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Regulation of Cardiac Excitability Protein Kinase C Isozymes

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Abstract

Cardiac excitability and electrical activity are determined by the sum of individual ion channels, gap junctions and exchanger activities. Electrophysiological remodeling during heart disease involves changes in membrane properties of cardiomyocytes and is related to higher prevalence of arrhythmia-associated morbidity and mortality. Pharmacological and genetic manipulation of cardiac cells as well as animal models of cardiovascular diseases are used to identity changes in electrophysiological properties and the molecular mechanisms associated with the disease. Protein kinase C (PKC) and several other kinases play a pivotal role in cardiac electrophysiological remodeling. Therefore, identifying specific therapies that regulate these kinases is the main focus of current research. PKC, a family of serine/threonine kinases, has been implicated as potential signaling nodes associated with biochemical and biophysical stress in cardiovascular diseases. Thus, the role of PKC isozymes in regulating cardiac excitability has been a subject of great attention. In this review, we describe the role of PKC isozymes that are involved in cardiac excitability and discuss both genetic and pharmacological tools that were used, their attributes and limitations. Selective and effective pharmacological interventions to normalize cardiac electrical activities and correct cardiac arrhythmias will be of great clinical benefit.

INTRODUCTION

The final common pathway of electrical excitability in the heart is the generation of conducted action potentials, whereas voltage-gated ion channels and gap junctions play an important role in the action potential electrogenesis (1, 2). Cardiovascular diseases (i.e. coronary artery disease, stroke and heart failure) are associated with changes of cardiac electrical excitability due to transcriptional and post-translational modifications of ion channels and gap junction proteins (3, 4).

Conceptual and technical breakthroughs during the last decade have led to revolutionary advances in our knowledge of allosteric regulation of ion channels, whereas several studies have focused on identifying cellular nodes where signals converge and serve as multi-effector brakes to re-establish the normal electrophysiological properties inside the damaged heart. Nowadays, there is abundant evidence showing that channel phosphorylation can influence profoundly the electrical properties of cardiomyocytes and other cells (5, 6), whereas various kinases have been described as candidate mediators of the pathological cardiac electrophysiological remodeling (6–8). Among different kinases, activation of PKC isozymes has been a subject of great attention in the cardiovascular field since it is

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implicated in pathological settings such as heart failure (9, 10), ischemia (11, 12) and ischemic preconditioning (13–15).

We first demonstrated the relevance of PKC isozymes to cardiac excitability by showing that prolonged exposure of isolated neonatal cardiomyocytes to 4-β phorbol ester 12-myristate-13-acetate (PMA, 1–100nM, a non-selective PKC activator) enhanced the rate of contraction in a dose-dependent fashion (16). Indeed, several studies have reported the contribution of specific PKC isozymes to cardiac excitability by regulating ion channel expression (17–19) and activity (20–22), and inotropic and chronotropic effects (23). In this review, we focus on insights gained from the rational design of small molecule inhibitors and activators of selective PKC isozymes to understand their critical role in modulating cardiac excitability (Figure 1).

PKC family

PKC is a group of ten closely related phospholipid-dependent serine-threonine protein kinases (24). These isozymes are classified based on their structure and activation requirements into three subgroups: conventional PKCs (α, βI, βII and γ), which are Ca-dependent and activated by diacylglycerol (DAG) and phosphatidylserine (PS); novel PKCs (ε, δ, η and θ), which are Ca-independent but activated by DAG and PS; and atypical PKCs (ζ and ι/λ), which are Ca and DAG-independent but activated by PS.

Of these PKC isozymes, α, β_I, β_{II}, ε, θ, λ, δ, ζ and γ PKC have been found in the heart in different animal species (25, 26), and high levels of PKC isozymes α, β_I, β_{II}, δ and ε have been reported in tsA201 and HL-1 cell lines (27). The presence of multiple PKC isozymes in the same tissue and even in the same cell type suggests that individual isozymes mediate distinct cellular functions. The effort to dissect the roles of individual PKC isozymes in cell functions has been hampered by the lack of isozyme-specific activators and inhibitors.

General PKC regulators (non-isozyme selective)

A number of small molecule drugs that regulate PKC isozymes has been generated over the last decades. Many of these compounds are competitive inhibitors of ATP binding to the catalytic site of the kinase (Table 1). However, because the catalytic site within the PKC family as well as in other protein kinases is highly conserved, generating a truly selective inhibitor of one protein kinase has proven to be a challenge (28) (Table 1). Small molecule activators of PKC isozymes are also available. Those compounds mimic the second messenger DAG and are related to the tumor promoter phorbol ester (PMA) (29), to bryostatin (30) and some other natural compounds. Some activators have higher affinity for one specific PKC subgroup over others in a dose-dependent manner (16). However, considering that these compounds are not able to distinguish the activation of PKC isozymes in the same subgroup, they are usually described as non-selective PKC activators. Furthermore, because there are multiple diacylglycerol-binding proteins (31), using them as PKC isozyme selective tools works only in a narrow concentration range and can activate proteins other than PKCs.

Isozyme-selective PKC regulators

Over twenty years ago, we suggested that protein-protein interactions play a critical role in determining the selectivity of individual PKC isozymes. We identified a family of proteins, collectively termed RACKs (Receptors for Activated C Kinase) that anchor individual activated PKC isozymes near their substrates and away from others (32). We then designed peptide regulators of these protein-protein interactions as isozyme-selective inhibitors (Figure 2). The rationale used for the design of the peptides and the data used to demonstrate

their selectivity were provided in over 200 publications from a variety of labs. For recent review see (33). In short, the peptides, 6–10 amino acids in length, correspond to unique sequences within the PKC isozyme-binding site on the RACK or the RACK-binding site on that PKC isozyme. Using this approach, we have identified selective inhibitors for all the PKC isozymes (see Table 1).

