes by phosphorylation of cellular proteins on serine or threonine residues. Molecular cloning has revealed that PKC family can be divided into three major groups (Fig. 3). The conventional PKCs or cPKC consist

of α, βI, βII and γ and have activity strongly dependent on calcium (Ca), phospholipid, and diacylglycerol (DG) (Nishizuka, 1988). The second group (novel PKC or nPKC) comprises the ε, δ, η and θ isoforms and exhibits Ca-independent activity. And finally, members of the atypical PKC group or aPKC, ζ and λ/ι are insensitive to DG, phorbol esters and Ca. There is also a μPKC that does not fit into any of the above groups and is sensitive to phospholipids and phorbol esters but insensitive to Ca. Remarkable progress has been made in understanding how extracellular stimuli act as upstream triggers to induce activation of particular PKC isoforms in cardiomyocytes (endothelin, phenylephrine, etc.) (Clerk et al., 1994; Clerk & Sugden, 2001; Rybin et al., 1999). Moreover, there is much evidence supporting the concept that PKC functions in an isoform-dependent manner and that specific cardiac manifestations depend on the activation of individual isoforms, not the activity of the entire PKC family (Chen et al., 2001a; Clerk & Sugden, 2001; Hahn et al., 2002; Mochly-Rosen & Gordon, 1998). Indeed, the successful development of isoform-specific modulator peptides and their use in combination with patch clamp technology (Hu et al., 2000; Xiao et al., 2001; Xiao et al., 2003) have empowered the investigation of isoform-selective roles of PKC family in ion channels modulation. Briefly, activator peptides by binding to one particular PKC isoform cause a conformational change of the enzyme structure which exposes its catalytic site, and enables its anchoring to the target molecule. On the opposite side, inhibitor peptides mimic structurally the PKC isoform binding site on the target protein or the target protein binding site on PKC and therefore inhibit the function of that particular PKC isoform. The specificity and sequences of the activator and inhibitor peptides of PKC isoforms are listed in Table 1. These peptides can be obtained through commercial custom peptide synthesis.

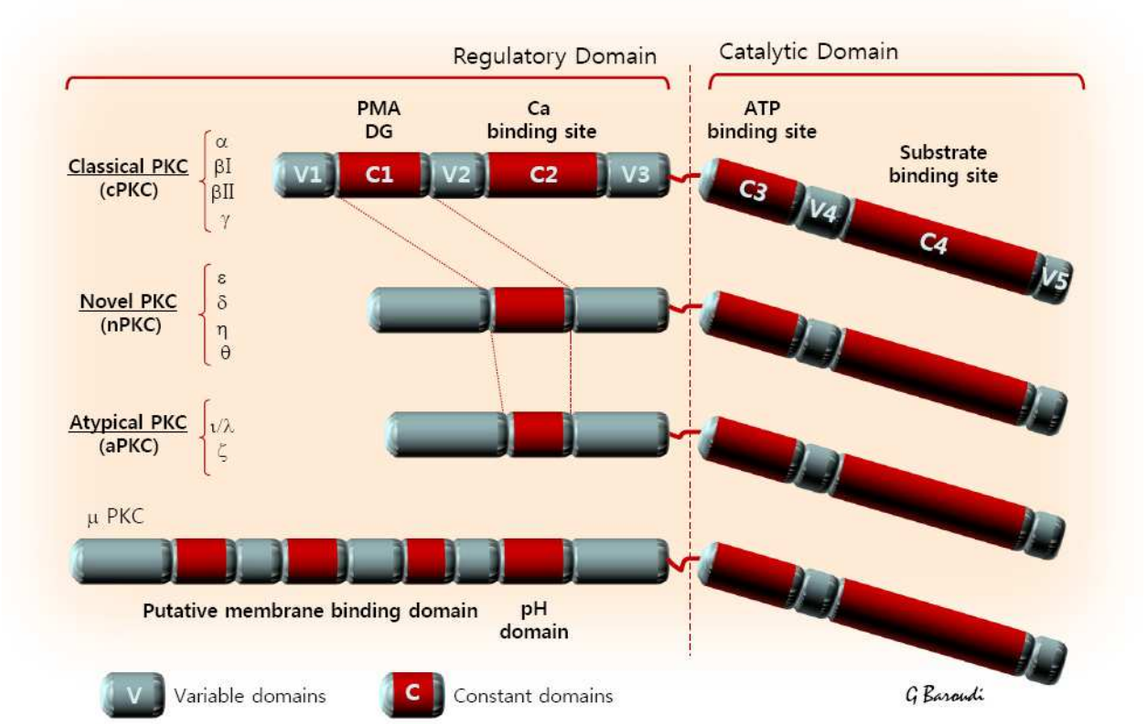

Fig. 3. Protein kinase C superfamily, classes of various PKC isoforms with corresponding structures.

Peptide	Specificity	Sequence
αC2-4	αPKC inhibitor	S-L-N-P-Q-W-N-E-T
βIV5-3	βIPKC inhibitor	K-L-F-I-M-N
βIIV5-3	βIIPKC inhibitor	Q-E-V-I-R-N
βC2-2	βPKC activator	S-V-E-I-W
δV1-1	δPKC inhibitor	S-F-N-S-Y-E-L-G-S-L
εV1-2	εPKC inhibitor	E-A-V-S-L-K-P-T
εV1-7	εPKC activator	H-D-A-P-I-G-Y-D
ηV1-2	ηPKC inhibitor	E-A-V-G-L-Q-P-T
Pentalysine	control	K-K-K-K-K

Table 1. PKC modulator peptides. List of the available PKC isoform-specific modulator peptides with their sequences and specificity.

2.5 Previous findings and implication for the current study

Previous data from several laboratories show that selective translocation/activation of the εPKC isoform provides protection against ischemic injury during ischemic/hypoxic preconditioning (Chan et al., 1995; Chou & Messing, 2005; Dorn et al., 1999; Gray et al., 1997; Liu et al., 1999; Ytrehus et al., 1994) and post-conditioning (Penna et al., 2006b; Philipp et al., 2006; Zatta et al., 2006) in different experimental paradigms. On the basis of the beneficial effects of εPKC translocation/activation and the fact that it can form a complex with Cx43 and phosphorylate the protein (Ping et al., 2001), we put forth that this particular isoform is critically involved in protection against ischemic injury. Therefore, the elucidation of the functional protective role of εPKC may allow for the identification of pharmacological agents that mimic the PKC isoform action on one particular target, i.e. Cx43Hc, without affecting other key molecules in cellular function. Certainly, other isozymes have been demonstrated to phosphorylate Cx43, notably αPKC (Bowling et al., 2001), but the functional implications in Cx43Hc regulation has not been assessed.

