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## COMMENTARY

# Physiological Inhibitors of Protein Kinase C

*Michael H. Melner**

DEPARTMENTS OF OBSTETRICS AND GYNECOLOGY AND OF CELL BIOLOGY, VANDERBILT UNIVERSITY SCHOOL OF MEDICINE, NASHVILLE, TN 37232-2515, U.S.A.

**ABSTRACT.** Increasing numbers of proteins that have the capacity of interacting with protein kinase C isozymes *in vitro* and inhibiting their enzymatic activity in a noncompetitive manner have been purified. While these proteins can be hypothesized to be part of a tight regulatory system for protein kinase C enzymatic activity, critical examinations of the roles of these proteins in the context of whole cells have not yet been performed. Interesting new data suggest that some of the classes of protein kinase C inhibitors may have a much broader role of interacting with multiple types of kinases and proto-oncogene products. cDNAs encoding a number of these inhibitor proteins have been isolated, which will allow the design and implementation of experiments on their cell biology and help address their function outside of the context of their operational definitions. BIOCHEM PHARMACOL 51;7: 869–877, 1996.

**KEY WORDS.** protein kinase C; inhibitor; signal transduction

The protein kinase C family of enzymes was defined originally as a homologous group of serine/threonine kinases that were activated by $Ca^{2+}$, phospholipid, and diacylglycerol [1–3]. Based upon sequence homology, the mammalian protein kinase C family now consists of 12 different isozymes that are named by the greek letters α, βI, βII, γ, δ, ε, ζ, η, θ, ι, λ, and μ. These isozymes are further classified into sub-groups based upon both sequence homology and enzymatic properties [1–3]. In contrast to the original classification, some of the isozymes, such as λ, are $Ca^{2+}$-independent, phospholipid-variable forms with mechanisms of regulation that have not been elucidated clearly. The major focus of research into the regulation of protein kinase C activity has been upon the mechanisms that stimulate enzyme activation. However, the important roles that protein kinases play in regulating key cellular processes, such as proliferation, have indicated that the control of these enzymes must be regulated very tightly and have raised the potential that there are also inhibitory mechanisms that may function in controlling their enzymatic activity.

The evidence for the existence of physiological inhibitors of multiple classes of protein kinases has been accumulating. As early as 1965, an endogenous inhibitor of the cAMP-dependent protein kinase, protein kinase A, was discovered in rabbit skeletal muscle and shown to be a heat-stable protein [4]. Subsequent characterization of this PKI† indicated that it is a highly specific and potent competitive inhibitor of the catalytic subunit of protein kinase A with a $K_i$ of 0.5 to 5.4 nM [5–7]. The evolutionary importance of this protein is underscored by the finding that the deduced amino acid sequences of the human and rabbit proteins are identical [8].

More recently, inhibitors of other classes of protein kinases have been discovered, which may have critical functions in controlling the cell cycle. The CDKs are involved in controlling the transition between steps in the cell cycle. A group of CDI proteins that bind and inactivate CDKs have been isolated [9, 10]. The CDIs are relatively small proteins designated by their sizes in kilodaltons p15, p16, p21, and p27 [9]. Although there are stimulatory mechanisms that regulate CDKs, the existence and importance of CDIs have now become evident.

These examples demonstrate the existence of inhibitor proteins that play important roles in regulating the activities of multiple classes of key protein kinases, even though stimulatory mechanisms exist to also regulate these enzymes. The interaction between positive and negative control mechanisms provides the potential for very tightly regulating the activities of important protein kinases. Interesting new data suggest that endogenous inhibitors of protein kinase C may also function to regulate the activities of this family of protein kinases. These proteins have been operationally defined as protein kinase C inhibitors based upon the action of the purified proteins in an *in vitro* assay or upon the effects of the purified proteins in patch-clamped cells. One of the critical questions that remain to

* Corresponding author: Dr. Michael H. Melner, Department of Obstetrics and Gynecology, Vanderbilt University School of Medicine, C1100 Medical Center North, Nashville, TN 37232-2515. Tel. (615) 343-2036; FAX (615) 343-2940.

