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Does Protein Kinase C Play a Pivotal Role in the Mechanisms of Ischemic Preconditioning?

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Summary. **This communication reviews the evidence for the pivotal role of protein kinase C in ischemic myocardial preconditioning. It is believed that several intracellular signalling pathways via receptor-coupled phospholipase C and its "cross-talk" with phospholipase D converge to activation of protein kinase C isotypes which is followed by phosphorylation of until now (a number of) unknown target proteins which produce the protective state of ischemic preconditioning.**

After briefly introducing the general biochemical properties of protein kinase C, its isotypes and the limitations of the methodology used to investigate the role of protein kinase C, studies are discussed in which pharmacological inhibition and activation and (immunore)activity and/or isotypes measurements of protein kinase C isotypes were applied to assess the role of activation of protein kinase C in ischemic myocardial preconditioning.

It is concluded that definitive proof for the involvement of protein kinase C in preconditioning requires future studies which must focus on the isotype(s) of protein kinase C that are activated, the duration of action, cellular translocation sites and the identity and stability (of covalently bound phosphate) of phosphorylated substrate proteins.

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Ischemic preconditioning is not an organ specific phenomenon, as it does not only occur in the heart [1,2], but also in kidney [3], liver [4] and skeletal muscle [5], while the brain is protected against the consequence of a new ischemic event at 24 hours after the preconditioning stimulus is applied [6]. Furthermore, brief ischemia in organs other than the heart may also limit irreversible damage produced by a subsequent coronary artery occlusion. Thus, in rats a 15-minute occlusion of the mesenteric artery with 10 minutes of reperfusion prior to a 60-minute coronary artery occlusion limited myocardial infarct size by 40% [7]. Since ganglion blockade abolished myocardial protection by mesenteric artery occlusion-reperfusion but not by brief coronary artery occlusion-reperfusion, the mechanism of protection by brief ischemia-reperfusion in other organs appears to differ from that by brief myocardial ischemia-reperfusion [7].

Initially, ischemic preconditioning studies focussed on time characteristics and the search for extracellular endogenous and exogenous factors that either mimicked or inhibited the phenomenon. It proved that protection occurred during two distinct episodes: a classical preconditioning period (CPP) that lasted 2 to 3 hours after the preconditioning stimulus was applied [1,2], and a second window of protection (SWOP) between 24 and 72 hours [8–10]. The mechanisms of protection for these two windows are most likely not the same. Rapidly produced endogenous factors may activate intracellular pathways during CPP, while the slower process of induction of heat-shock proteins may be involved during the SWOP. Initially, attention focussed on activation of adenosine A_1-receptors [11,12] or K^+_{ATP} channel opening [13,14] as the mechanisms for protection during CPP. More recently activation of protein kinase C has received wide attention [15]. Prior to reviewing the role of protein kinase C, we review the current state of knowledge of the molecular mechanism(s) of ischemic preconditioning. introduce the generally known biochemical properties of protein kinase C and discuss the limitations of the techniques used to investigate the potential role of protein kinase C. Finally, the evidence that activation of protein kinase C and the intracellular signalling pathways leading to its activation play a pivotal role in the mechanism of ischemic preconditioning is sum-

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marized. However, not all studies support a role for protein kinase C in preconditioning and this issue remains therefore controversial at the present time.

Most studies on ischemic preconditioning used infarct size as endpoint, but other endpoints such as recovery of contractile function, and protection against reperfusion-induced ventricular arrhythmias have also been used. Because these other endpoints require different experimental conditions (i.e. shorter duration of the sustained occlusions) we have limited ourselves to studies which used infarct size as endpoint. For this same reason we have excluded studies on ischemic preconditioning in other organs.

Current State of Knowledge of the Molecular Mechanism(s) of Ischemic Preconditioning

Activation of receptors by exogenously administered stimuli such as adenosine [11,12], bradykinin [16,17], noradrenaline [18,19], acetylcholine [20,21], endothelin-1 [22] or opiates [23] mimic myocardial protection by ischemic preconditioning. Intracellular signalling by these stimuli, via GTP-binding-protein-linked receptors and phospholipase C and possibly phospholipase D (see later) [24,25], leads to activation of one or more isotypes of the protein kinase C family which then phosphorylate putative target proteins [15,26]. Possible target proteins are those that regulate opening of K^+_{ATP} channels [13,14,27], activate ecto-5'-nucleotidase [28] (during CPP) or modulate transcriptional regulation of the expression of heat shock proteins [29,30] (during SWOP). For instance, K^+_{ATP} channels are opened when an ischemic preconditioning stimulus is applied, while blockade of K^+_{ATP} channels prevents ischemic preconditioning [13,14]. It is likely that modulation of K^+_{ATP} channels in the mitochondria, sarcoplasmic reticulum or the nucleus are involved in the mechanism of protection as blockade of the action potential shortening by dofetilide does not abolish protection by ischemic preconditioning [31]. Since protein kinase C can be activated via various receptors linked to phospholipase C- and possibly phospholipase D-mediated signalling pathways, these receptors may act synergistically [15]. Opening of K^+_{ATP} channels by pharmacological substances lowers the threshold for ischemic preconditioning [32], which is consistent with the hypothesis that K^+_{ATP} channels are target proteins for protein kinase C. Kitakaze et al [28] reported that ischemic preconditioning increased ecto-5'-nucleotidase activity and that activation of protein kinase C increases ecto-5'-nucleotidase activity in isolated rat cardiomyocytes, supporting the candidacy of ecto-5'-nucleotidase as another target protein of protein kinase C.