Selective 6–10 amino acid-long peptide activators of PKC isozymes were also rationally designed. These peptides are derived from intra-molecular interaction sites within each PKC isozyme (34). Each peptide activator interferes with an isozyme-specific (or sub-family-specific) inhibitory intra-molecular interaction within that PKC; each peptide is identical in sequence to one of the two intra-molecular interaction sites. By acting as a competitive inhibitor of the inhibitory intra-molecular interactions, the peptide acts as a selective activator of that isozyme. The intra-molecular interaction that we focused on is the pseudo-RACK site, the site that covers the RACK-binding site when PKC is inactive. Peptides derived from the pseudo-RACK binding site are selective activators of the corresponding PKC. Using this approach, we have identified a selective activator for all the conventional PKC isozymes, ψβRACK, and isozyme-selective activators for δ, ε, η and θPKCs (see Table 1).

Peptides that act intracellularly need to be conjugated to carrier peptides to deliver them across biological membranes. A number of such short peptide carriers were identified. We used mainly $TAT_{47–57}$ (35). All the peptide regulators of PKC conjugated to such peptide carriers showed high selectivity for the corresponding isozyme. They were efficacious *in vitro* and *in vivo* at low nanomolar concentrations and were well-tolerated when acutely injected intravenously, intraperitoneally, and subcutaneously. They were also effective when delivered in a sustained fashion, using subcutaneous Alzet pumps to obtain weeks of constant delivery (3–10mg/kg/day).

PKC and Ca Channels

Ca channels consist of a central α_1 subunit, which forms the ion-conducting pore, and the auxiliary subunits α_2-δ, β and γ, which modulate surface expression and biophysical properties. In the sarcolemma of cardiac cells, two types of Ca channels have been identified: the high-voltage, L-type Ca channel, and the low-voltage, T-type Ca channel (2). They play an important role in normal and diseased myocytes (1).

L-type Ca channels

The influx of Ca through L-type Ca channels plays an essential role in both cardiac excitability and excitation-contraction coupling. The depolarizing current through L-type Ca channels contributes to the cardiac action potential plateau and to Ca-induced Ca release from the sarcoplasmic reticulum. Four genes encode L-type Ca channel α_1 subunits in mammals: Cav1.2 (α_{1C}), Cav1.3 (α_{1D}), Cav1.1 (α_{1S}) and Cav1.4 (α_{1F}) (36). α_{1S} and α_{1F} expression is restricted to the skeletal muscle and the retina. α_{1C} is assumed to be the most abundant isoform in the cardiovascular system, whereas α_{1D} is expressed in neurons and neuroendocrine cells (1, 37). It is therefore believed that the contribution of L-type Ca currents to the physiology/pathophysiology of the heart and the therapeutic effects of Ca channel antagonists are mainly mediated through α_{1C} Ca channels. This assumption has been challenged by recent findings that α_{1D} knockout mice unexpectedly exhibited intrinsic sino-atrial dysfunction and predisposition to atrial fibrillation (38–40), suggesting an important functional role for the α_{1D} Ca channel in the supraventricular tissue.

Multiple G protein-coupled receptors in the heart act through phospholipase C-dependent hydrolysis of membrane phosphoinositides to regulate many cellular proteins, including the

L-type Ca channels. Early studies suggested that neurohormones (i.e. endothelin-1 and angiotensin II) modulate L-type Ca channels in a PKC isozyme-dependent manner. For example, acute endothelin 1 treatment (which activates PKC) resulted in increased, decreased or no change in basal Ca influx in isolated cardiomyocytes under different conditions (41–43).

Disparate results have also been found in studies using non-selective PKC activators. The published work from our group showed that PMA (a non-selective PKC activator) consistently inhibited Ca channels in rat ventricular cells (22, 44). However, PMA has also been reported to inhibit, have no effect, stimulate, or initially stimulate but later inhibit L-type Ca currents in cardiac myocytes (45). Non-selective PKC activators produce inconsistent responses in non-cardiac Ca channels as well. Stimulation of Ca channels by phorbol esters has been reported in Aplysia bag cell neurons (46), neuroblastomas (47), secretory RINm5f cells (48) and frog sympathetic neurons (49). Conversely, inhibition of Ca channels by phorbol esters has been reported in chick DRG cells (50), and PC-12 cells (51). Because specific PKC isozymes contribute to a wide variety of cellular responses and sometimes exert even opposite effects inside the cell, the usage of non-selective PKC activators might provide misleading information on the relative contribution of PKC isozymes to Ca channels. It seems therefore that the outcome of PKC activation is species and tissue-dependent, probably because of the isozyme type present in each cell type.

Over the last decade, the rational design of selective PKC modulators as well as the use of genetic models has contributed to better understanding the role of selective PKC isozymes on both α_{1C} and α_{1D} L-type Ca channels. The PKC phosphorylation sites of the cardiac α_{1C} subunit of the L-type Ca channel have been mapped to threonines 27 and 31 of the N-terminus, in which conversion of the threonines 27 and 31 to alanine abolishes the PKC-mediated downregulation of α_{1C} Ca channel current ($I_{Ca\text{-}L}$) in tsA-201 cells (52). In parallel, mutating the threonines 27 and 31 to aspartate restored the PKC sensitivity of α_{1C} $I_{Ca\text{-}L}$, suggesting that change in net charge by phosphorylation of threonines 27 and 31 of the N-terminus is responsible for the α_{1C} $I_{Ca\text{-}L}$ inhibition (52). In order to dissect the role of specific PKC isozymes in the regulation of cardiac Ca channels, we took advantage of PKC-regulating peptides developed by our lab (24, 33). Intracellular delivery of selective εPKC activator peptide (ε V1-7) resulted in significant inhibition of $I_{Ca\text{-}L}$ in ventricular myocytes isolated from adult rats (22). The inhibitory effect of εV1-7 was completely prevented by the εPKC peptide inhibitor (εV1-2). Because sustained εPKC activation has been associated with cardiac dysfunction and heart failure in different animal models (53–55), we tested whether chronic *in vivo* modulation of εPKC leads to altered $I_{Ca\text{-}L}$. Using transgenic mice with specific cardiac constitutive activation of εPKC (expressing the pseudo-RACK sequence of εPKC, ψεRACK), we found that sustained εPKC resulted in reduced basal $I_{Ca\text{-}L}$ density as well as blunted the β-adrenergic activation of $I_{Ca\text{-}L}$ (56).