† *Abbreviations:* PKI, protein kinase inhibitor; CDK, cyclin-dependent kinase; CDI, cyclin-dependent kinase inhibitor; PKCI-1, protein kinase C inhibitor 1; DAG, diacylglycerol; KCIP-1, kinase C inhibitor protein 1; RACKS, receptors for activated C kinase; HIV-1, human immunodeficiency virus 1; and HTLV-1, human T-cell leukemia virus 1.

be addressed is whether these endogenous inhibitors act *in vivo* in this capacity or whether they function primarily in other capacities within the cell.

Another mechanism for regulating the cellular effects of protein kinases is the involvement of protein phosphatases that remove phosphates from phosphorylated protein substrates. Although these enzymes represent an important regulatory mechanism, this commentary will focus primarily upon endogenous proteins with inhibitory activity to protein kinase C.

## ENDOGENOUS PROTEIN INHIBITORS OF PROTEIN KINASE C

### *PKCI-1*

The existence of endogenous proteins/peptides that inhibit protein kinase C became evident in the initial purification of $Ca^{2+}$-binding proteins and isozymes of protein kinase C by multiple laboratories. McDonald and Walsh [11] identified a 17-kDa protein from bovine brain using $Ca^{2+}$-dependent hydrophobic-interaction chromatography on phenyl-Sepharose, which demonstrated potent inhibitory activity on a preparation of protein kinase C isozymes [12]. Although the protein was purified using $Ca^{2+}$-dependent hydrophobic-interaction chromatography, it did not display a $Ca^{2+}$-dependent mobility shift on SDS-polyacrylamide gels as seen with high affinity $Ca^{2+}$-binding proteins such as calmodulin [11]. Subsequent purification and characterization of the protein designated PKC-1 yielded amino acid sequence data with no significant homology to any other known proteins [13]. More recently, two genes with significant homology to PKC-1 have been identified, a cDNA in a maize endosperm cDNA library [14] and an open reading frame on a cyanobacterial dicistronic message [15]. The function of these two genes is unknown at this time but indicates the evolutionary conservation of these proteins and their potential importance. Very recently, the maize protein has been expressed in bacteria, and some preliminary studies report that it has very little activity at inhibiting mammalian brain protein kinase C but that its effects in the presence of another protein kinase C inhibitor, 14-3-3, may be synergistic [16]. It will be interesting to see how this system develops and especially whether there may be interactions between different classes of protein kinase C inhibitor proteins.

PKCI-1 has a number of unique properties as a potential physiological inhibitor of protein kinase C. The inhibitory activity of purified PKCI-1 is heat-stable to 100° for 2 min [17] and is specific for protein kinase C. It has no apparent effects on calmodulin-dependent myosin light-chain kinase, protein kinase A catalytic subunit, casein kinase I, calmodulin-dependent cyclic nucleotide phosphodiesterase, or epidermal growth factor receptor tyrosine kinase [17–20]. Purified PKCI-1 has no apparent phosphatase activity, and its inhibitory effects are independent of $Ca^{2+}$ and phospholipid and are not due to interactions with protein kinase C substrates or cofactors [18–20]. Structurally, this protein contains a histidine-rich region, His-Val-His-Leu-His, which has been shown to be a $Zn^{2+}$-binding domain [13, 21]. This region represents a novel $Zn^{2+}$-binding sequence that differs significantly from the $Zn^{2+}$-finger domains of transcription factors, the $Zn^{2+}$-coordination sites of metalloproteinases, and the $Zn^{2+}$-containing catalytic sites of enzymes. The role of this domain in the function of PKCI-1 is not known but, in general, represents an interesting structural feature that is shared among multiple kinases including the isozymes of the protein kinase C. The cysteine-rich region of the regulatory domain of the protein kinase C isozymes contains zinc finger motifs that are similar to those found in transcription factors such as GAL4 (reviewed in Ref. 22). The zinc finger motifs bind $Zn^{2+}$, although these isozymes demonstrate no apparent DNA binding activity. Other kinases such as the c-Raf kinase isozymes and DAG kinase have zinc finger motifs but exhibit no DNA-binding activity (reviewed in Ref. 22). Presumably, the zinc finger motifs are stabilizing the conformation of an important domain essential for the function of these enzymes. However, further characterization of these domains is needed to elucidate their mechanistic roles.