General Biochemical Properties of Protein Kinase C

In general, the conformation of protein kinases consists of two regions which are connected by a region functioning as a hinge. The protein substrate fits into the groove between the two regions and interacts with a catalytic domain and cofactors interacting with the regulatory domain [33]. The specificity of protein kinases such as cyclic AMP dependent protein kinase, Ca^{2+}-calmodulin dependent protein kinase (CaM-PK) and protein kinase C for their substrate proteins is determined by both the primary sequence of these proteins around their phosphorylation site and the capacity of these sites to interact with the catalytic domain of the protein kinase. Generally, protein kinases are inactivated by the interaction between a pseudo substrate region in the protein kinase's primary sequence and the active site [33]. This restraint is removed during activation by changes in protein kinase conformation due to interaction of second messengers (e.g. cyclic AMP, 1,2-diacylglycerol (1,2-DAG) and Ca^{2+}) with the protein kinase's regulatory sites and by competition between the protein substrates and the pseudo substrate site, all present at the N-terminal region of the primary structure of the protein kinases (Fig. 1). Indeed, activation of most protein Serine/Threonine (Ser/Thr) kinases is preceded by receptor activation followed by synthesis or release of low-molecular-weight protein kinase effectors or second messengers.

Protein kinases can be divided in several types. Protein kinase A is a cyclic AMP dependent protein-Ser/Thr kinase, while protein kinase C is a group of protein-Ser/Thr kinases which are phosphatidylserine (PtdSer-), 1,2-DAG- and/or Ca^{2+}-dependent. Recently, protein kinase D was discovered in COS cells to be dependent on 1,2-DAG and phorbol esters, but information on this enzyme in myocardium is not yet available [35]. In myocardial cells protein kinase C regulates various processes, including myocardial contraction, ion transport, energy metabolism, gene expression and hypertrophic growth [25,36,37]. The role of protein kinase C in growth and proliferation has been implied by its identification as a high-affinity intracellular receptor for tumor-promoting phorbol esters which directly activate most protein kinase C isotypes in a relatively unspecific manner. Phosphorylation of target proteins by protein kinase C isotypes depends on their intracellular location at the time of action. This compartmentalization may be caused by the architecture and intracellular localization of anchor proteins e.g. the so-called receptors for activated C kinase (RACK) [38,39]. Therefore, after protein kinase C is activated it translocates to other cellular compartments such as the sarcolemma where it exerts its principal action (Fig. 1). However, several other compartments such as mitochondria, myofibrils, sarcoplasmic