Besides the negative effects of εPKC on α_{1C} $I_{Ca\text{-}L}$ by phosphorylating amino acid residues at N-terminal domain, Yang *et al.* (2005) have demonstrated in HEK293 cells that α, ε and ζ PKC isozymes phosphorylated serine 1928 at the C-terminal domain of the α_{1C} L-type Ca channel, resulting in increased α_{1C} L-type Ca channel activity (57). The serine 1928 phosphorylation has been previously related to up-regulation of α_{1C} L-type Ca channel activity in a protein kinase A-dependent manner (58). Furthermore, Yang *et al.* (2009) have showed in Langendorff-perfused heart that both serines 1674 and 1928 were phosphorylated in response to PMA (non-selective PKC activator) (59). Using GST fusion proteins, the authors provided evidence that α, βI, βII, γ and θ PKC isozymes were able to phosphorylate the serine 1674 site in HEK293 cells. Therefore, isolated hearts from transgenic mice over-expressing αPKC displayed increased phosphorylation of serine 1674 and 1928 (59). Conversely, αPKC knockout mice presented decreased cardiac levels of serines 1674 and

1928 phosphorylation (59). Besides its effect on cardiomyocyte excitability, αPKC may affect cardiac excitation-contraction coupling through a negative regulation of sarcoplasmic reticulum Ca load, thereby affecting cardiac performance during cardiac dysfunction and heart failure (60).

Unlike the well-characterized α_{1C} L-type Ca channels, α_{1D} L-type Ca channels have not been highlighted in earlier studies. We have demonstrated in the tsA201 cell line, using single channel analysis, that PMA (non-selective PKC activator) treatment resulted in a decrease in open probability and increase in closed-time without any significant effect on the conductance of the α_{1D} L-type Ca channel (61). More recently, we showed that phosphorylation of serine 81 in the N-terminal region plays a negative role in α_{1D} Ca channel activity in tsA201 cell line (62). This response was mediated by non-selective PKC activation. The introduction of a negatively charged residue at position 81, by converting serine to aspartate, mimicked the PKC phosphorylation effect on the α_{1D} Ca channel. The modulation of the α_{1D} Ca channel by PKC was prevented by dialyzing cells with a 35 amino acid peptide mimicking the α_{1D} N-terminal region comprising serine 81. In addition, we evaluated, in the tsA201 cell line, the role of specific PKC isozymes on α_{1D} $I_{Ca\text{-}L}$ by using different PKC isozyme-selective inhibitor peptides. We first demonstrated that βIIV5-3 (selective βIIPKC inhibitor) and εV1-2 (selective εPKC inhibitor) peptides antagonized the negative effects of PMA on α_{1D} $I_{Ca\text{-}L}$ (62). These results indicate that phosphorylation of serine 81 by βIIPKC and εPKC is required for down-regulation of α_{1D} Ca channel activity. Parallel to their effects on cardiomyocyte excitability, βIIPKC and εPKC may affect cardiac excitation-contraction coupling through a negative myofilament Ca-sensitivity and cardiac hypertrophy, respectively, thereby affecting cardiac function during ventricular remodeling and heart failure (10, 54, 63).

T-type Ca channels

Transient low-voltage activated currents (I_{CaT}) characterize T-type channels. They have been identified in the sinoatrial node, AV node, Purkinje system, vascular smooth cells, and in neurons (2, 37). The T-type Ca channel has been involved in pacemaking and in Ca signaling in secretory cells and vascular smooth muscle (37). I_{CaT} has been implicated in triggering Ca release from the sarcoplasmic reticulum (64) and in contraction of Purkinje cells (65). There is evidence indicating that T-type Ca channels also promote cell proliferation and growth (66). In this regard, increased levels of re-expression of T-type Ca channels in certain pathologic states, such as ventricular hypertrophy, have been reported by others (67) and us (68). Only a few studies reported on the interaction of PKC with T-type Ca channels in the heart. Tseng and Boyden (69) demonstrated that PKC activation inhibited I_{CaT} in canine cardiac cells. Similarly, Zhang *et al.* (2000) showed that indirect activation of PKC by arachidonic acid attenuated the recently cloned human T-type α_{1H} current expressed in HEK 293 cells (70). At the molecular level, Cribbs *et al.* (1998) showed the existence of several PKC consensus motifs on the T-type Ca channel α_{1H} subunit (71). Two sites are located on the domain I–II cytoplasmic loop and one site on the domain III–IV connector. Zheng *et al.* (2010) recently showed that lysophosphatidylcholine up-regulated T-type Ca channels in a PKC-dependent manner (72). However, the identity of the PKC isozyme which regulates T-type Ca channels is not yet known.

Based on the findings described above, we summarize that integration of different regulatory mechanisms controlled by specific PKC isozymes may provide sensitive modulation of Ca channel activity in response to cardiac physiopathology. In fact, it is possible that different PKC isozymes may have opposing effects on Ca channels, as previously suggested by the effect of PMA on the L-type Ca channel activity. Therefore, considering that Ca channels are the main mediators of Ca influx and are pivotal in cardiac function, further studies

clarifying the physiological contribution of PKC isozyme-mediated Ca channel function need to be performed in normal and diseased hearts.