We recently isolated human, rat, macaque, and bovine cDNA clones encoding PKCI-1 [23, 24]. The cDNAs indicate a high degree of deduced amino acid sequence homology among different species (e.g. 93.6% identity in human vs rat). This high degree of sequence homology is particularly evident in domains such as the zinc-binding domain where the histidine-rich sequence is maintained. Interestingly, Southern blot analysis has suggested the potential existence of multiple genes or pseudogenes related to PKCI-1. If these multiple genes are functional and encode closely related PKCI proteins, there is potential for differing regulatory functions such as specificity for individual or groups of protein kinase C isozymes. This information will require the cloning and characterization of multiple members of this gene family. The tissue distribution of PKCI-1 mRNA expression is fairly broad, which would be expected since protein kinase C isozymes are also widely expressed. However, there is a high degree of cell-specific localization of PKCI-1 mRNA within tissues. For example, in the rat ovary, high levels of PKCI-1 mRNA are expressed predominantly within proliferating granulosa cells of actively growing follicles, whereas expression is relatively low in differentiated corpora lutea [24]. In the brain, where high levels of the protein were first examined, *in situ* hybridization studies indicate differential expression of PKCI-1 in specific regions and within specific hypothalamic nuclei (unpublished data). The subcellular localization of PKCI-1 is not known but will prove interesting to evaluate in comparison to the localization of the individual isozymes of protein kinase C.

Relatively little information has been obtained on the cellular functions of PKCI-1. One very interesting paper by Rane *et al.* [20] demonstrated that purified PKCI-1 protein inhibited norepinephrine-induced modulation of the calcium current when administered in patch pipette solution

to patch clamped primary cultures of chick sensory neurons. Another peptide based upon 13 amino acids of the pseudosubstrate region of protein kinase C was also effective at blocking this neurotransmitter-induced modulation of the neuronal calcium current. These data suggested that PKCI-1 protein was highly effective at inhibiting protein kinase C activity within the context of live cells, and this inhibition resulted in altered neuronal function. These studies are some of the few that have investigated the cell biology of the protein kinase C inhibitors. The isolated cDNA clones encoding PKCI-1 allow the potential for performing transfection/transformation studies to begin to investigate the cellular functions of PKCI-1.

## PKCI-2

A second protein with inhibitory activity to protein kinase C was also identified in bovine brain using $Ca^{2+}$-dependent hydrophobic-interaction chromatography on phenyl-Sepharose and named the 12-kDa inhibitor [25]. Some early data concerning this protein suggested that it may have been generated by proteolytic activity of the 17-kDa PKCI-1 [17]. Subsequent purification and characterization of the 12-kDa protein initially indicated that the amino acid sequence was 97% identical to the human FK506-binding protein, a cytosolic immunophilin that binds the immunosuppressant FK506 [26]. Further ion-exchange chromatography partially separated the protein kinase C inhibitory activity from the FK506-binding protein [26], indicating that the FK506-binding protein is not an inhibitor of protein kinase C and that the 12-kDa protein has a different identity.