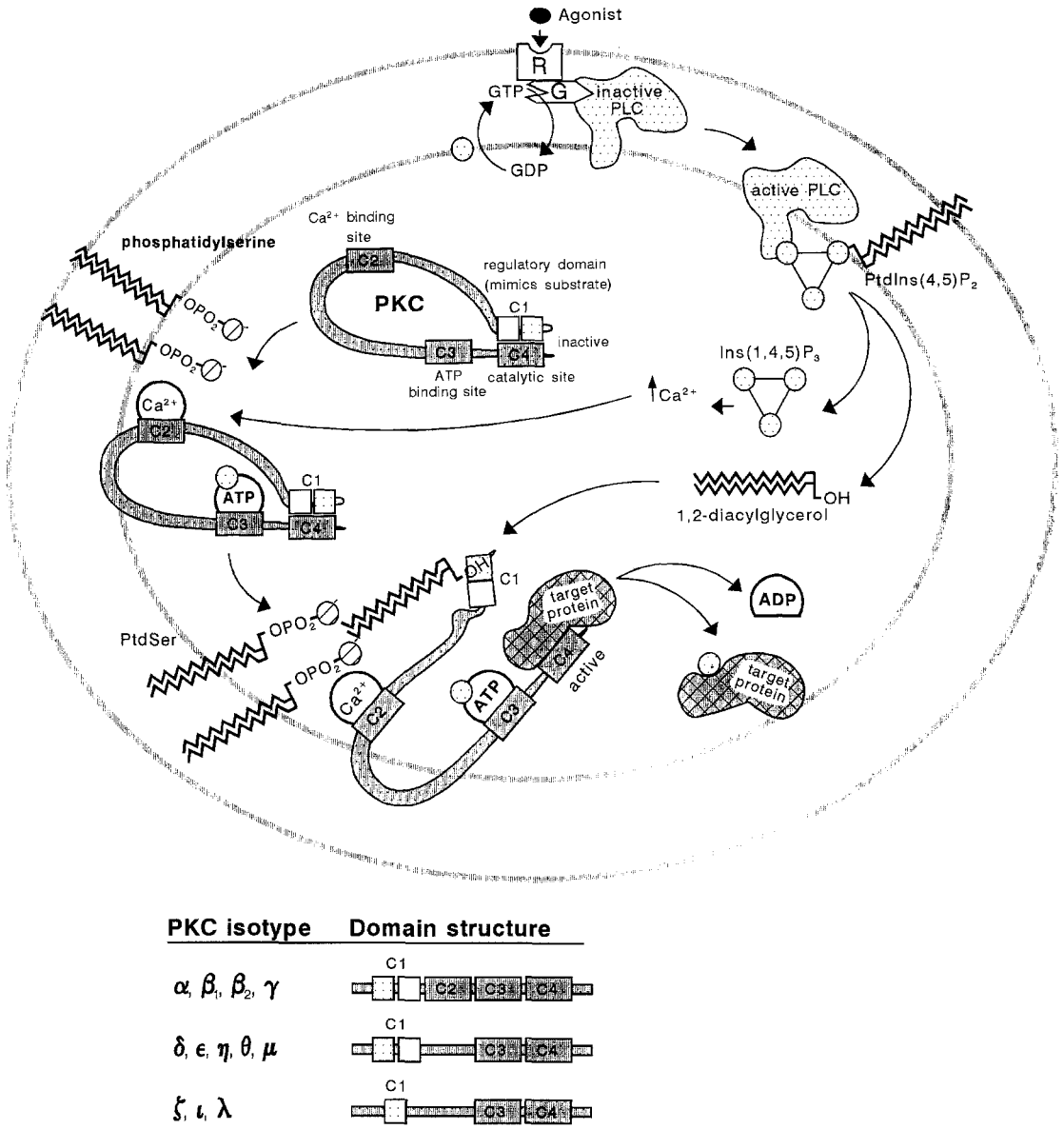

Fig. 1 *Isotypes of protein kinase C, the functional domains in their primary structure, and receptor-coupled phospholipase C-mediated signal transduction leading to protein kinase C activation. The various protein kinase C isotypes share some sequence homology and have all a common ATP-binding site (C3) and catalytic site (C4). Only protein kinase C – α, – β_1, – β_2 and – γ have a Ca^{2+} binding site (C2) and 1,2-diacylglycerol (1,2-DAG)–binding site (C1). In the inactivated state the isotypes of protein kinase C are folded so that an endogenous "pseudo substrate" region on the N-terminal part of the protein occupies the catalytic site (C-terminal part). When agonists (see text) bind to their specific receptors linked to phospholipase C in the cardiac sarcolemma, receptor activation is followed by phospholipase C catalyzed hydrolysis of PtdIns(4,5)P_2 to form inositol-1,4,5-trisphosphate (Ins(1,4,5)P_3). Ins(1,4,5)P_3 releases Ca^{2+} from the Ins (1,4,5)P_3 receptor-sensitive Ca^{2+} storage sites in the cardiac sarcoplasmic reticulum. PtdIns(4,5)P_2 hydrolysis also forms 1,2-diacylglycerol (1,2-DAG), which increases the affinity of some isotypes for Ca^{2+}. When the intracellular free Ca^{2+} concentration increases, some isotypes become more tightly associated with membranes containing the negatively charged head groups of PtdSer, allowing 1,2-DAG to reach its binding site (C1) on the protein kinase C. The 1,2-DAG-protein kinase C complex approaches the membrane by placing the kinase in a pocket of negatively-charged head groups of PtdSer in which Ca^{2+} remains attracted. When this occurs, the conformation of protein kinase C changes, exposing the unoccupied catalytic site, thereby allowing the kinase to phosphorylate cellular proteins. There is also evidence that specific binding proteins (e.g. MARCKS and RACK) determine the cellular translocation process of protein kinase C isotypes. The membrane-bound protein kinase C-DAG-(Ca^{2+})-membrane complex only slowly dissociates and this property is the basis for the commonly used "translocation assay" for assessment of PKC activation. Figure adapted from Zeisel et al [34], with permission of the FASEB Journal.*

reticulum and the perinuclear zone also possess specific receptor sites for protein kinase C isotypes [40].

Protein Kinase C Isotypes

The protein kinase C family can be divided into three distinct subfamilies on basis of their structure and catalytic and regulatory properties (Fig. 1). Classical protein kinase C isotypes (cPKC's) such as protein kinase C-α, -β_1, -β_2 and -γ are activated by Ca^{2+}, PtdSer and 1,2-DAG or phorbol esters such as phorbol-12-myristate-13-acetate (PMA). Novel protein kinase C isotypes (nPKC's) such as protein kinase C-δ, -ε, -η, -θ and -μ are Ca^{2+} independent and only need PtdSer and 1,2-DAG (or PMA) to become activated. Atypical protein kinase C isotypes (aPKC's) are protein kinase C-ζ, -ι and -λ which are also Ca^{2+} independent and only require PtdSer to become activated (Fig. 1). At present, the still growing number of isotypes can be discriminated by immunoblot or immunohistofluorescence analysis. Most investigators use histone III-S as substrate and γ-^{32}P-labelled ATP as phosphate donor to measure protein kinase C activity, which reflects the activity of some of the isotypes present in the cellular fraction. Histone III-S is a poor substrate for some nPKC's (δ, ε and η) compared to the other isotypes (α, β_1, β_2, γ) [41]. Moreover, techniques such as hydroxylapatite high-pressure-liquid-chromatography can be used to determine the activity of protein kinase C isotypes after separation [42]. Measurement of the mRNA concentration using specific cDNA probes on Northern blotting can also be used for the detection of protein kinase C isotypes [43], but mRNA levels offer only limited information because these do not always reflect the functional activities of the isotypes.