2.6 Structural Cx-mimetic peptides as inhibitors of CxHc function

Another experimental strategy using a different set of peptides has been utilized in the course of this study: the structural mimetics of Cx43. The conventional gap junction blockers (as heptanol) that are available to date act on both whole gap junction channels and unapposed Hc. Interestingly, Cx-mimetic peptides have recently emerged as powerful tools capable of blocking primarily the unapposed Cx43Hc by mimicking short amino acids sequences on the extracellular loops of Cx (references (Braet et al., 2003a; Evans et al., 2006; Leybaert et al., 2003; Martin et al., 2005; Pearson et al., 2005) testing ATP release from various non-cardiac cell types). Here, we investigated the consequent effect of two Cx43-mimetic peptides, Gap26 and Gap 27, on the electrophysiology of Cx43Hc exogenously expressed in the tsA201 cells using the patch clamp technique. In addition, we have designed multiple experimental protocols to assess the therapeutic potentials of these peptides against ischemia/perfusion injury in various settings including isolated cell model and intact hearts.

Initially, the Cx-mimetic peptides were designed with the intention of selectively blocking gap junction channels by intercepting the apposition of pairs of Hc from adjacent cells (Dahl et al., 1994; Warner et al., 1995), but have subsequently emerged as potent blockers for the

unapposed CxHc with little or no immediate effect on gap junction (Braet et al., 2003a; Evans et al., 2006; Leybaert et al., 2003; Martin et al., 2005; Pearson et al., 2005).

Particularly, Gap26 and Gap27 peptides have recently emerged as powerful tools that block unapposed Cx43Hc (Braet et al., 2003a; Evans et al., 2006; Leybaert et al., 2003; Martin et al., 2005; Pearson et al., 2005) by mimicking short amino acids sequences on the first (VCYDKSFPISHVR) and second (SRPTEKTIFII) extracellular loops of Cx43, respectively (Fig. 4). Both sequences contain conserved motifs that are involved in connexin-connexin interaction in gap junction channels (Dahl et al., 1992; Warner et al., 1995). These motifs are not consistently found in other cell surface proteins suggesting that Gap26 and Gap 27 specifically interact with Cx43 without interfering with other Cx or surface proteins (Braet et al., 2003a; Isakson et al., 2006; Warner et al., 1995). In fact, the selective affinity of Gap26 and Gap27 toward Cx43Hc was first reported in 2003 by Braet and collaborators (Braet et al., 2003a; Braet et al., 2003b). In their studies, Gap26 and Gap27 were shown to selectively block the uptake of a reporter dye and the release of ATP in brain endothelial cells and Cx43-expressing HeLa cells without reducing gap junctional coupling. Subsequently, Cx-mimetic peptides were shown to consistently suppress the bi-directional permeability, i.e. dye uptake and ATP release, of the unapposed CxHc without affecting gap junctional coupling in several cell types including brain endothelium, retinal epithelium, and bladder cancer

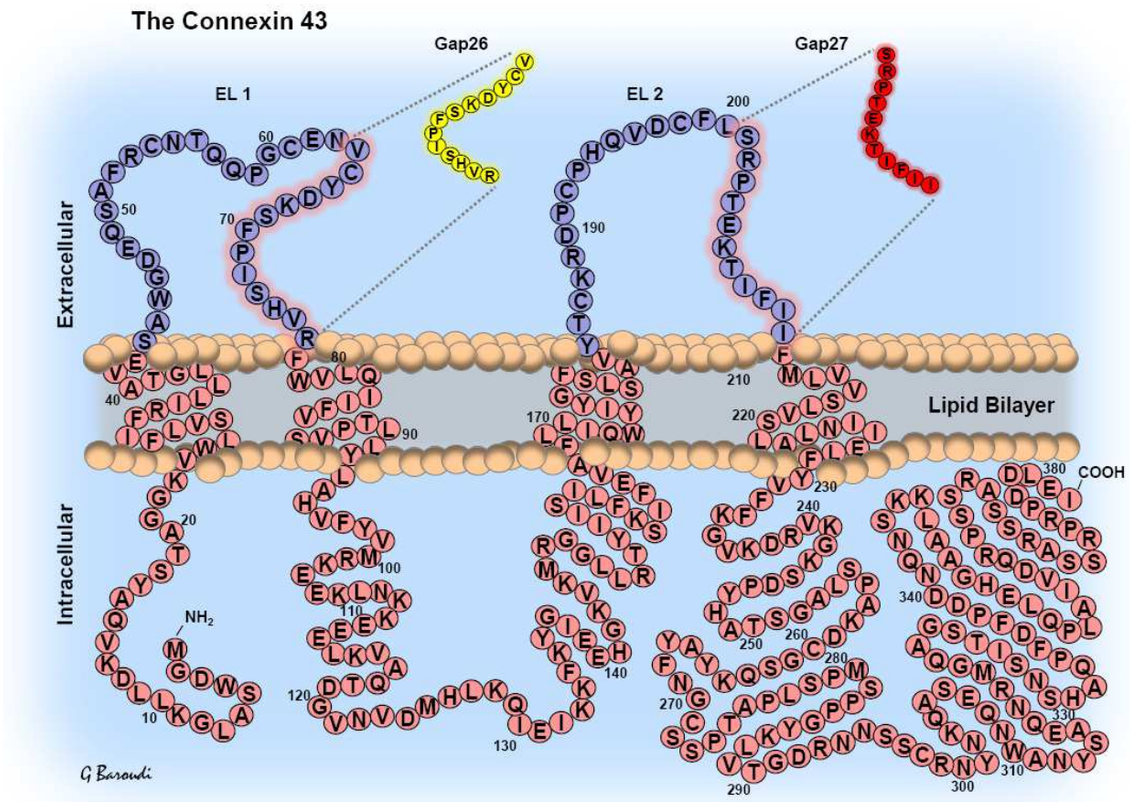

Fig. 4. Connexin 43 and its structural-mimetic peptides. Gap26 and Gap27 mimic the structure of first (EL 1) and second (EL 2) extracellular loops of Cx43, respectively.

epithelium cells, as well as transfected HEK293, HeLa, C6 glioma and ECV304 cell lines (De Vuyst et al., 2006; Leybaert et al., 2003; Pearson et al., 2005). Although, the mechanism that underlies the affinity of Cx-mimetic peptides toward Hc is currently not known, a possible explanation could be the accessibility of the extracellular loops of unapposed Hc compared to those of gap junction channels which are engaged in the Hc-Hc apposition and are therefore less accessible for interactions with exogenous peptides. On the other hand, it is still not clear how binding of these peptides to connexins causes a block of the Hc.