PKC and Na Channels

Voltage-gated Na channels are the primary channels responsible for the rising phase of the action potential in excitable cells. The regulation of Na channels by PKC has been studied using general PKC activators such as PMA (73) and OAG (1-oleoyl-2-acetyl-*sn*-glycerol) (74). In general, activation of PKC by these non-isozyme specific activators leads to a reduction in Na current in both brain and heart (75–77). Heterologously expressed rat brain (rBIIA) (73, 77) and human cardiac Na channel currents (hH1) (76) were reduced upon PKC activation. While both rBIIA and hH1 contain consensus sites for phosphorylation by PKC, most of the sites are not conserved between these two isozymes. In one study (76), elimination of conserved consensus PKC sites in the hH1 interdomain III–IV linker, which contains the putative PKC site (Ser^{1503}) does not completely eliminate the PMA-induced Na current inhibition, implying that other phosphorylation site(s) may exist. Considering that Na channels are associated with other proteins forming a multi-protein complex (78), the remaining Na current may be due to PKC regulation of other kinases or phosphatases, and not necessarily to phosphorylation of another Na channel residue.

While these previous studies implicated PKC in the regulation of Na channels, the role and the identity of the isozyme(s) responsible for this regulation remained largely unexplored. Our laboratory has utilized rationally designed PKC modulators to gain insight into the Na channel regulation by PKC. Studies from our lab using Xenopus oocytes showed that the Na current is inhibited by PMA, a general PKC activator. In addition, the use of the peptide specific activator of εPKC, ψεRACK (Table 1), mimicked PMA effects on I_{Na} and the use of the peptide specific inhibitor of εPKC, εV1-2 (Table 1), prevented these effects, thus establishing the involvement of at least εPKC in the regulation of Na channels. Similarly, the decrease in the neuronal Nav1.8 peak current induced by PMA was prevented by a specific εPKC isozyme peptide antagonist, whereas the PMA effect on Nav1.7 was prevented by εPKC and βIIPKC peptide inhibitors (79).

Considering that βIIPKC and εPKC are key molecules involved in cardiac hypertrophy, heart failure [for recent review see (24)], and disrupted cardiac excitability (i.e. down-regulation of Ca and Na channel currents), it may be possible to consider using βIIPKC and εPKC isozyme-selective inhibitors as therapeutic tools for the treatment of chronic cardiac diseases.

PKC and K Channels

K channels are of importance in determining the shape and mostly the repolarization of the cardiac action potential (80). In cardiac myocytes, at least eleven K currents have been characterized pharmacologically or by molecular cloning. These currents include the inward rectifier K current, I_{Kl}; the transient outward K current, I_{to}; the ultra-rapidly activating delayed rectifier K current, I_{Kur}; and the rapidly I_{Kr} and slowly I_{Ks} activating delayed rectifier K currents (4). In general, the initial and rapid phase (phase 2) of the repolarization is caused by outward K movement (transient outward current, I_{to}) through rapidly activating and inactivating K channels, and the late phase (phase 3) is under the control of the delayed rectifier current (I_K). As such, K currents can modulate the duration of the action potential and refractoriness of the myocardium. It is noteworthy to emphasize that there is considerable heterogeneity in the morphology of action potentials and underlying K currents from various areas of the heart and between species.

The human ether-a-go-go-related gene hERG encodes the voltage-gated K channel underlying I_{Kr}, which initiates repolarization and terminates the plateau phase of the action potential (81). Several papers have reported that either α-adrenergic or non-selective PKC stimulation by PMA reduces hERG currents in cardiomyocyte (82–85). However, this phenomenon seems to be independent of direct PKC phosphorylation of the channel, since deletion by mutagenesis of PKC-dependent phosphorylation sites in hERG does not abolish PMA-induced effects on the channel (85). The cellular mechanisms of this PKC-mediated are still not completely understood. Of interest, the non-selective PKC inhibitor, BIM-I blocks hERG currents in a PKC-independent manner (86). Thus, PKC-independent effects have to be carefully considered when using non-selective PKC inhibitors in models involving I_{Kr} currents. Other K currents such as I_{to1}, I_{Kl} and Kv4.2/Kv4.3 currents are also negatively regulated by non-selective PKC activation in the heart (87), whereas increased εPKC translocation (and not δPKC) indirectly attenuates K currents by suppressing the synthesis of K channel proteins in the heart (88).

I_{Ks} channel is encoded by KvLQT1 and minK genes. KvLQT1/MinK channel contributes to the phase 2 slow repolarization of cardiac action potential. PKC has been suggested as a key mediator of KvLQT1/MinK channel regulation. The inhibition of the KvLQT1/MinK channel in ventricular myocytes by angiotensin II occurs in a PKC-dependent manner (89). In contrast, PKC is implicated in KvLQT1/MinK channel activation upon beta-3 adrenergic stimulation in Xenopus oocytes (90). Considering that different PKC isozymes have opposite effects in the cell (91), future studies are required in order to better understand the contribution of specific PKC isozymes in these processes. Mouse and rat I_{Ks} currents were decreased by PKC activation (92), whereas guinea pig I_{Ks} was activated by PKC (93). These findings suggest that regulation of I_{Ks} by PKC is also species dependent. In mouse and rat minK K channel, there is a putative PKC phosphorylation site at Ser102. Position Ser102 in the minK protein has been shown to be critical in determining the effect of PKC. Work in our lab has focused on the use of rationally designed small molecules to gain insight into the modulatory actions of PKC on the delayed rectifier, I_{Ks}. The data from our lab showed that in Xenopus oocytes, activation of PKC by PMA activates the human cardiac I_{Ks} channel and that selective inhibition of βIIPKC, but not βIPKC (using βIIV5-3 and βIV5-3, respectively; Table 1) abolishes PMA-induced I_{Ks} activation. These findings support the hypothesis of specialized function of only βIIPKC (21). However, additional work is required to better understand the behavior of K channels upon βIIPKC activation during cardiac stress such as ventricular dysfunction and heart failure. In addition, the identification of the particular PKC isozymes that mediate the regulation of K currents is of importance for the understanding of the mechanism of ion channel regulation and the development of new therapeutic agents. Future studies utilizing rationally designed PKC modulators may help to answer this question.