## *14-3-3*

Another family of proteins was isolated from ovine brain based upon their ability to inhibit $Ca^{2+}$-phospholipid-dependent protein kinase C. These were acidic proteins ranging from 29 to 33 kDa, which were designated KCIP-1 based upon their ability to function as potent noncompetitive inhibitors of protein kinase C, independent of substrates, ATP, or co-factors [27]. Peptides from KCIP-1 isoforms were sequenced, and comparisons with the EMBL computer database revealed a high degree of homology with bovine brain 14-3-3 protein [28–30]. The 14-3-3 proteins have an unclear functional role although they are identical to an activator protein of the rate-limiting enzymes of catecholamine and serotonin biosynthesis, tyrosine and tryptophan hydroxylases [31]. The 14-3-3 proteins are present in the brain in relatively high levels where 13.3 μg/mL of soluble protein (~1%) has been measured (reviewed in Ref. 30), although they show a wide tissue distribution. They exist as a family of closely related isoforms that are derived from distinct gene products and are designated by the greek letters α, β, γ, δ, ε, ζ, η, τ, and σ [30, 32–34]. Homologous proteins have also been found in a broad range of species such as *Drosophila* and yeast and in plants such as spinach, *Oenothera*, and *Arabidopsis* [34–37]. While the action of these proteins on protein kinase C activity has been overwhelmingly described as inhibitory, there have been reports of a stimulatory effect on protein kinase C [38]. In contrast to PKCI-1, the 14-3-3 proteins are larger in molecular weight and are heat-labile. However, in common with PKCI-1, these proteins appear to act independently of substrates, cofactors, and ATP and act noncompetitively.

Several putative functions have been proposed for the 14-3-3 proteins, although definitive and specific mechanistic roles are still not clear [34]. The proteins have been proposed to be activator proteins of the rate-limiting enzymes of catecholamine and serotonin biosynthesis, tyrosine and tryptophan hydroxylases, although the proteins are expressed in tissues and cells that do not contain these enzymes [34]. A protein designated Exo 1 with putative roles in $Ca^{2+}$-dependent exocytosis of secretory granules in adrenal chromaffin cells has been shown to be a member of the 14-3-3 family [39]. In these studies, Exo 1 has been shown to stimulate $Ca^{2+}$-dependent exocytosis in permeabilized adrenal chromaffin cells [40]. A role for Exo 1 as an endogenous inhibitor of protein kinase C seems unlikely since this protein synergizes with protein kinase C in stimulating exocytosis [41]. This property is associated with multiple different isoforms of the 14-3-3 protein family [42]. In *Arabidopsis*, the 14-3-3 protein was isolated as part of a DNA binding complex to a *cis*-acting element, the protein/G box complex [37]. These studies have implied a role for 14-3-3 in regulating transcription. In yeast, disruption of the 14-3-3 homologue elicited a 30% reduction in the growth rate under specific media, indicating a putative role in growth regulation [43]. In yet another system, a *Xenopus* homologue was cloned that was coordinately expressed with the proopiomelanocortin (POMC) gene in melanotrope cells of the intermediate pituitary during background adaptation [44]. This regulated expression in pituitary cells has implied a potential role in pituitary hormone secretion. Human cDNA clones have been isolated from keratinocytes, mammary epithelial cells, and T-cells, which are members of the 14-3-3 family [45–47]. Interestingly, the expression of the epithelial forms (designated stratifin and HME1) is down-regulated in SV-40 virus-transformed keratinocytes and in human mammary carcinomas compared with normal cells [45, 46]. There are thus a number of putative functions for the 14-3-3 proteins including their potential roles as intracellular regulators of protein kinase C activity. The diversity of cellular functions in which the 14-3-3 proteins have been implicated to be involved is wide, and careful examinations of their roles as specific *in vivo* inhibitors of protein kinase C within cells are needed.

A controversial point has arisen as to whether the ζ isoform of 14-3-3 has phospholipase $A_2$ activity. A human platelet ζ isoform of 14-3-3 has been cloned, and the protein expressed in *Escherichia coli* was shown to cleave the *sn*-2 fatty acid of choline and ethanolamine and form a stable arachidinoyl-enzyme intermediate [48]. However, Robinson *et al.* [49] did not find phospholipase $A_2$ activity

associated with any of multiple isoforms of 14-3-3 including the ζ isoform. They have suggested that the activity of the recombinant protein expressed in *E. coli* could represent a separate phospholipase $A_2$ activity in *E. coli* [49].