3. Experimental design and methods

In summary, this study was designed to test the hypothesis that the cardioprotective effect attributed to PKC activation is mediated, at least in part, through the closure of the unapposed Cx43Hc and their decreased conductance. Specifically, the study aims to assess the following:

1. Unapposed Cx43Hc function is selectively and differentially regulated by various isoforms of PKC. Particularly, phosphorylation of Cx43 by εPKC inhibits unapposed Cx43Hc.
2. Inhibition of the Cx43Hc, using the synthetic structural Cx43-mimetic peptides Gap26, confers sustained cardioprotection against ischemia/reperfusion injury by counteracting the ischemia-induced dephosphorylation effect on the unapposed Cx43Hc.

Therefore, our specific objectives are summarized as follows:

1. To dissect functional roles that individual PKC isoforms exert in the modulation of Cx43Hc using the unique matrix of PKC isoform-specific activator and inhibitor peptides in tsA201 cells transiently transfected with Cx43.
2. To test whether Cx43-mimetic peptide, Gap26, confers resistance to isolated rat ventricular myocytes against ischemia-induced cell death.
3. To test whether Gap26 confers cardioprotection against ischemia/reperfusion to the perfused intact heart.

3.1 Dissection of functional roles of PKC isoform

The matrix of PKC isoforms-specific modulator peptides (Table 1) was utilized to dissect and interrogate the individual PKC isoform roles in the modulation of Cx43Hc. Cx43Hc-mediated currents are recorded from transfected tsA201 cells using the patch clamp technique in whole cell configuration as described (Hawat & Baroudi, 2008). Cx43Hc currents were induced by incubating cells in a low-Ca bath solution. The selective inhibitor and activator peptides of PKC isoforms were individually applied to the intracellular space through the patch pipette. For each experiment, the cell was dialyzed with a different activator or inhibitor peptide (0.1 μM) for at least five minutes to allow for peptide to access the cytoplasm. The implication of a specific PKC isoform in Cx43Hc modulation is predicted by the capability of the corresponding activator peptide to reduce currents or the inhibitor peptide to abolish current inhibition induced by a general PKC activator, the phorbol myristate acetate (PMA, 10 nM applied in the bath solution). The PMA-induced PKC activation effect on Cx43Hc currents was assessed in the presence of each of the inhibitor peptides separately. To substantiate the specificity of PMA vis-à-vis PKC activation, the effect of PMA was compared with an inactive phorbol ester, the 4αPDD, that possesses all characteristics of PMA except for its capacity to activate PKC. Appropriate time was allowed for steady-state current levels to be reached

before the application of PMA or 4αPDD. As negative controls, we used a scrambled version of active peptides to assess their effect on Cx43Hc-induced currents.

3.2 Study on isolated cardiomyocytes

To assess the functional effect of the Cx43-mimetic peptide Gap26 directly on Cx43Hc, currents were recorded using patch clamp from transfected tsA201 cells. Since peptides exert their effect by binding to the extracellular loops, these were added to the extracellular bath solution (0.5 μM). To determine the sensitivity of isolated myocytes to simulated ischemia/reperfusion, freshly isolated cardiac myocytes were incubated with saline, Gap26 or scrambled GAP26 (sGap26) 10 min before or 30 min after the initiation of ischemia as described (Hawat et al., 2010). Briefly, cells were pelleted by low speed centrifugation for 1 min and were washed twice with incubation buffer. Under these conditions, most of the non-myocyte cells remain in the supernatant, producing a pellet that contains >95% myocytes (Chen et al., 1999; Hawat et al., 2010). To simulate ischemic stress, cells were transferred to microcentrifuge tubes, washed twice with degassed glucose-free incubation buffer and pelleted as before. After centrifugation, 90% of supernatant was removed and microballoons were layered over the remaining buffer to create an air-tight environment. Tubes were incubated at 37^{o}C for 180 min the damage to cardiac myocytes was assessed by trypan blue dye exclusion assay as previously described (Armstrong et al., 1994; Armstrong et al., 1997). The staining should correlate with myocytes rounding indicating cell death.

3.3 Study on intact heart

Using Langendorff technique, isolated Sprague-Dawley rat hearts were subjected to 20-min control stabilization followed by 40 min regional ischemia by ligation of the left anterior descending (LAD) coronary artery and then 180 min reperfusion by removing the ligation. The Cx43 structural mimetic peptide (0.5 μM) was administered either 10 min prior to the onset of ischemia or 30 min after (this is 10 min before reperfusion), as described (Hawat et al., 2010). For control, s Gap26 was utilized. Evans blue perfusion was used to determine the region at risk. The left ventricle was sectioned into 2-mm slices and incubated with triphenyltetrazolium chloride (TTC) to estimate the injured (infracting) myocardial zone. The results were confirmed by histology. Myocardial perfusate flow was also determined by measuring the volume of perfusate recovered from Langendorff-perfused heart during 1 min at 10 min after LAD occlusion or 10 min after reperfusion and compared to basal MPF (BMPF) as previously desrcibed (Hawat et al., 2010).