PKC and Gap Junction

In the heart, the gap junction plays an essential role in the electrical cell-to-cell coupling and impulse propagation between cells. Gap junction proteins are encoded by the connexin multigene family, whereas connexin 43 (Cx43) is the most abundant and widespread of the connexins in the heart. Cx43 phosphorylation influences its expression, degradation and functional properties such as conductance and open-probability of channels. Thus, dysfunction of the cardiac gap junction due to Cx43 post-translational changes mainly contributes to ventricular arrhythmia. Over the last decade, PKC-mediated phosphorylation of Cx43 has been reported and associated with both increased (94, 95) and decreased conductance (7, 96). However, the contribution of specific PKC isozymes to this phenomenon remains poorly understood due to the utilization of non-selective PKC isozyme inhibitors.

Among different PKC isozymes, only αPKC and εPKC have been described to co-localize with Cx43 in the heart (3, 97, 98). Bowling *et al.* (2001) showed that both εPKC and αPKC co-localize with Cx43 in non-failing and failing hearts, however, only εPKC directly phosphorylates Cx43 (99). Doble *et al.* (2000) demonstrated that non-specific PKC activation by either fibroblast growth-factor 2 (FGF-2) or PMA increased εPKC but not αPKC co-localization with Cx43 in myocytes, resulting in decreased gap junction permeability, whereas chelerythrine treatment (a non-selective PKC inhibitor) blocked the effects of FGF-2 on Cx43 phosphorylation and permeability (7, 13). Furthermore, evidence that the εPKC isozyme is directly involved in Cx43 phosphorylation was provided by showing that over-expression of the mutated dominant-negative form of εPKC decreased myocyte Cx43 phosphorylation and permeability (7, 13). Therefore, Cx43 phosphorylation by εPKC has been associated with decreased gap junctional intercellular communication. Bao *et al.* (2004) proposed that phosphorylation of Ser368 by PKC produces a conformational change in the C-terminal domain that leads to a decrease in Cx43 permeability (100).

PKC and Na/Ca Exchanger

The cardiac Na/Ca exchanger (NCX) plays a pivotal role in regulating intracellular Ca homeostasis to maintain normal electrical and mechanical activities of the heart. NCX1 is particularly important to excitability because during each action potential, NCX1 rapidly extrudes the Ca which entered the myocytes and brings Ca into the myocytes during cardiac depolarization.

Ruknudin *et al.* (2007) have shown that NCX is a member of a macromolecular complex that includes different kinases and phosphatases (101). However, the role of protein phosphorylation in the NCX has not been well defined. Iwamoto *et al.* (1996, 1998) first provided evidence that the cardiac isoform of NCX can be phosphorylated by PKC in CCL39 fibroblasts, resulting in increased NCX activity (102, 103). They found that NCX1 was phosphorylated upon incubation with PMA (non-selective PKC activator). Of interest, when all the consensus sites of the PKC phosphorylation were mutated in the NCX1, the protein still showed response after PMA stimulation (102), suggesting that either PMA stimulation could result in non-specific kinase activation or that there could be nonconsensus sites for PKC on NCX1.

Zhang *et al.* (2001, 2009) have reported that chelerythrine (a non-selective PKC inhibitor) abolished the deleterious effect of endothelin-1 treatment on NCX1 activity in guinea-pig ventricular myocytes (8, 104). Using an animal model, Katanosaka *et al.* (2007) showed increased NCX phosphorylation and association of NCX1 with εPKC in the hypertrophic hearts of thoracic aortic-banded (TAB) mice (105). Indeed, transgenic mice over-expressing an NCX1 mutation in specific-PKC phosphorylation sites (NCX1-S249A/S250A/S357A) were more resistant to TAB-induced cardiac hypertrophy (105). To date, it is well-established in the literature that NCX level and activity are increased in heart failure as consequence of sarcoplasmic reticulum dysfunction and cytosolic calcium accumulation during diastole (106). Direct PKC-mediated NCX phosphorylation contributes to NCX activation. In fact, cardiac NCX1 was shown to be stimulated by acute treatment with protein kinase C (PKC) activators, such as PMA and Gq-coupled receptor agonists (103, 107). In that sense, the use of rationally designed inhibitor peptides of selective PKC isozymes might contribute to correct NCX hyper activation and improves heart failure prognosis.

Besides its direct effect on NCX activity, PKC indirectly regulates NCX function by phosphorylating phospholemman, a small transmembrane protein which belongs to a family

of proteins (FXYD gene family) that bind to and regulate NCX. Many papers have reported that regulation of cardiac contractility and NCX1 activity by phospholemman is critically dependent of its phosphorylation at serines 63 and 68 by PKC in cardiac myocytes (108–110). In fact, overexpression of S68E or S63A mutations in phospholemman inhibited NCX activity. Indeed, NCX1 activity is known to be modulated by ε-adrenergic stimulation in a PKC-dependent manner (103, 111). NCX1 activity is also regulated by PKA phosphorylation at serine 68.

Phospholemman phosphorylation also regulates the membrane Na/K ATPase (Na/K pump) activity, a vital component for the maintenance of normal electrical activity. Han *et al.* have demonstrated that non-selective PKC activation (using PMA) increases Na/K pump Vmax (112). Of interest, PKC-associated Na/K pump activation is abrogated in phospholemman deficient mice, providing evidences that PKC regulates Na/K pump by phosphorylating phospholemman (113). Phospholemman phosphorylation at both Ser63 and Ser68 is necessary for completely relieving the phospholemman-induced Na/K pump alteration (114). Considering that NCX and the Na/K pump are found together in a functional complex in cardiac myocytes (115), the contribution of PKC-mediated phospholemman phosphorylation to this complex needs to be better exploited. Therefore, futures studies using rationally designed peptides regulators may help to clarify the contribution of PKC isozymes to direct and indirect NCX and Na/K pump regulation.