Very recently, the 14-3-3 proteins have been demonstrated to bind to and regulate the activities of a number of other proteins in signal transduction pathways including proto-oncogene and oncogene products [50]. Members of the 14-3-3 protein family have been shown to interact with Raf-1, Bcr-Abl, and the polyomavirus middle tumor antigen [51–56]. The evidence that 14-3-3 proteins interact with these cellular targets is convincing in that it was obtained using multiple different approaches including the yeast two-hybrid interaction system with Raf-1 as bait, co-precipitation using antibodies to Raf-1 or polyomavirus middle tumor antigen, and screening cDNA expression libraries with a purified fragment of Bcr. These data raise interesting possibilities for the 14-3-3 proteins as a broad class of modulators of important signal transduction proteins. However, they raise an equal number of questions concerning the specific roles of these proteins. For example, proteins of the 14-3-3 family have been demonstrated to be the previously purified proteins that stimulate the import of newly synthesized proteins into mitochondria and termed mitochondrial import stimulation factor (MSF) [57]. These authors interpret the 14-3-3 proteins as potential cytosolic chaperones that may stabilize the conformation of other proteins for mitochondrial import. This same hypothesis was presented for the role of 14-3-3 proteins in stabilizing active conformations of signal transduction proteins [50]. A broad role such as protein stabilization would help explain the diverse roles that these proteins have been hypothesized to fill.

## ENDOGENOUS $Ca^{2+}$-BINDING PROTEINS THAT ARE INHIBITORY TO PROTEIN KINASE C

### *Annexin V*

The endogenous proteins discussed to this point are not characterized as high affinity $Ca^{2+}$-binding proteins. However, the annexins are a family of $Ca^{2+}$-binding proteins that demonstrate reversible, $Ca^{2+}$-dependent binding to cell membrane phospholipids [58]. Recent studies suggest that annexin V is a highly potent and specific inhibitor of protein kinase C [59]. Schlaepfer *et al.* [59] have demonstrated that annexin V inhibits the protein kinase C-mediated phosphorylation of some specific protein substrates (e.g. annexin I, myosin light chain kinase) with half-maximal inhibition occurring at 0.4 μM. In an *in vitro* system, this is one of the most effective endogenous inhibitors of protein kinase C described to date. The inhibitory effects of annexin V were not due to its effects at sequestration of $Ca^{2+}$ or phospholipids, and the effects were specific for protein kinase C with no effects on epidermal growth factor receptor/kinase or cAMP-dependent protein kinase [59]. Since a protein kinase C preparation from rat brain was used in these studies, it is not clear if annexin V demonstrates any specificity for the different protein kinase C isozymes or subclasses of isozymes. It will be of interest to determine whether annexin V exhibits such a specificity for the different isozymes of protein kinase C. Annexin V is present within cells at levels greater than that needed for the inhibition of protein kinase C, so it is reasonable to hypothesize that this protein may have functional roles in regulating intracellular protein kinase C activity.

It is interesting that the 14-3-3 proteins share some limited amino acid sequence homology with one of the annexins, annexin I [60]. Annexin I is believed to interact with protein kinase C and has been characterized as one of the intracellular receptors for activated C kinase (acronym: RACKS; [60]). A synthetic peptide based upon a sequence within annexin I and with homology to the 14-3-3 proteins was shown to inhibit insulin-induced protein kinase C translocation and oocyte maturation when injected into *Xenopus* oocytes [61]. These studies indicate a potentially important domain recognized by protein kinase C which may serve as an intracellular target for protein kinase C or as a regulatory domain for the enzyme.