4. Results

4.1 PKC-mediated inhibition of Cx43Hc currents is primarily mediated through εPKC isoform

Application of the general PKC activator, PMA, in the bath solution consistently inhibited Cx43Hc currents (Fig. 5A, 5B, 5C). Averaged data show that PMA resulted in Cx43Hc current reduction by 74.0±4.3% (n=6, P<0.05). Superfusion of tsA201 cells with 4αPDD, an inactive phorbol ester analog that does not activate PKC, did not significantly affect Cx43Hc current amplitude (increase of 2.0±1.5%, n=5, P=NS; Fig. 5D), indicating that the PMA effect reported above is indeed mediated via PKC activation. To dissect the role of individual PKC isoforms in the regulation of Cx43Hc, we tested the ability of five PKC isoform-specific inhibitor peptides

to antagonize the effect of PMA, i.e. αC2–4, βIV5–3, βIIV5–3, δV1–7, and εV1–2 targeting α-, βI-, βII-, δ-, and ε-PKC isoforms, respectively. Each of the tested peptides targets a corresponding native PKC isoform in tsA201 cells. Importantly, when cells were dialysed with the specific inhibitor peptide of the 'cardioprotective' εPKC, the PMA-induced current inhibition was completely abolished (118.5±15.8%, n=6, significantly different from the reference PMA effect; Fig. 5E) , this was in contrast with the other tested PKC isoforms inhibitors. In the presence of scrambled εV1–2, the inhibitory effect of PMA on Cx43Hc currents was not significantly different compared with the PMA effect alone (69.4±4.7%, n=3, *P*=NS). On the other hand, using an εPKC activator peptide εV1–7 (0.1 μM) alone was able to cause a 75.7±2.6% reduction in the basal Cx43Hc current (n=3, *P*<0.05; Fig. 5F), thus confirming the involvement of εPKC in Cx43Hc inhibition. Detailed results are reported (Hawat & Baroudi, 2008).

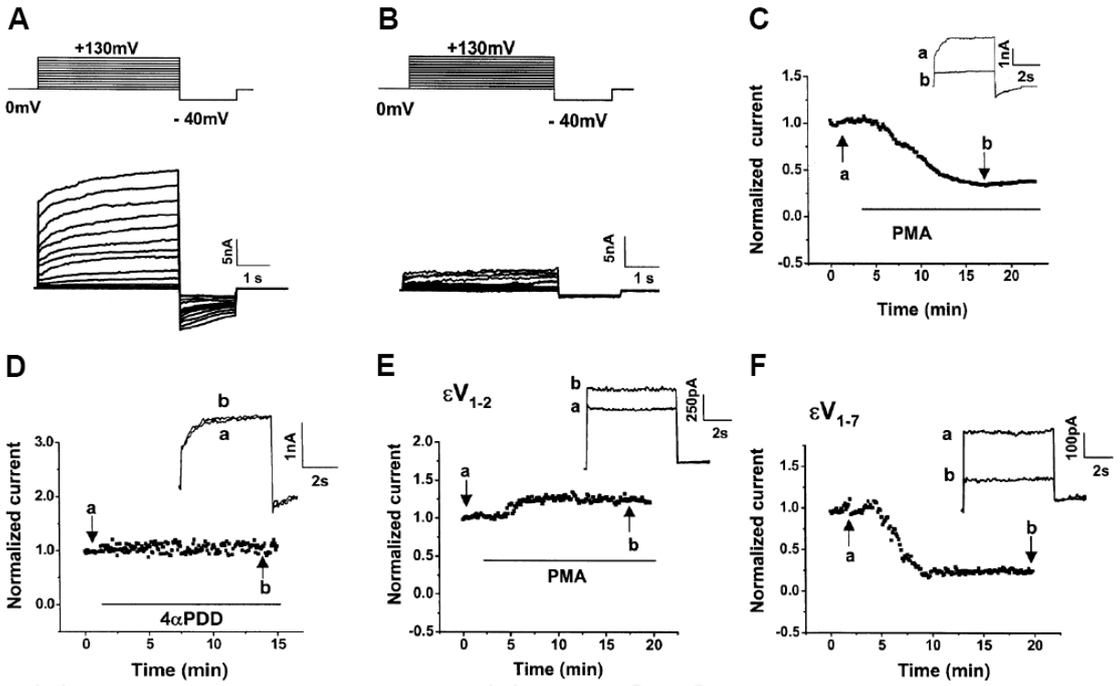

Fig. 5. Cx43Hc modulation by PMA and εPKC. A, whole-cell currents recorded from a typical tsA201 cell transfected with Cx43. B, traces from a non-transfected cell. Time course recording plotting Cx43Hc current amplitudes against time in the presence of C, PMA;. D, 4αPDD; E, PMA and the εPKC-specific inhibitor peptide; and F, εPKC activator peptide.

4.2 Gap26 inhibits Cx43Hc currents

Because we believed inhibition of Cx43Hc by εPKC may represent the basic key mechanism of cardioprotection during ischemic preconditioning, we next investigated the functional effect of the presumably specific inhibitor of Cx43Hc, the mimetic Gap26 peptide, on Cx43Hc mediated currents recorded from individual tsA201 cells transiently expressing Cx43. In Fig. 6A, a representative time course recording shows rapid Cx43Hc currents reduction when Gap26 was introduced in the bath solution. A steady-state inhibition was reached in all experiments. Averaged data indicate that Gap26 caused 60.1±4.6% (n=5, *P*<0.05) current reduction. In the presence of sGap26 peptide, the amplitude of Cx43Hc

currents did not vary over a similar time frame (99.0±1.1%, n=5, *P*>0.05) (Fig. 6B). The histogram (Fig. 6C) summarizes the effects of both peptides on normalized Cx43Hc currents. Curves representing the current–voltage (I–V) relationship for Cx43Hc in the absence and in the presence of Gap26 are illustrated in Fig. 6D. I–V data were elicited by depolarizing cells with voltage steps ranging from +0 to +90 mV.

Because connexin 40 (Cx40) and connexin 45 (Cx45) may also form hemichannels in the heart, we examined whether Gap26 has effects on currents recorded from tsA201 cells transfected with either connexin isoforms. Importantly, the application of Gap26 did not cause a significant reduction in currents mediated by Cx40Hc (0.7±1.6%, n=4, *P*>0.05) or Cx45Hc (0.2±0.7%, n=4, *P*>0.05) over a time frame similar to that used in Cx43Hc experiments (Hawat et al., 2010).