PKC and Arrhythmias

Ischemic heart disease is the leading cause of congestive heart failure and death in the Western world (116). Early reperfusion after coronary occlusion improves cardiac function and reduces infarct size, but it is invariably accompanied by an overload of intracellular Ca that mediates cellular damage and contractile dysfunction (117, 118) and fatal ventricular arrhythmias, such as ventricular fibrillation. Efforts have thus been made to minimize the adverse arrhythmic events related to myocardial ischemia-related reperfusion. While the protective role of εPKC activation in myocardial ischemia-reperfusion injury was extensively characterized regarding the alteration in hemodymamics, cellular damage, and infarction size (119), the potential protective effect of εPKC activation on the fatal ventricular arrhythmia associated with ischemia-reperfusion is just emerging.

We used optical mapping techniques to focus on the potential protective role of εPKC modulation on ischemia-reperfusion arrhythmias in two lines of transgenic mice which moderately over-express the εPKC (termed εPKC agonist mice) activator peptide (ψεRACK, Table 1) (120) resulting in a 20% increase in εPKC activity, and εPKC inhibitor peptide (εV1-2; Table 1) resulting in a 15% inhibition of εPKC activity (termed εPKC antagonist mice) in the heart only (expression of the peptides was restricted to cardiac myocytes) (121). Mice were subjected to 10 min global ischemia and 30 min reperfusion and action potentials and intracellular Ca transients were recorded simultaneously at 37 °C. In the εPKC antagonist group, in which εPKC activity was down-regulated, 10 out of 13 (76.9%) TG mice developed VT, of which six (46.2%) degenerated into sustained VF upon reperfusion (121). Interestingly, in εPKC agonist mice, in which the activity of εPKC was up-regulated, no VF was observed and only 1 out of 12 mice showed only transient VT during reperfusion. During ischemia and reperfusion, intracellular Ca transient decay was exceedingly slower in the antagonist mice compared to the agonist TG mice. The data showed that moderate *in vivo* activation of εPKC exerts beneficial anti-arrhythmic effects vis a vis the lethal reperfusion arrhythmias (121). Similarly, *in vivo* treatment of pigs with the εPKC activator, ψεRACK, early during ischemia completely blocked post-ischemic arrhythmia (122). It is interesting to note that although both εPKC inhibition (by treatment with δV1-1) and εPKC activation (by treatment with ψεRACK) resulted in similar

reduction in infarct size in this transient ischemic model in pigs, only εPKC activation prevented post-ischemic arrhythmia (123). These findings have important implications for the development of PKC isozyme targeted therapeutics and subsequently for the treatment of ischemic heart disease. Further studies are required in order to identify the specific targets involved in this anti-arrhythmic PKC-mediated phenomenon.

Summary and Perspectives

In this review, we describe a continuum of responses emanating from different PKC isozymes that contribute to cardiac excitability. It is clear that PKC isozymes play critical roles in the individual ion channels, gap junction and exchangers, which ultimately interfere in the initiation and propagation of the action potential and thus cardiac excitability (Figure 1). The conflicting data on the role of individual PKC isozymes in the regulation of cardiac excitability may be due not only to the differences between species and tissue distribution, but also to the pharmacological and genetic tools used in the studies. Because PKC isozymes have unique, and sometimes, opposing functions, the use of isozyme-selective tools is critical. Using either genetic manipulation or pharmacological tools able to deliver selective agonist and antagonist peptides for the same PKC isozyme in the heart is particularly useful; it helps to determine the contribution of selective PKC isozyme(s) to cardiac excitability-related processes. Clearly, PKC isozymes are central in regulating the electrical activity of the heart. Using these isozyme-selective inhibitors and activators, we demonstrated a role for specific PKC isozymes in the regulation of Ca, Na and K channels [1–3]. It may be possible now to investigate the positive and negative contributions of specific PKC isozymes to action potential electrogenesis and excitation-contraction coupling in different pathological conditions such as ischemia, cardiac dysfunction and heart failure. Therefore, *in vivo* studies using different animal models will help to point out the relevance of specific PKC isozymes as therapeutic targets for the treatment of electrochemical changes induced by cardiovascular diseases.