## EXOGENOUS PROTEINS THAT INHIBIT PROTEIN KINASE C

Another group of proteins are exogenous viral proteins present within cells as a result of retroviral infection, which may have interesting mechanisms of altering the host cell response to infection and thus may be important regulators of the pathophysiology of the cell. One such protein is the HIV-1 envelope glycoprotein gp41. Ruegg and Strand [62] have demonstrated that synthetic peptides based upon amino acids 581–597 of gp41, which inhibit the proliferation of human lymphocytes, inhibit protein kinase C-mediated phosphorylation of the CD3 γ-chain in intact cells and inhibit partially purified protein kinase C. This inhibition was further shown to be noncompetitive with respect to the substrates histone and ATP and independent of the regulatory domain of protein kinase C [62]. These data have raised the hypothesis that gp41 may contribute to immunosuppression in HIV-1-infected individuals by inhibiting protein kinase C activity and blocking protein kinase C-dependent immune function in lymphocytes [62]. Similar results were obtained by others using a synthetic heptadecapeptide based upon the homologous region of the transmembrane envelope proteins of murine, feline, and the human retroviruses HTLV-1 and HTLV-2 [63, 64]. Similar to the results obtained with the HIV-1-derived peptide, the HTLV-1 homologous peptide inhibits protein kinase C and the inhibition is independent of $Ca^{2+}$, phospholipid, or ATP and the peptide does not function as a competitive inhibitor. In addition, the inhibition is specific with no effects on cAMP-dependent protein kinase or the calcium and phospholipid-independent form of protein kinase C (PK-M; [63, 64]). These studies suggest that envelope proteins derived from retroviruses may exert regulatory influences on intracellular protein kinase C, which may be im-

portant to the pathophysiology of the retroviral-induced diseases. Although these two retroviral envelope proteins could have regulatory effects on the host cells, the pathophysiology of these two retroviruses is dramatically different with HIV-1 eliciting immunosuppression and HTLV-1 associated with adult T-cell leukemia. Clearly, other mechanisms most likely interact with the envelope protein-induced changes to elicit the unique pathophysiology of each retrovirus. It is of further interest that the mechanisms by which these retroviral proteins inhibit protein kinase C are similar to the mechanisms by which the endogenous protein inhibitors function, noncompetitive mechanisms that are independent of the substrates and cofactors of the enzyme.

## UNCHARACTERIZED ENDOGENOUS INHIBITORS OF PROTEIN KINASE C

Numerous other endogenous inhibitors of protein kinase C have been reported in multiple systems that have not been characterized fully, but interesting preliminary data suggest their existence. For example, defensins are low molecular weight cationic peptides (mol wt <3500) present in human neutrophils, which have apparent antimicrobial activity against many bacteria, fungi, and viruses [65]. Charp *et al.* [65] have determined that defensins from human neutrophils are highly potent and specific inhibitors of protein kinase C. In common with other endogenous inhibitors of protein kinase C, these peptides inhibit protein kinase C by a noncompetitive mechanism with respect to substrate, $Ca^{2+}$, phospholipid, or ATP with $K_i$ values of 1.2 to 1.7 μM. The defensins tested also demonstrated specificity in having no effects on myosin light chain kinase activity or the holoenzyme or catalytic subunit of cAMP-dependent protein kinase [65].

Other studies have suggested the existence of endogenous protein kinase C inhibitors in neutrophils. Balazovich *et al.* [66] described an endogenous inhibitor of protein kinase C in neutrophil-specific granule membranes, which was protease- and heat-sensitive. Further examination of this inhibitor has suggested that it is a protein of 41 kDa [67]. The primary structure, identity, and relationship of this protein inhibitor to other described inhibitors will be of interest to examine. Another group has indicated the existence of an inhibitor of protein kinase C in rabbit peritoneal neutrophils [68]. The inhibitory activity was heat-labile, destroyed by freeze–thawing, and eluted as two separate peaks on Sephadex G-150 gel exclusion chromatography that did not coincide with phosphatase activity [68]. Gandini *et al.* [69] indicated the existence of an endogenous inhibitor of protein kinase C in microsomes from peripheral blood mononuclear cells. This inhibitor was labile to trypsin treatment, but it apparently acted at the regulatory domain of protein kinase C since inhibition could be overcome by treatment with phorbol esters.