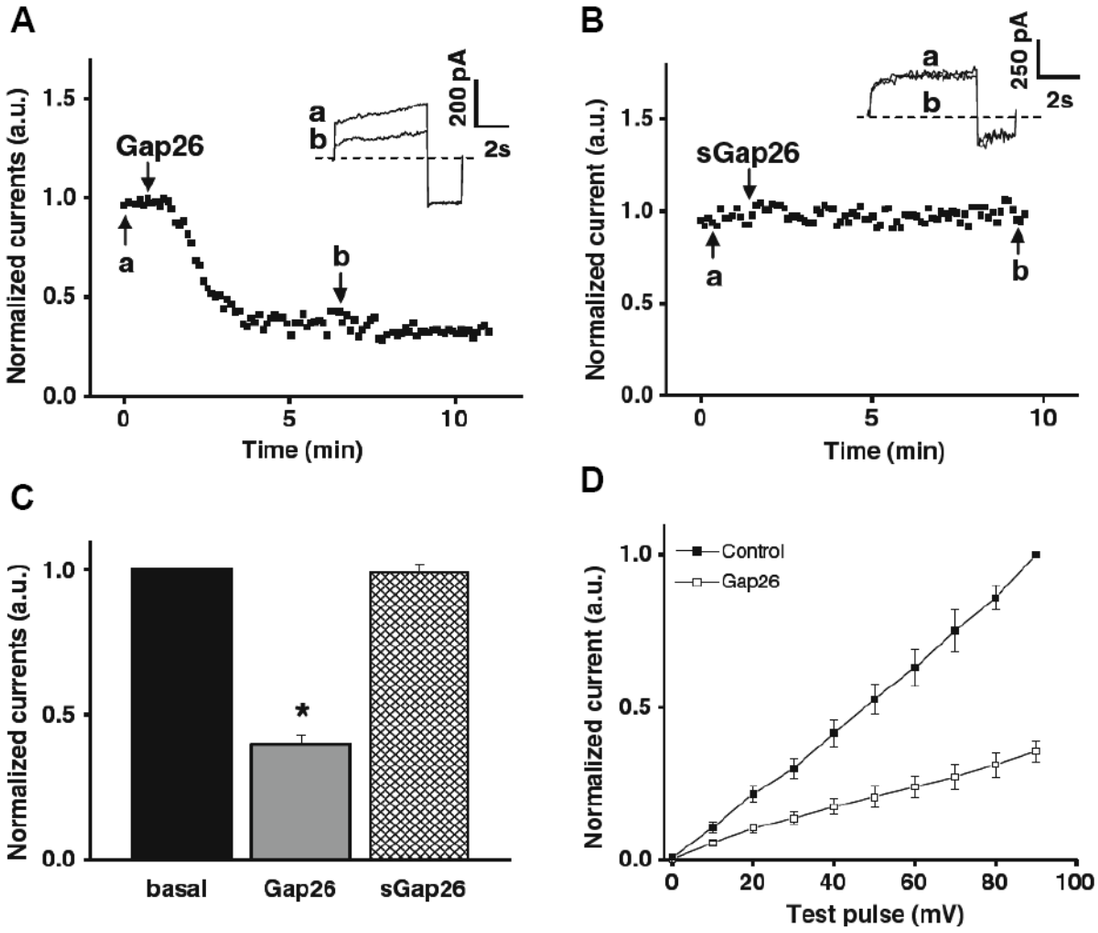

Fig. 6. Cx43Hc-mediated whole-cell current inhibition by Gap26 recorded from individual tsA201 cells. A, time course recording plotting current amplitudes in presence of Gap26 against time. B, a control time course recording in presence of sGap26. Insert in A and B show current traces recorded at time points indicated by arrows a and b. Current amplitudes are normalized against control and are represented in arbitrary units (a.u.). C, a histogram summarizing effects of Gap26 and sGap26 in comparison to basal current. D, current-voltage relationship obtained from tsA201 cells in absence and in presence of Gap26.

4.3 Connexin 43 mimetic peptide confers resistance to intact heart against ischemia injury

We next assessed the effect of Gap26 on intact ischemic heart. Administration of Gap26 before ischemia significantly decreased infarct size from 36.3±2.1 of area at risk in untreated hearts (n=7) to 18.8±1.9% (n=6, $P<0.05$) (Fig. 7A). In order to assess the peptide capability to counteract an existing ischemia, a separate group of hearts was studied in which Gap26 was introduced 30 min after the LAD coronary occlusion. Similarly, the infarct size was significantly reduced to 16.2±0.8% (n=7, $P<0.05$) of area at risk. These results correspond to infarct size reductions by 48.2% and 55.4% when Gap26 was introduced before or during ischemia, respectively. The size of area at risk to total ventricles did not change significantly in both experimental groups (41.3±4.5%, n=6, $P>0.05$ and 40.6±3.2%, n=7, $P>0.05$, respectively) compared to the group of untreated hearts (44.8±2.0%, n=7) (Fig. 7B). As negative control,