In a preliminary study it was found that α, β, ε and ζ are the most prominent isotypes in the rat heart [44]. Similar observations have been made in cultured neonatal rat cardiomyocytes [45] and adult rat cardiomyocytes [46]. However, in a more recent study in adult rat ventricular myocytes, protein kinase C-ε was abundantly present and protein kinase C-α could not be detected [47,48], while in another study protein kinase C-α, -δ, -ε, -η, -θ, -ζ, -λ and -ι were detected in adult rat heart [49]. In the canine heart the presence of protein kinase C-α, -β_1, -β_2, -γ, -δ, -ε, -ι, -θ and -ζ has been described [50]. Thus, the species and the type of assays determine the pattern of protein kinase C isotypes. Furthermore, the affinity and the specificity of the antibodies used to detect the protein kinase C isotypes determine the abundancy of detection but not the absolute concentration of the isotype in vivo. In the pig, using rabbit polyclonal antibodies, we have demonstrated the presence of protein kinase C-α and -ε, while the isotypes-δ and -ζ were barely detectable. Other isotypes were not studied [51]. Myocardial homogenates do not only contain homogenized myocytes but also homogenized fibroblasts, smooth muscle cells and endothelial cells. Consequently, the protein kinase C isotypes of these cells in these homogenates are measured all together. So far, in only one study immunohistochemistry was used to detect the isotypes in situ [48]. In that study it was concluded that protein kinase C-δ is probably the most important isoform involved in preconditioning in the rat heart [48].

Protein Kinase C in Coupling Phospholipase C- to Phospholipase D-activation

Endogenous ligands such as adenosine A_1-, α_1-adrenergic- and muscarinic agonists, bradykinin, angiotensin II, endothelin-1 or opiates stimulate, via the receptor-G-protein coupled to phospholipase C, the intracellular signalling pathway [25,48,52–56]. Phospholipase C catalyzes hydrolysis of phosphatidylinositol-4,5-biophosphate (Ptdlns (4,5)P_2) which leads to formation of the second messengers inositol-1,4,5-triophosphate (Ins(1,4,5)P_3) and 1,2-DAG (Fig. 1) [25]. These messengers, directly or indirectly, activate Ca^{2+}-independent and/or Ca^{2+}-dependent protein kinase C isotypes and calmodulin-dependent protein kinase (CaM-PK). The activated protein kinase C isotypes and/or CaM-PK phosphorylate specific target proteins which may be responsible for CPP. Phospholipase D, another enzyme of which a stimulation leads to 1,2-DAG formation can be strongly stimulated by phorbol esters [36,45,51,57,58]. It uses phosphatidylcholine (PtdCho) as substrate and its activation leads initially to formation of phosphatidic acid (PtdOH) and choline [59]. PtdOH is rapidly hydrolysed to 1,2-DAG and inorganic phosphate (P_i) by PtdOH hydrolase. The 1,2-DAG formed by phospholipase D potentially contributes to activation of protein kinase C isotypes [51]. Stimuli such as noradrenaline, angiotensin II and endothelin-1 stimulate both phospholipase C and phospholipase D [45,58,60]. In fact, protein kinase C has been proposed to function as a switch which reduces the rate of PtdIns(4,5)P_2 hydrolysis catalyzed by phospholipase C and stimulates the rate of PtdCho hydrolysis catalyzed by phospholipase D [57,58,59]. Through this "cross talk" mechanism between phospholipase C and D, the cardiomyocytes may be continuously supplied with 1,2-DAG after receptor stimulation, because the cellular concentration of PtdCho is about 100 times higher than that of PtdIns(4,5)P_2. The continuous production of 1,2-DAG could be of importance for maintenance of activation of the protein kinase C isotypes involved in ischemic preconditioning.

Evidence for a Role of Protein Kinase C in Ischemic Preconditioning

Inhibition of protein kinase C (Table 1)

Selective inhibition of protein kinase C activation by administration of inhibitors prior to or after applying the cardioprotective stimulus is one approach to investigate the role of protein kinase C in ischemic preconditioning. Depending on the inhibitor, protein kinase C function can be blocked at its catalytic or regulatory sites (Fig. 1). It is also possible to downregulate protein kinase C activity by prolonged (1-2 days) stimulation with phorbol ester [61]. This last approach has not been used in ischemic preconditioning, but is of interest in view of the development of tolerance to ischemic preconditioning when a very large number of brief occlusion-reperfusion sequences are applied [62].