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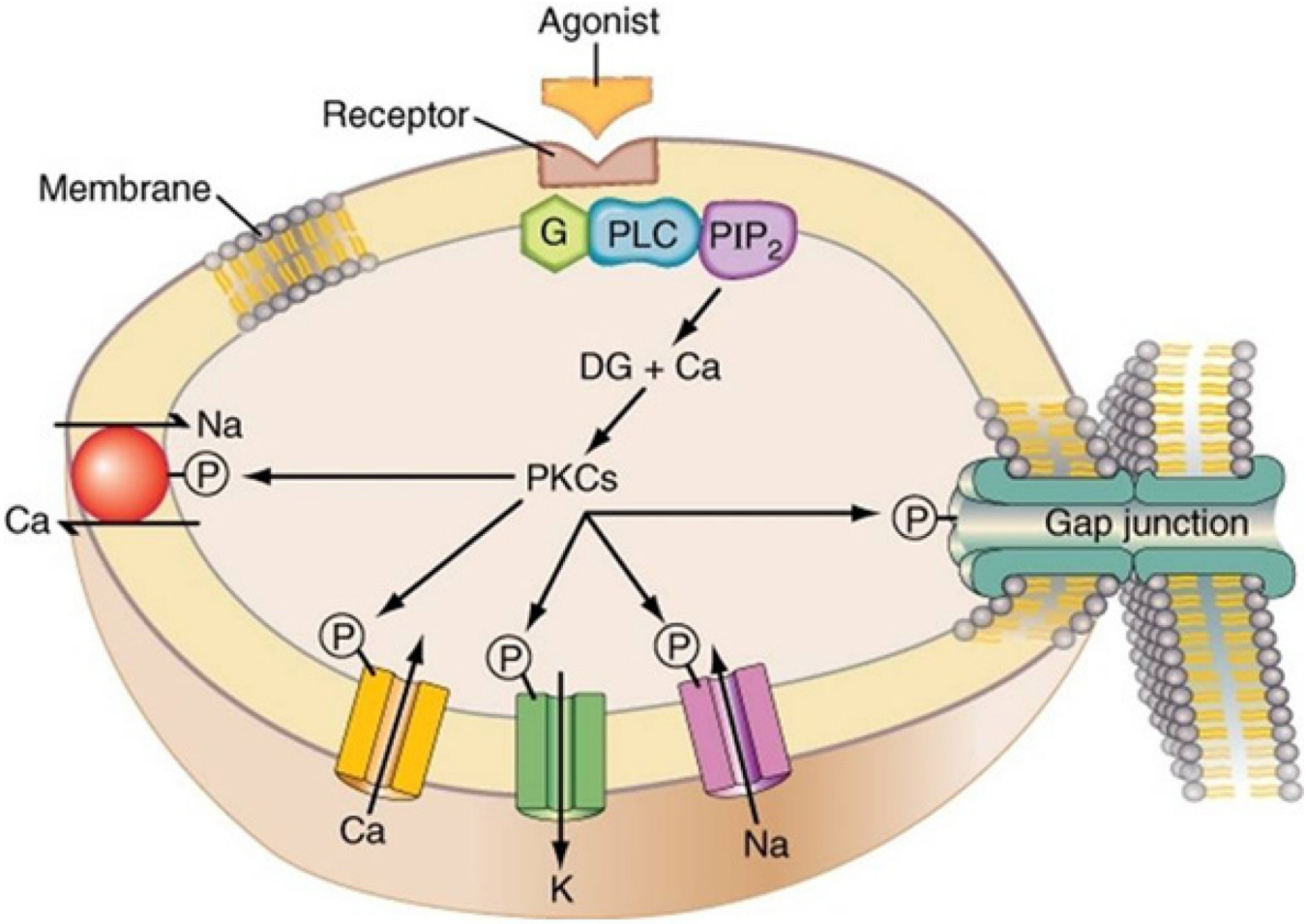

Figure 1.
Schematic representation of protein kinase C (PKC) regulation of cardiac excitability and membrane associated function through phosphorylation mechanisms. Ca: Ca channel; K: K channel; Na: Na channel; Na/Ca: Na/Ca exchanger; DG: Diacyl glycerol; G: G-protein; PLC: Phospholipase C; PIP2: Phosphoinositol di-phosphate; P: Phosphate.

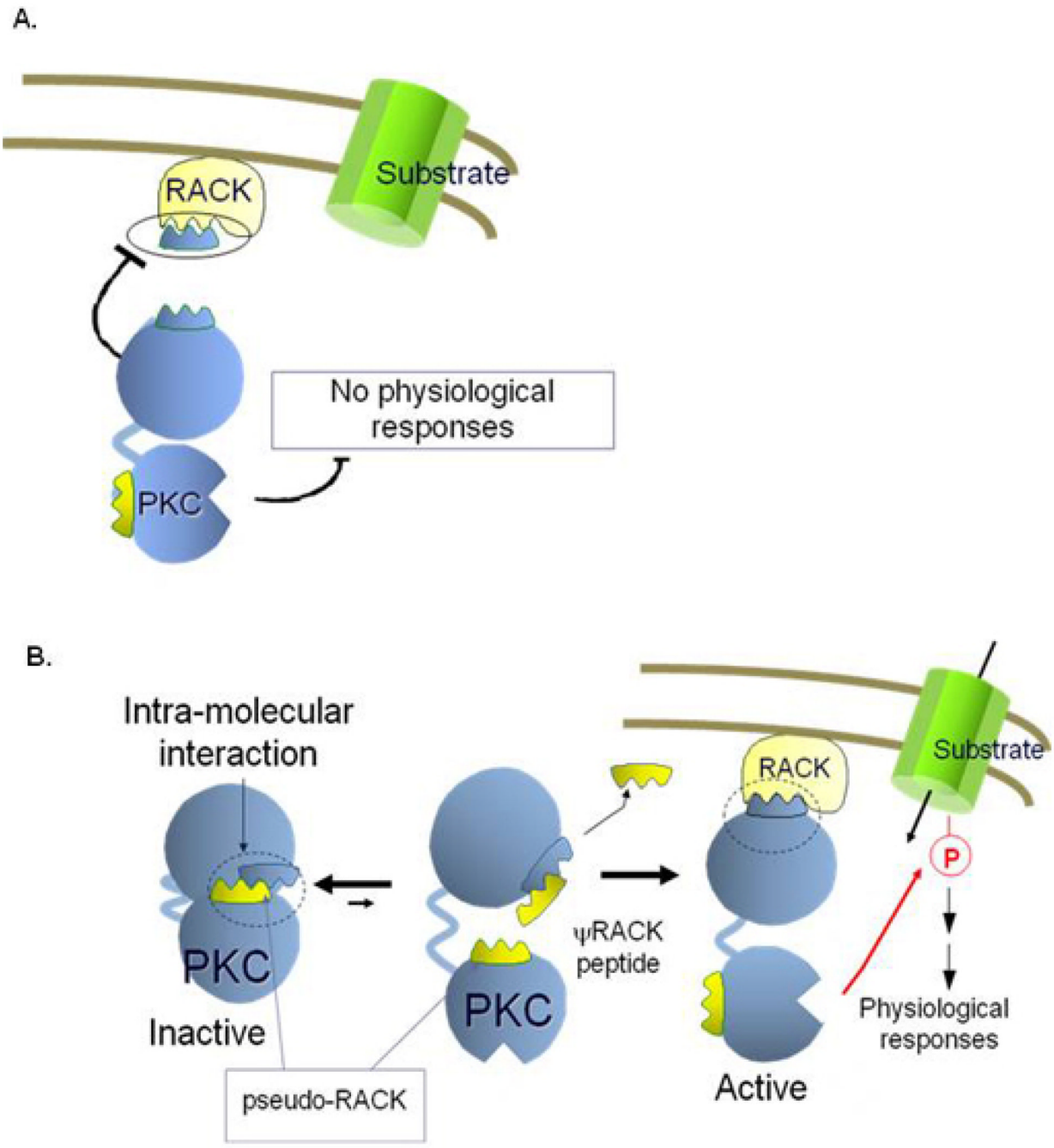

Figure 2.