The potential existence of other endogenous inhibitors in the brain has been described by multiple laboratories, although these activities have not been characterized further. Pribilla *et al.* [70] have described a low molecular weight ($M_r$ 600–700), heat-resistant inhibitor of protein kinase C from bovine brain. This inhibitor does not act at the regulatory domain of protein kinase C and has been shown to be a heat-, alkali-, and acid-resistant molecule that is not a protein. Others have described an endogenous inhibitor of protein kinase C from rat brain with properties different from those of the more characterized inhibitor proteins. Schwantke and Le Peuch [71] have presented preliminary evidence for the presence of an inhibitor in rat brain homogenate that is independent of ATP, $Ca^{2+}$, and phospholipid but competitive with respect to substrate. This protein has gel exclusion chromatography characteristics of a 20-kDa protein, assuming that it has a globular shape.

The potential existence of endogenous inhibitors of protein kinase C was also described in cytosols from the pseudopregnant rat ovary [72]. However, further studies have suggested that this specific inhibitory activity may have been a phosphatase [73].

These studies have suggested the existence of other proteins and compounds that may be endogenous inhibitors of protein kinase C. More definitive characterization of the function of these putative inhibitors awaits their purification and identification of their structures.

## OVERVIEW AND FUTURE DIRECTIONS

The preliminary data accumulated to date are intriguing in implicating the potential involvement of endogenous inhibitor proteins in regulating protein kinase C. The existence of specific protein inhibitors of other classes or protein kinases supports the concept that analogous proteins could exist for regulating protein kinase C. It is interesting that some generalizations can be made about most of the inhibitor proteins that have been characterized to date (i.e. PKCI-1, 14-3-3 isoforms, and annexin V) (Table 1). They all appear to be interacting noncompetitively with protein kinase C, independently of substrate, ATP, $Ca^{2+}$, and phospholipid. As such, they appear to be interacting at domains of protein kinase C other than the regulatory domain. It will be of interest to examine further this interaction and to determine whether these interactions occur *in vivo* within cells or whether this interaction *in vitro* is an artifact of cell disruption. In general, the inhibitors are relatively smaller proteins that may allow translocation within the cell.

The mechanisms by which protein kinase C is affected within cells may be hypothesized to involve one (or multiple) of many different steps. These hypothetical mechanisms of action of the endogenous inhibitor proteins are summarized diagrammatically in Fig. 1. First, the inhibitors may interact with protein kinase C and inhibit enzymatic activity (shown as 1 in Fig. 1). This has been shown to occur with many of the characterized protein inhibitors *in vitro* using either purified protein substrates or common protein substrates. Accordingly, the inhibitor proteins and

**TABLE 1. Endogenous protein inhibitors of protein kinase C**

| Designation | | Mol wt | Tissue source | References |
|---|---|---|---|---|
| PKCI-1 | | 14,000 (calculated)<br>17,000 ($M_r$) | Brain | 11–15, 17–21, 23, 24 |
| 14-3-3 | α | 29,500 | Brain | 27–30, 32, 34 |
| | β | 29,000 | Brain | 27–30, 32, 34 |
| | γ | 30,000 | Brain | 27–30, 32, 34 |
| | δ | 29,500 | Brain | 27–30, 32, 34 |
| | ε | 33,000 | Brain | 33 |
| | ξ | 29,000 | Brain | 33 |
| | η | 29,000 | Brain | 27–30, 32, 34 |
| | τ | 28,000 | T-cells | 47 |
| Stratifin HME1 | σ | 30,000 | Epithelial cells | 45, 46 |
| Annexin I peptide | | | | 61 |
| Annexin V | | 32,000 | Human placenta recombinant | 59 |
| HIV-1 gp41 | | 41,000 | | 62 |
| HTLV-1 CSK-17 | | 15,000 | | 63, 64 |