The most prominent drugs used to inhibit protein kinase activation or translocation are staurosporine [17,48,52,63], 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) [27,64], chelerythrine [53,65–67], calphostin-C [68], polymyxin B [17,52,55,56,64,66], bisindolylmaleimide [28,69] and colchicine [63]. Staurosporine, H-7, and polymyxin B are non-specific inhibitors of protein kinase C compared to cyclic AMP-dependent protein kinase, CaM-PK or protein-Tyr kinases [70]. All non-selective inhibitors act on the catalytic domain of protein kinase C, which contains a high degree of sequence homology with other protein kinases. The more specific inhibitor calphostin-C [71] acts on the regulatory domain (Fig. 1). Chelerythrine, another specific inhibitor [67] interacts with the catalytic domain but also competes with the classically used protein substrates of protein kinase C [57]. Furthermore, polymyxin B directly blocks K^+_{ATP} channels, one of the possible target proteins of protein kinase C and is therefore inappropriate to investigate the role of protein kinase C in ischemic preconditioning [72,73]. Moreover, it is unknown whether protein kinase C inhibitors are equipotent for all enzyme isotypes. It is quite feasible that the degree of inhibition depends on both the isotype [60,74–76] and species. Table 1 shows that polymyxin B [66], staurosporine [48] and chelerythrine [48] abolished the protective effect in isolated rat hearts and calphostin-C [68] and chelerythrine [53] in the in vivo rat model. Polymyxin B [17,52,55,56] and staurosporine [17] abolished protection in in vitro and polymyxin B [52], staurosporine [52,63], chelerythrine [67] and colchicine [63] in in vivo rabbit model. In dogs, the data are different as Przyklenk et al [64] observed that polymyxin B and H-7 did not abolish cardioprotection by ischemic preconditioning. In a preliminary study in pigs, staurosporine and bisindolylmaleimide limited infarct size [69]. These studies suggest a different role for protein kinase C in rats and rabbits than in dogs and pigs. The different results in rat, rabbit and pig could be related to species-dependent differences in myocardial expression, in the task performed and the site of translocation of the various isotypes [38,39,48,64,77]. Moreover, the various isozymes might be activated differently by Ca^{2+}, 1,2-DAG and free fatty acids [24,25,36,37].

Reviewing the studies using pharmacological blockade one is tempted to conclude that the protein kinase C family is involved in the mechanism of ischemic preconditioning in rat and rabbit. However, none of the studies investigated whether blockade of protein kinase C actually occurred in the in vivo model. In vitro assays of enzyme activity will not provide conclusive answers also because these have to be performed in subcellular fractions in the absence of the inhibitor due to the isolation procedure and in the presence of optimal amounts of 1,2-DAG and/or Ca^{2+}. The ideal experimental design would be to study the enzyme activity in vivo by measuring phosphorylation degrees of one or more of the unknown specific target proteins.

Activation of protein kinase C (Table 2)

Activation of protein kinase C by administration of phorbol esters, such as 12-O-tetradecanoyl phorbol-13-acetate (TPA) and PMA [27,66] or 1,2-DAG analogues such as 1-stearoyl-2-arachidonoyl glycerol (SAG) [48], 1,2-dioctanoyl sn-glycerol (DOG) [65] and oleyl acetylglycerol (OAG) [64] prior to a sustained coronary artery occlusion has been a second approach to investigate the role of protein kinase C in ischemic preconditioning. The advantage of using phorbol esters over 1,2-DAG as activating substances is that they are not metabolized and produce prolonged protein kinase C activation. Protein kinase C translocation takes place after phorbol ester (or the 1,2-DAG analog) is bound to the enzyme's regulatory domain whereby it obtains not only an increased affinity for acidic membrane phospholipids (PtdSer) but also an increased activity (Fig. 1).

PMA and several 1,2-DAG analogs mimic preconditioning in the rat [53] and rabbit [52,63], but PMA failed to limit infarct size in pigs [69]. Przyklenk et al [64] measured protein kinase C translocation after administration of PMA in dogs. Although these studies lack information regarding activation (translocation) of the isotype(s) in relation to the protective effect, the results with activators suggest a role for protein kinase C in ischemic preconditioning in rat and rabbit. Furthermore, the route of administration and the dose used may be different for the large animal studies and the in vitro and in vivo studies of small animals. For instance, Vogt et al [69] used intramyocardial administration of PMA (1 μM) to activate protein kinase C but failed to mimic the protective effect of ischemic preconditioning. However, the dose could have been too high because Cohen et al [78] found that 0.2 nM PMA was protective, while 2 nM PMA was damaging in the isolated rabbit heart. It is feasible

Table 1. *Protein kinase C inhibitors and protection by ischemic preconditioning.*