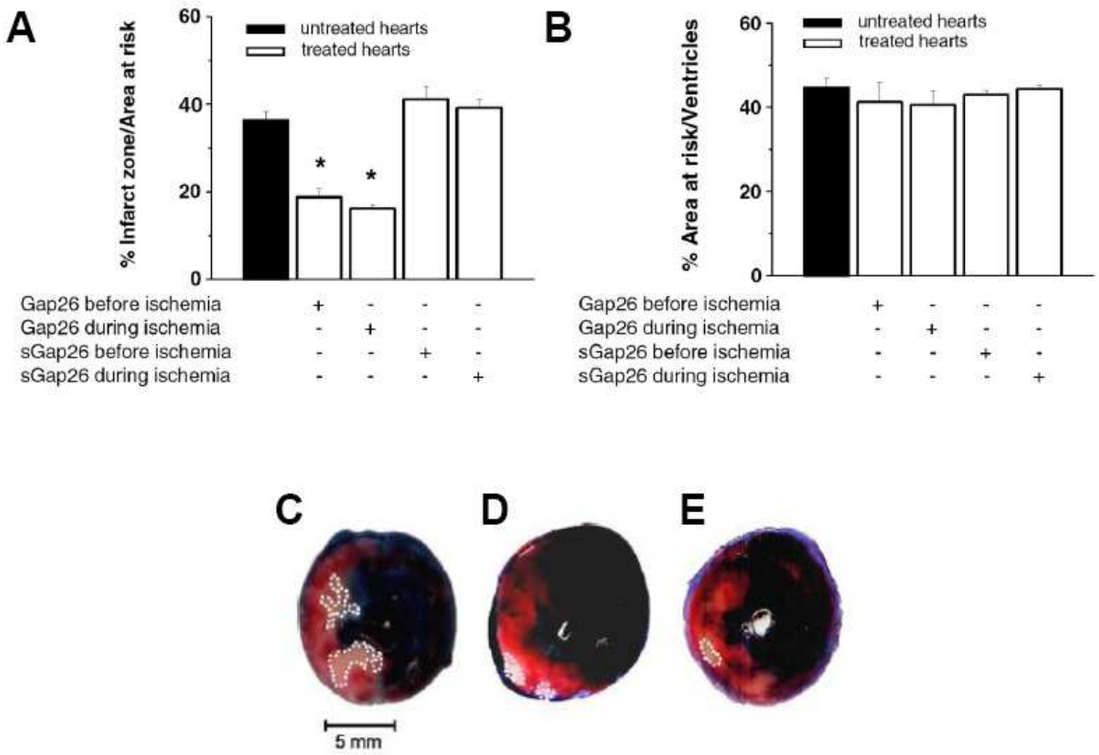

Fig. 7. Effect of synthetic peptides on intact hearts subjected to regional ischemia by LAD ligation. a Histogram representing the proportion of infarct zone to the area at risk, b histogram representing the proportion of area at risk to total ventricles. Values obtained from ischemic hearts in the presence of Gap26 or sGap26 added either 10 min before (n=6 and n=3, respectively) or 30 min after (n=7 and n=4, respectively) the occlusion of LAD are reported. *P<0.05 indicates statistically significant difference in comparison to untreated hearts (n=7). Photographs of TTC-stained heart sections after 40 min ischemia and 180 min reperfusion representing c untreated heart, d heart treated with Gap26 introduced 10 min before LAD occlusion, and e Gap26 introduced 30 min after LAD occlusion. c–e The infarct zone is the whitish area delimited with white dashed lines; the area at risk corresponds to the red region including the infarct zone; and the perfused area is in dark blue

sGap26 was also tested. Administration of sGap26 did not statistically affect the size of infarct area to area at risk when introduced before ischemia (41.1±3.0%, n=3, *P*>0.05) or during ischemia (39.2±2.0%, n=4, *P*>0.05) in comparison to untreated hearts (36.3±2.1%, n=7). The global size of total ventricles was comparable between groups of untreated hearts and hearts treated with Gap26 or sGap26 (data not shown). Percentage values for different heart areas obtained from various experimental groups are listed in Table 1. Transversal slices obtained from hearts representing the groups of untreated hearts, hearts treated with Gap26 before ischemia, and hearts treated with Gap26 during ischemia are shown in Fig. 7C, 7D, 7E.

In order to investigate the effect of Gap26 on the function of Langendorff-perfused hearts, we compared the MPF measured 10 min after LAD occlusion and 10 min after reperfusion to the BMPF. The BMPF is determined for each heart during normal conditions, i.e., before LAD occlusion in the absence of Gap26. Application of Gap26 to non-ischemic hearts during 1 h did not cause significant changes to MPF (data not shown). Importantly, addition of Gap26 in the perfusion solution increased MPF during reperfusion of ischemic hearts from 65.7±5.1% (n=7) of BMPF in untreated hearts to 89.8±4.9% (n=7, *P*<0.05) and 86.7±4.8% (n=4, *P*<0.05) in hearts treated with Gap26 before or during ischemia, respectively (Fig. 8). As negative control, sGap26 did not affect MPF when introduced before or during ischemia. Values for MPF variations in different experimental groups are listed in Table 2.

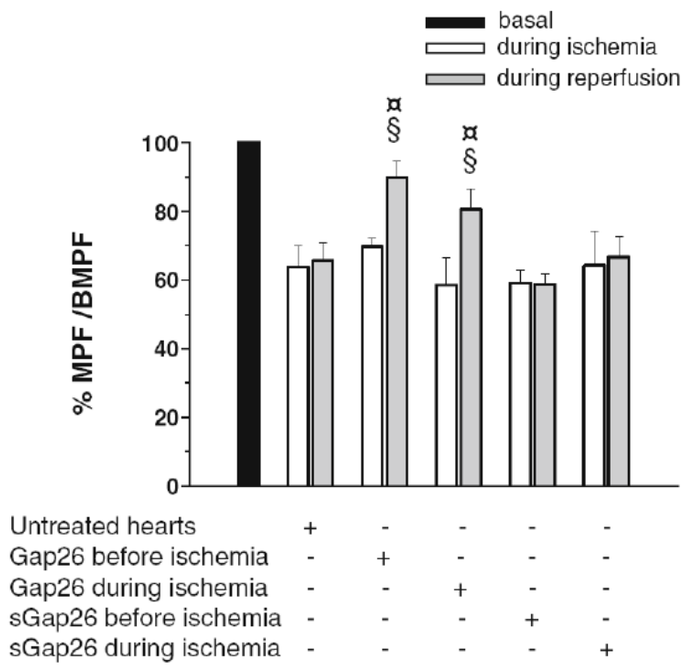

Fig. 8. Percentages of MPF measured at 10 min after LAD occlusion (open columns) or at 10 min after reperfusion (filled columns) to BMPF. The groups shown correspond to the untreated hearts (n=7), hearts treated with Gap26 before ischemia (n=7), hearts treated with Gap26 during ischemia (n=4), hearts treated with the inactive sGap26 before ischemia (n=3), and heart treated with sGap26 during ischemia (n=4). ¤*P*<0.05 indicates data significantly different from untreated hearts. §P<0.05 indicates data significantly different from MPF during ischemia.

Groups	IZ/AAR (%)	AAR/V (%)	N	MPFI/BMPF (%)	MPFR/BMPF (%)	N
Untreated hearts	36.3±2.1	44.8±2.0	7	63.8±6.2	65.7±5.1	7
Gap26 before ischemia	18.8±1.9	41.3±4.5	6	69.9±2.3	89.8±4.9	7
Gap26 during ischemia	16.2±0.8	40.6±3.2	7	58.5±7.4	86.7±4.8	4
sGap26 before ischemia	41.1±3.0	43.1±0.3	3	59.1±3.7	57.1±3.1	3
sGap26 during ischemia	39.2±2.0	44.4±1.0	4	64.2±9.9	66.7±5.9	4

IZ infarct zone, AAR area at risk, V ventricles, MPFI myocardial perfusate flow measured during ischemia, MPFR myocardial perfusate flow measured during reperfusion, BMPF basal myocardial flow

[a] Statistically different in comparison to untreated group

[b] Statistically different in comparison to MPF during ischemia

Table 2. Percentage values for heart areas and myocardial perfusate flows.