Schematic representation of protein kinase C (PKC) inhibitors and activators. A: A peptide, derived from the RACK-binding site on PKC binds to the RACK and inhibits binding of the activated PKC to its RACK. Under these conditions, a substrate near the RACK (e.g., an ion channel) is not phosphorylated and therefore the physiological response mediated by this phosphorylation does not occur. B: An intra-molecular interaction between the RACK-binding site on PKC and a sequence in the enzyme that mimics the PKC-binding site on the RACK (hence pseudo-RACK), keeps the enzyme in the inactive state. A peptide corresponding to the pseudo-RACK sequence binds to the RACK-binding site on PKC and renders the enzyme active. Because the affinity of the pseudo-RACK peptide for the RACK-

binding site on PKC is lower than that of the RACK, the pseudo-RACK peptide is replaced by the RACK. That results in anchoring of the active PKC near its substrate (e.g., ion channel), leading to the channel's phosphorylation and the consequent physiological response (e.g., ion influx).

Table 1

Pharmacological tools that regulate PKC isozymes

Drug	Biological function	Chemical characteristics	Specificity/selectivity	Kinase binding site	Refs
PMA	PKC activator	Phorbol ester	Non-specific	DAG-binding site	(29)
Bryostatin	PKC activator	Macrolactones	Non-specific	DAG-binding site Some synthetic analogs are specific	(124, 125)
ψβRACK	PKC activator	PKC-derived peptide	cPKC-specific	Regulatory domain βPKC aa 241–246	(126)
ψδRACK	PKC activator	PKC-derived peptide	δPKC	Regulatory domain δPKC aa 74–81	(127)
ψθRACK	PKC activator	PKC-derived peptide	θPKC	Regulatory domain θPKC aa 73–82	
ψεRACK	PKC activator	PKC-derived peptide	εPKC	Regulatory domain εPKC aa 85–92	(120)
ψηRACK	PKC activator	PKC-derived peptide	ηPKC	Regulatory domain ηPKC aa 88–95	
Staurosporine	PKC inhibitor	indolo-carbazole	Non-specific	Catalytic domain ATP site, affects also other kinases	(128)
Go6076	PKC inhibitor	nonglycosidic indolocarbazole	cPKC, nPKC	Catalytic domain ATP site	(129, 130)
Bisindolmaleimide (LY333531)	PKC inhibitor	macrocyclic bis(indolyl) maleimide	βPKC	Catalytic domain ATP site, affects also other protein kinases	(131, 132)
GF109203X	PKC inhibitor	Bisindolylmaleimide	PKC selective	Catalytic domain ATP site, affects also other protein kinases	(133)
Ro31-8820	PKC inhibitor	bis(indolyl)maleimide	PKC selective	Catalytic domain ATP site, affects also other protein kinases	(134)
Ro32-0423	PKC inhibitor	bisindolylmaleimide	αPKC and βIPKC	Catalytic domain ATP site, affects also other protein kinases	(135)
PKC412	PKC inhibitor	indolo-carbazole	Ser/Thr and Tyr protein kinases	Catalytic domain ATP site, affects also other protein kinases	(136)
UNC01	PKC inhibitor	indolo-carbazole	cPKC>nKC	Catalytic domain ATP site, affects also other protein kinases	(137)
Rottlerin	PKC inhibitor	polyphenols	δPKC	Catalytic domain ATP site, inhibits also a calcium calmodulin kinases at the same IC_{50}	(138)
ISIS 3521	PKC inhibitor	Anti-sense	αPKC	αPKC	(139)
ISIS9606	PKC inhibitor	Anti-sense	αPKC	αPKC	(140)
βIV5-3	PKC inhibitor	PKC-derived peptide	βIPKC	Regulatory domain βIPKC aa 646–651	(141)
βIIV5-3	PKC inhibitor	PKC-derived peptide	βIIPKC	Regulatory domain βII PKC aa 645–650	(141)
γV5-3	PKC inhibitor	PKC-derived peptide	γPKC	Regulatory domain γPKC aa 659–664	(142)
δV1-1	PKC inhibitor	PKC-derived peptide	δPKC	Regulatory domain δPKC aa 8–17	(127)
εV1-2	PKC inhibitor	PKC-derived peptide	εPKC	Regulatory domain εPKC aa 14–21	(143)
θV1-1	PKC inhibitor	PKC-derived peptide	θPKC	Regulatory domain θPKC aa 8–13	
ηV1-2	PKC inhibitor	PKC-derived peptide	ηPKC	Regulatory domain ηPKC aa 18–25	

Drug	Biological function	Chemical characteristics	Specificity/selectivity	Kinase binding site	Refs
β-pseudosubstrate	PKC inhibitor	PKC-derived peptide	Pan PKC	catalytic domain, substrate site; βPKC aa 19–31	(144)
ζ-pseudosubstrate	PKC inhibitor	PKC-derived peptide	aPKC subfamily	catalytic domain, substrate site; ζPKC 105–121	(145)
βC2-1, 2, 4	PKC inhibitor	PKC-derived peptide	cPKC subfamily	Regulatory domain βIIPKC aa 209–216; 186–198; 218–226 respectively	(126)