Model	Species	CP-Stimulus x(I + R)(min)	Prolonged-Ischemia I(R)(min)	Protein kinase C inhibitor	Evaluation (ISL/FR)	Result	Reference
Isolated heart	Rat	3x(5I + 5R)	30I(120R)	Polymyxin B, before CP-stim	ISL	Abolishes	[66]
		3x(5I + 5R)	30I(120R)	Chelerythrine, before CP-stim	ISL	Abolishes	
		1x(2I + 10R)	20I(40R)	Staurosporine, before CP-stim	FR	Abolishes	[48]
		Phenylephrine + 10R	20I(40R)	Staurosporine, before CP-stim	FR	Abolishes	
		1x(2I + 10R)	20I(40R)	Chelerythrine, before CP-stim	FR	Abolishes	
		Phenylephrine + 10R	20I(40R)	Chelerythrine, before CP-stim	FR	Abolishes	
	Rabbit	1x(5I + 10R)	30I(120R)	Staurosporine, before and after CP-stim	ISL	Failed to abolish	[17]
		1x(5I + 10R)	30I(120R)	Staurosporine, after CP-stim	ISL	Abolishes	
		Bradykinin	30I(120R)	Staurosporine, after CP-stim	ISL	Abolishes	
		Bradykinin	30I(120R)	Polymyxin B, 50 min starting 5 min before CP-stim	ISL	Abolishes	
		1x(5I + 10R)	30I(180R)	Polymyxin B, 5 min after CP-stim	ISL	Abolishes	[52]
		Phenylephrine	30I(120R)	Polymyxin B, before and after CP-stim	ISL	Abolishes	[55]
		Angiotensin II	30I(120R)	Polymyxin B, 50 min starting 5 min before CP-stim	ISL	Abolishes	[56]
In Vivo	Rat	1x(5I + 10R)	45I(150R)	Cheleythrine, after CP-stim	ISL	Abolishes	[53]
		3x(3I + 5R)	90I(240R)	Calphostin C, before and after CP-stim	ISL	Abolishes	[68]
	Rabbit	1x(5I + 10R)	30I(180R)	Staurosporine, 5 min after CP-stim	ISL	Abolishes	[52]
		1x(5I + 10R)	30I(180R)	Polymyxin B, 5 min after CP-stim	ISL	Abolishes	
		1x(5I + 10R)	30I(180R)	Chelerythrine, 8 min after CP-stim	ISL	Abolishes	[67]
		1x(5I + 10R)	30I(180R)	Staurosporine, before CP-stim	ISL	Attenuates	[63]
		1x(5I + 10R)	30I(180R)	Colchicine, 30 min before before CP-stim	ISL	Abolishes	
	Dog	4x(5I + 10R)	60I(240R)	H-7 (IV), before, during and after CP-stim	ISL	Failed to abolish	[64]
		4x(5I + 10R)	60I(240R)	H-7 (IC), before, during and after CP-stim	ISL	Failed to abolish	
		4x(5I + 10R)	60I(240R)	Polymyxin B, before, during and after CP-stim	ISL	Failed to abolish	
		4x(5I + 5R)	90I(360R)	Polymyxin B, 5 min before and during CP-stim	ISL	Abolishes	[28]
		4x(5I + 5R)	90I(360R)	GF109203X, 5 min before and during CP-stim	ISL	Abolishes	
		Methoxamine	90I(360R)	Polymyxin B, 5 min before and during CP-stim	ISL	Abolishes	
		Methoxamine	90I(360R)	GF109203X, 5 min before and during CP-stim	ISL	Abolishes	
	Pig	2x(10I + 30R)	60I(120R)	Staurosporine	ISL	Failed to abolish	[69]
		BIS	60I(120R)	Bisindolylmaleimide	ISL	Mimics	

I = ischemia; R = reperfusion; CP = cardioprotective; BIS = bisindolylmaleimide; H-7 = 1-(5-isoquinolinesulfonyl)-2-methylpiperazine; ISL = infarct size limitation; FR = functional recovery.

Table 2. *Protein kinase C activators and myocardial infarct size.*

Model	Species	CP-Stimulus	Prolonged-Ischemia I(R) (min)	Evaluation (ISL/FR)	Result	Reference
Isolated heart	Rat	SAG	20I(40R)	FR	Mimics	[48]
	Rabbit	PMA	30I(180R)	ISL	Mimics	[52]
		OAG	30I(180R)	ISL	Mimics	
		PMA	30I(120R)	ISL	Mimics	[63]
In Vivo	Rat	DOG	45I(150R)	ISL	Mimics	[53]
	Rabbit	PMA	30I(180R)	ISL	Mimics	[63]
	Pig	PMA	None	ISL	Failed to mimic	[69]

I = ischemia; R = reperfusion; CP = cardioprotective; SAG = 1-stearoyl-2-arachidonoyl glycerol; PMA = phorbol-12-myristate-13-acetate; OAG = oleyl acetyl-glycerol; DOG = 1,2-dioctanoyl sn-glycerol; ISL = infarct size limitation; FR = functional recovery.

Table 3. *Protein kinase C translocation/activation and protection by ischemic preconditioning.*

Model	Species	CP-Stimulus x(I + R) (min)	Prolonged-Ischemia I(R) (min)	PKC assay	Result	Reference
Isolated heart	Rat	1x(2I)	None	Immunohistofluorescence	PKC-δ to sarcolemma, ϵ to nucleus, no ζ, α, β_1	[48]
		Phenylephrine	None	Immunohistofluorescence	PKC-δ to sarcolemma, ζ to nucleus, no ϵ, α, β_1, η	
In Vivo	Dog	4x(5I + 10R)	None	Fluorescent to binding by confocal microscopy	No PKC translocation	[64]
		4x(5I + 10R)	None	Activity by protein phosphorylation	No PKC activation	
		None	10I	Activity by protein phosphorylation	PKC activation vs Con	
		4x(5I + 10R)	10I	Activity by protein phosphorylation	PKC activation vs Con	
		PMA	None	Activity by protein phosphorylation	PKC activation	
	Dog	4x(5I + 5R)	None	Activity by protein phosphorylation	PKC activation	[28]
	Pig	1x(10I + 7.5R)	None	Immunoreactivity on Western blot	PKC-ϵ translocation	[58]
		1x(10I + 7.5R)	None	Activity by protein phosphorylation	PKC-ϵ translocation	
	Pig	2x(10I + 30R)	None	Activity by protein phosphorylation	PKC activation	[69]
		PMA	None	Activity by protein phosphorylation	PKC activation	

I = ischemia; R = reperfusion; CP = cardioprotective; PMA = phorbol-12-myristate-13-acetate; PKC = protein kinase C

that in that study the low dose of PMA activated an isotype that is protective, while at the higher dose an isotype was activated that was damaging [78].