4.4 Effect of Gap26 on isolated cardiomyocytes

In order to elucidate the cellular basis for the Gap26-mediated cardioprotection observed in intact hearts, we investigated the effect of the peptide on isolated cardiomyocytes from adult rat hearts subjected to simulated ischemia–reperfusion. Application of Gap26 in the bath solution either 10 min before or 30 min after the initiation of simulated ischemia increased the number of surviving cardiomyocytes after 180 min reperfusion from 28.4±6.5% of total cells (n=8) to 53.1±6.8% (n=8, P<0.05) and 55.0±6.5% (n=8, *P*<0.05), respectively (Fig. 9A). The percentage of surviving cells did not differ statistically between groups treated with Gap26 before or during simulated ischemia. As negative control, the percentage of surviving cells following application of sGap26 before ischemia (22.9±6.9%, n=8) or during ischemia (26.0±6.6%, n=8) did not differ significantly in comparison to untreated cells (28.4±6.5%,

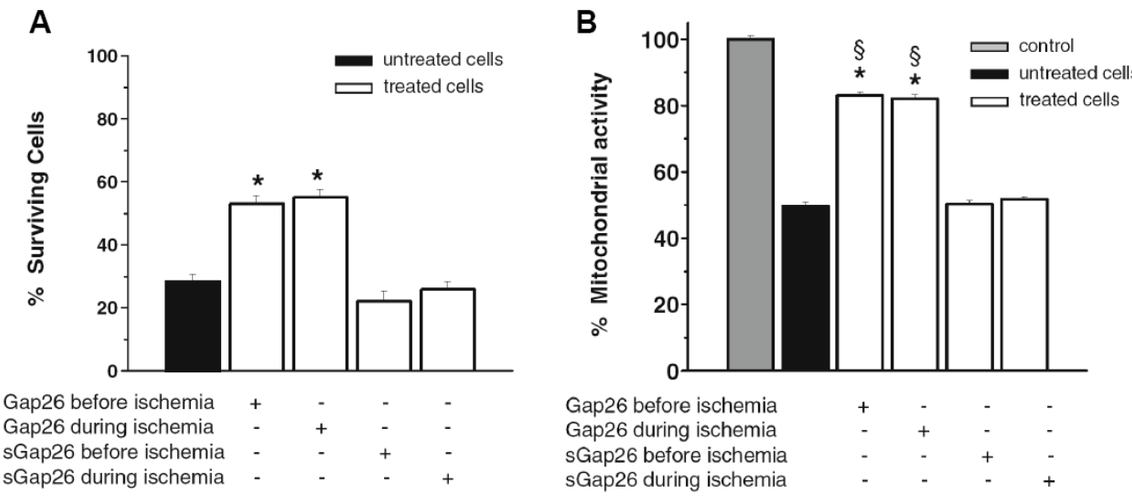

Fig. 9. Effect of Gap26 on viability of isolated adult rat cardiomyocytes during simulated ischemia–reperfusion. a The percentage of surviving cells to total cells after reperfusion was determined in the absence or presence of Gap26 or sGap26 added to the bath solution before or during ischemia (n=8 in each group). Data are expressed as mean ± SEM of eight experiments using myocytes from three different animals. b Mitochondrial dysfunction was determined with the MTT test (n=5 in each group, obtained from a single rat heart). *P<0.05 indicates significant difference in comparison to untreated cells. §P<0.05 indicates significant difference in comparison to control.

n=8, *P*>0.05). In order to substantiate these findings, we assessed mitochondrial activity in each experimental group of cardiomyocytes using the colorimetric MTT assay. To alleviate comparison, results are expressed as percentages of the mitochondrial activity measured in a control group of cardiomyocytes maintained at normoxic conditions during a similar time frame. Simulated ischemia–reperfusion reduced mitochondrial activity in untreated cardiomyocytes to 49.7±1.2%, n=5, *P*<0.05 in comparison to the control cardiomyocytes 100.0±1.2%, n=5. Treatment of cardiomyocytes with Gap26 prevented partially but significantly this mitochondrial dysfunction when introduced before or during ischemia (83.0±1.1%, n=5, *P*<0.05 and 82.1±1.1%, n=5, P<0.05, respectively). Application of sGap26 did not prevent the mitochondrial dysfunction when applied before ischemia (50.3±1.0%, n=5, P>0.05) or during ischemia (51.8±0.9%, n=5, *P*>0.05) in comparison to untreated cells. Results obtained from the different experimental groups are illustrated in the histogram (Fig. 9B).

5. Discussion

5.1 PKC isoform selective modulation of Cx43Hc

The first important observation here was the unraveling of selective and differential implication of PKC isoforms in the modulation of Cx43Hc conductance. The εPKC was found to be the isoform predominantly involved in the modulation of Cx43Hc conductance after PMA superfusion.

Cx43Hc dephosphorylation (Beardslee et al., 2000; Jain et al., 2003; Jeyaraman et al., 2003; Miura et al., 2004; Schulz et al., 2003) and opening (John et al., 1999b; Kondo et al., 2000; Shintani-Ishida et al., 2007) have been proposed as key mechanisms underlying cell injury in response to simulated ischemia in cardiomyocytes. Preservation of Cx43 phosphorylation by selective activation of the εPKC isoform occurs in response to ischemic pre- (Chou & Messing, 2005; Liu et al., 1999; Ytrehus et al., 1994) and postconditioning (Penna et al., 2006a; Philipp et al., 2006; Zatta et al., 2006), in which brief episodes of ischemia reduce the adverse effects of subsequent or preceding myocardial ischemia, respectively. As the function of multiple proteins may be affected by εPKC, delineation of the isoform-specific regulation of downstream targets, e.g. Cx43Hc, is indeed important to improve our understanding of the pathogenesis of ischemic heart disease and to identify new opportunities for drug development.

5.2 Cx43 mimetic peptide protects heart against myocardial infarction

In this context, another important finding was the rapid depression of Cx43Hc conductance in response to the application of the Cx43 mimetic peptide Gap26. To date, the involvement of unequivocally identified unapposed Hc in CxMPs-mediated phenomena, such as regulation of ATP release and calcium propagation, is still debated (Dahl, 2007). Therefore, we demonstrated here for the first time that superfusion of the ion channel-deficient tsA201 cells, transiently expressing Cx43, with Gap26 readily inhibits Cx43Hc-mediated currents recorded from individual cells. This observation complements with previous studies reporting on the inhibitory effect of Gap26 on the permeability of Cx43Hc (Braet et al., 2003b; Gomes et al., 2005; Leybaert et al., 2003; Pearson et al., 2005), the second major functional characteristic of connexin channels besides conductance. Although not

surprisingly, this result represents the most direct evidence reported so far for the Gap26 inhibitory effect on Cx43Hc. Curiously, this inhibition occurred using a peptide concentration that is 1,000 times lower than what has previously been utilized to block Hc currents (0.5 µM in this study versus 500 µM in (Romanov et al., 2007)). Occasionally, the specificity of CxMPs toward Hc has been challenged (Wang et al., 2007). Therefore, we tested the effect of Gap26 on Hc of Cx40 and Cx45 which are also present in the heart. Importantly, Gap26 did not affect currents from Cx40Hc or Cx45Hc.