Measurements of protein kinase C-translocation (activation) (Table 3)

Translocation of protein kinase C from the cytosol to the membrane has been investigated employing: (1) immunoblot analysis using protein kinase C isotype-specific antibodies of SDS-electrophoretically separated subcellular fractions isolated from myocardial homogenates [44,51,69]; (2) immunohistofluorescence detection (with confocal microscopy) of protein kinase C isotypes in sections of myocardial tissue [48,64]; (3) assay of total protein kinase C activity in subcellular fractions isolated from myocardial homogenates by measurement of Ca^{2+}- and/or 1,2-DAG-dependent ^{32}P incorporation from γ-^{32}P-labelled ATP into histone III-S or a protein kinase C isoenzyme-specific substrate protein such as peptide-ϵ [51,54,64,79] or other peptides [28,64]. All three methods have their limitations. For instance, in protein phosphorylation or immunoreactivity assays, cardiac biopsies are usually rapidly frozen in liquid N_2,

followed by preparation of particulate fractions from the homogenates. It can not be excluded, however, that redistribution or (in) activation occurs during isolation of the subcellular fractions. In the subfractions both the basal rate and the maximum rate of histone III-S (or other substrate protein) phosphorylation are measured in the presence of Ca^{2+}, PtdSer and 1,2-DAG. The results of these measurements only reflect the total protein kinase C activities in the subcellular fractions. Moreover, histone III-S is a relatively poor substrate for some nPKC's (δ, ε and η) compared to the cPKC's [41]. Therefore, measurements of the rate of ^{32}P incorporation into the synthetic protein kinase C-ε-specific substrate peptide-ε may provide the required information about the ε isotype [51]. It should also be noted that mixed micelles of Ca^{2+}-1,2-DAG-PtdSer embedded in Triton-X-100 micelles, used to activate protein kinase C in the ^{32}P incorporation assays, only mimic the cellular membrane environment of protein kinase C in the intact cell. It is unknown whether myocardium is preconditioned homogenously or heterogeneously. In the latter case, the sampling site of the biopsy may pose a restriction. Because protein kinase C assayed in subcellular fractions isolated from homogenates of whole myocardial tissue represents a mixture of activities of myocytes, fibroblasts, smooth muscle cells and endothelial cells, immunohistofluorescence measurements must therefore be performed to provide information on the cell type involved in protein kinase C translocation/activation. Measurements of protein kinase C isotype activity by immunohistofluorescence must, however, be interpreted with caution, because the specific antibodies are not always capable to distinguish active from inactive protein kinase C isotypes.

Weinbrenner et al, using Western blotting, showed in rats a rapid translocation of the Ca^{2+}-dependent protein kinase C isotype α and the Ca^{2+}-independent isotypes (δ, ε and ζ) to the sarcolemma after brief ischemia and increased expression of the Ca^{2+}-independent protein kinases C-δ and -ε in the cytosol after prolonged ischemia [24]. Mitchell et al, using immunohistofluorescence, showed in rat hearts that protein kinase C-δ translocated from the cytosol to the sarcolemma after both brief ischemia and α_1-adrenergic stimulation [48]. Brief ischemia also caused translocation of protein kinase C-ε from the cytosol to the nuclear region. Measurements of other protein kinase C-isotypes (α, β_1, ζ and η) did not provide evidence for occurrence of translocation after brief ischemia or α_1-adrenergic stimulation [48]. These results provide the first evidence that (at least in the rat) specific protein kinase C isotypes are involved in ischemic preconditioning.

Przyklenk et al used a probe consisting of the protein kinase C inhibitor bisindolylmaleimide conjugated to fluorescein that selectively binds to active protein kinase C and observed no difference in the total amount and the cellular distribution of protein kinase C fluorescence with preconditioning in dogs [64]. The advantage of this method over immunofluorescence is that it distinguishes between active and inactive protein kinase C. In their study Przyklenk et al also obtained quantitative information on the changes in the amount and subcellular distribution of protein kinase C by measuring the rate of ^{32}P incorporation into the threonine group of a protein kinase C-specific peptide, which was not further specified [64]. A small rise in protein kinase C activity was found in the membrane fraction isolated from biopsies obtained after 10 min of ischemia compared to those isolated after four sequences of 5 min occlusion-reperfusion or no intervention [64]. However, no difference in protein kinase C activity between matched groups of controls and 'ischemic preconditioned' dogs could be measured at time points comparable to the onset of the long occlusion or at 10 min into sustained ischemia [64]. Using the same protein kinase C analysis, Vogt et al found a modest (10 to 20%) redistribution of protein kinase C from the cytosol to the membrane fraction in pig hearts subjected to 10 min of ischemia [69]. In contrast to the studies by Przyklenk et al [64], Kitakaze et al [28] observed in the same canine model a marked translocation of Ca^{2+}- and lipid-dependent protein kinase C activity in cytosol and membrane fractions isolated from preconditioned epi- as well as endomyocardium. These authors ascribed their positive findings to the time of measurements (5 min after the preconditioning stimulus against 10 min by Przyklenk et al [64]).