The most important finding however was the protection that Gap26 conferred to intact rat heart against regional ischemia induced by LAD coronary occlusion. We showed that administration of Gap26 prior to LAD occlusion resulted in more than 48% reduction of infarct size compared to untreated hearts. Similarly, Gap26 reduced infarct size by 55% when administered during ischemia. We also showed that the salutary effect of Gap26 extends to heart function by increasing the MPF during reperfusion by nearly 37% and 32% when the peptide was administered before or during LAD occlusion, respectively, in comparison to untreated hearts. These results indicate that whereas Gap26 can confer resistance to "normal" hearts against subsequent ischemia, it also has the capability to salvage injured hearts when administered after the occurrence of ischemia. Hypothetically, we ascribe these effects to the presumable Gap26-mediated inhibition of cardiomyocytic Cx43Hc opened by ischemic stress. Indeed, Gap26 is also known to inhibit Cx43 GJCs when used at relatively high concentrations and/or following prolonged exposure (Evans et al., 2006). Nonetheless, all experiments in this study were performed using low concentration of Gap26 (0.5 µM) previously shown to selectively inhibit Hc without directly affecting GJCs (Clarke et al., 2009; Verma et al., 2009). Therefore, we consider that neither the peptide concentration nor the duration of exposure to Gap26 (which varied depending on protocols between ~3 and 4 h) was sufficient to modulate GJCs in the studied hearts. In concordance with the fact that death of cardiomyocytes, and therefore myocardial injury, principally occurs during reperfusion (Gottlieb et al., 1994; Shintani-Ishida et al., 2007; Zhao et al., 2000), both structural and functional improvements in intact hearts did not differ significantly whether Gap26 was introduced before occlusion or before reperfusion.

To investigate the cellular basis of the protection of intact hearts, we assessed the effect of Gap26 on isolated cardiomyocytes subjected to simulated ischemia–reperfusion. We found a nearly twofold increase in the number of surviving cells when Gap26 was administered either before or during the simulated ischemia. A similar increase was previously noted in isolated neonatal rat cardiomyocytes treated with Gap26 prior to simulated ischemia (Shintani-Ishida et al., 2007). Clearly, both observations underscore the capability of Gap26 to confer protection to cardiomyocytes in the absence of direct intercellular communication, precisely through GJC, and therefore point to the inhibition of unapposed Hc as the underlying mechanism of cardioprotection. Altogether, these results strongly suggest that the observed cardioprotection conferred by Gap26 is most likely mediated by the specific inhibition of Cx43Hc opened by the ischemic stress.

6. Perspective

The non-junctional Cx43 have previously been associated with ischemic preconditioning-mediated cardioprotection (Li et al., 2004; Padilla et al., 2003). This effect has been related to

increase in Cx43 phosphorylation (Hatanaka et al., 2004; Hund et al., 2007; Miura et al., 2004; Schulz et al., 2003). Interestingly, different studies pointed to the isozyme εPKC as a principal mediator of ischemic preconditioning-mediated cardioprotection (Gray et al., 1997; Hund et al., 2007; Inagaki et al., 2003; Liu et al., 1999; Ping et al., 1997; Saurin et al., 2002). Here, we showed using a unique set of PKC isozyme-specific modulator peptides that εPKC, among other PKC isozymes, selectively inhibits the conductance of Cx43Hc. These data prompt us to believe that inhibition of Cx43Hc represents a fundamental basis for the cardioprotection conferred by ischemic preconditioning. In the light of our findings using Cx43 mimetic peptide Gap26 and given the ubiquitous expression of PKC and the abundance of its substrates within cells and throughout tissues, we put forth that the selective inhibition of the abnormally open Cx43Hc, which we believe are solely localized in the ischemic region of the heart, would be more suitable to mimic pharmacologically the cardioprotection conferred by ischemic preconditioning than to modulate PKC isozymes as previously proposed (Chen et al., 2001a; Chen et al., 2001b). The accessibility of CxMP binding sites from extracellular space, which circumvents the need of conjugating the peptides with transmembrane carriers and the limitations deriving from their use, is indeed another therapeutic advantage over the use of intracellular modulators. Certainly, studies using more elaborated experimental models are needed to substantiate the therapeutic potentials of Gap26.

7. Conclusion

In conclusion, we demonstrate for the first time that administration of Gap26 prior to ischemia prevents injury by making intact heart more resistant to ischemic stress. Moreover, usage of Gap26 as a treatment following occurrence of ischemia reduces cardiac tissue damage and improves intact heart function. The discovery of new agents capable to make heart more resistant to ischemia and/or to improve its recovery after injury caused by ischemia will certainly be promising tools to fight ischemic heart disease.

8. Acknowledgements

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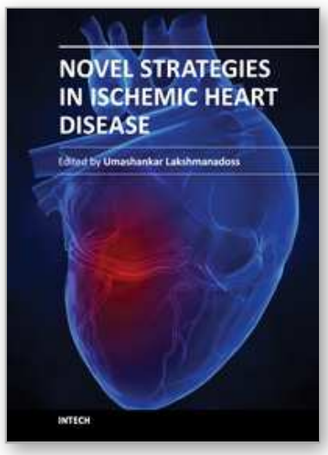

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The first edition of this book will provide a comprehensive overview of ischemic heart disease, including epidemiology, risk factors, pathogenesis, clinical presentation, diagnostic tests, differential diagnosis, treatment, complications and prognosis. Also discussed are current treatment options, protocols and diagnostic procedures, as well as the latest advances in the field. The book will serve as a cutting-edge point of reference for the basic or clinical researcher, and any clinician involved in the diagnosis and management of ischemic heart disease. This book is essentially designed to fill the vital gap existing between these practices, to provide a textbook that is substantial and readable, compact and reasonably comprehensive, and to provide an excellent blend of "basics to bedside and beyond" in the field of ischemic heart disease. The book also covers the future novel treatment strategies, focusing on the basic scientific and clinical aspects of the diagnosis and management of ischemic heart disease.

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