We studied translocation of protein kinase C enzyme activity by ^{32}P incorporation into histone III-S and ε-peptide and immunoreactivity of a number of protein kinase C isoforms (α, ε, δ and ζ) of cytosolic and membrane fractions isolated from biopsies of porcine myocardium preconditioned by a 10-minute coronary artery occlusion and 7.5-minutes of reperfusion [51,80]. Ca^{2+} and 1,2-DAG-stimulated protein kinase C activity with histone III-S as substrate was higher in the cytosolic and particulate fractions isolated from the preconditioned myocardium than from the control region. Significant Ca^{2+}-independent, 1,2-DAG-stimulated phosphorylation of ε-peptide was found in the cytosolic fractions, but not in the particulate fractions. However, no significant increase of 1,2-DAG-stimulated phosphorylation of ε-peptide in the cytosolic fraction from the preconditioned myocardium was observed. These were rather unexpected findings in view of our protein kinase C-ε immunoreactivity measurements (see below). The cytosolic and particulate fractions were also examined by immunoblot analysis using rabbit polyclonal antibodies specific for protein kinase C-α, -δ, -ε and -ζ isotypes [51]. This analysis revealed significant levels of expression of the Ca^{2+}-independent isotype protein kinase C-ε, the abundant presence of protein kinase C-α, while protein kinase C-δ and -ζ were barely undetectable. The immunoreactivity data also indicate that neither the

(Ca^{2+} and 1,2-DAG)-stimulated histone III-S and peptide-ε kinase activities of the cytosolic nor those of the particulate fractions reflect the relative immunoreactivities in the corresponding fractions. In contrast to the ^{32}P-incorporation data, the immunofluorescence data suggested that the total amount and subcellular distribution of protein kinase C-α and -ε was not altered in the preconditioned region compared to the non-ischemic region of the left circumflex coronary artery. Therefore, by using immunofluorescence we were unable to detect the occurrence of ischemia-induced expression of protein kinase C or ischemia-induced translocation of protein kinase C from the cytosolic to the particulate fraction of the protein kinase C isotype -α, -δ, -ε or ζ. On the other hand, binding proteins, such as myristoylated-alanine-rich-C-kinase-substrate (MARCKS) and RACK may determine whether activated protein kinase C isotypes translocate and are providing another mechanism for functional specificity to specific intracellular locations. Thus, in the in vitro phosphorylation assays of the subcellular fractions, different amounts of MARCKS- or RACK-bound protein kinase C isotypes can alter the protein kinase C activity measured [81]. This could cause the discrepancy between the activity assays and Western blotting. Nevertheless, our results on ^{32}P incorporation demonstrate an increase in cytosolic and membrane-bound protein kinase C activities due to brief ischemia and supports a role for protein kinase C in ischemic preconditioning in pigs [51].

Concluding Remarks

At present MARCKS is the only endogenous target protein for protein kinase C, that has been shown to be phosphorylated in preconditioned rabbit myocardium. However, the former is believed to be an intracellular location site rather than a protein factor intimately involved in the protective response [82]. Irrespective of the target protein(s) we are dealing with, its (their) covalently bound phosphates must be relatively stable during the 2 to 3 hours in which the cardioprotection is present (CPP). Furthermore, the precise time point that protein kinase C is maximally translocated (activated) during ischemia or reperfusion (preconditioning stimulus) is unknown and consequently also the time point at which the enzyme reaches the target proteins for catalyzing their phosphorylation. It is quite feasible that protein kinase C is removed from its translocation site or proteolytically degraded after it has performed its action and thereafter it is no longer detectable by immunoreactivity or activity measurements. It is therefore mandatory to determine the time course of translocation/activation and subsequent relocalization/inactivation or proteolytic degradation of the protein kinase C isotype and the time course(s) of phosphorylation and dephosphorylation or proteolytic degradation of the target protein(s). Because the time course of weaning of the protective effect of the CPP is roughly known, the time course of dephosphorylation/inactivation of the target protein could be correlated to the time course of weaning of protection. Candidate target proteins of protein kinase C involved in the CPP are e.g. the K^+_{ATP} channel [13,14] and/or the ecto-5'-nucleotidase [28], but experimental evidence for phosphate incorporation into these proteins or regulating proteins is lacking. If the K^+_{ATP} channel or the ecto-5'-nucleotides are target proteins the most likely translocation site for the protein kinase C isotype(s) involved in the CPP is the sarcolemma.

Protein kinase C is involved in the agonist-receptor interaction induced changes in gene expression of many cells [25,36,37,41,74,75,83]. Taking into account the time required for inducing heat shock/stress proteins [29,30] the former may only play a role in SWOP. Therefore, a transcription factor involved in the regulation of expression of heat shock/stress proteins could be another potential target protein of protein kinase C. If true, the nucleus may be the translocation site for the protein kinase C isotype(s).

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