the protein kinase C isozymes would be expected to interact *in vivo* within cells. If this is the case, interaction may become evident using a two-hybrid method of cloning interacting proteins [74, 75]. Another putative mechanism of inhibition is that the inhibitor proteins could block the translocation of protein kinase C isozymes to intracellular targets (shown as 2 in Fig. 1). Such targets could be the RACKS described by Mochly-Rosen and colleagues [60, 61]. By blocking the translocation to protein targets, the intracellular action(s) of protein kinase C isozymes could be disrupted. Alternatively, the inhibitor proteins could be interacting with a domain of protein kinase C, which interacts with the phospholipid membrane (shown as 3 in Fig. 1). While such an interaction would be expected to yield some indication of competitive inhibition with respect to phospholipids, such an interaction could be of a lower affinity and still be effective, depending upon the relative concentrations of protein kinase C and the inhibitor proteins. The inhibitor proteins could also act via inhibition of translocation of protein kinase C to the nucleus (shown as 4 in Fig. 1). Studies have indicated that certain isozymes of protein kinase C translocate to the nucleus upon activation (reviewed in Ref. 76). An inhibition of this step could abolish the phosphorylation of specific nuclear substrates and inhibit the action of protein kinase C. One other potential mechanism for regulating protein kinase C function exists although there are no data that directly address this putative function. Binding of the inhibitors to protein kinase C may alter their intracellular degradation (shown as 5 in Fig. 1). Chronic treatment with phorbol esters has been shown to increase the intracellular proteolysis of protein kinase C as a component of the down-regulation response [77]. It will be of interest to examine whether the intracellular inhibitor proteins alter the half-life of protein kinase C. And lastly, the proteins could be involved in multimeric interactions with other proteins that influence protein kinase C signal transduction (shown as 6 in Fig. 1). For example, by interacting with other proto-oncogenes and oncogene targets, these proteins could influence the way cells respond to a signal from protein kinase C. These putative mechanisms are hypotheses that are presented to offer a framework that will aid in the design of experiments to examine the mechanisms and cell biology of the endogenous inhibitors of protein kinase C.

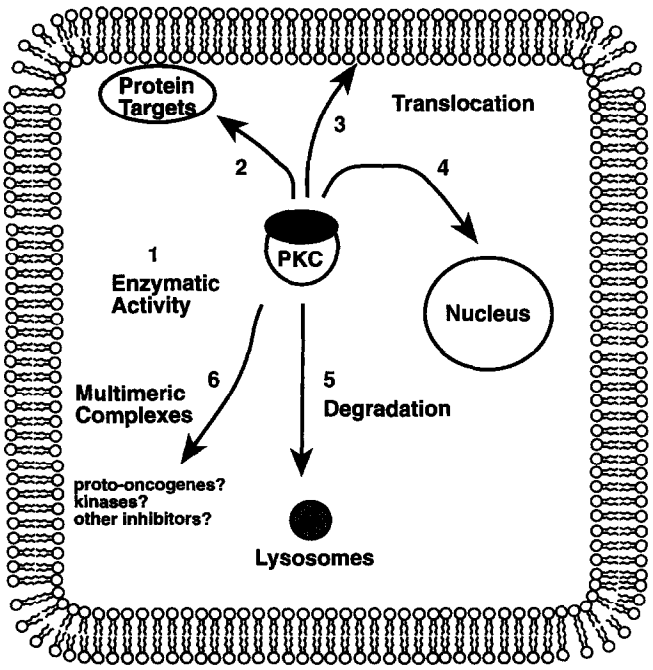


**FIG. 1. Hypothetical intracellular mechanisms by which endogenous inhibitors of protein kinase C could elicit their actions.**

One of the critical questions that must be addressed is whether these proteins, which have been characterized to date as inhibitors of protein kinase C, function primarily to regulate protein kinase C activity or are these proteins involved with other cellular functions. The proteins have been operationally defined as inhibitors based primarily upon *in vitro* assays although some preliminary *in vivo* data

suggest that they function within cells in this capacity [20]. Overexpression and gene knockout experiments will examine more critically the intracellular roles of these proteins. Another critical question is the isozyme specificity of the inhibitors. Are these broad spectrum inhibitors of this class of enzymes or is there isozyme- or subclass-specific action? Since the inhibitors in different families exist as multiple isoforms (PKCI-1, 14-3-3), it will be interesting to determine whether the different isoforms demonstrate different protein kinase C isozyme specificities.

It is clear that the main area in this field awaiting exploration is determining the cellular and physiological roles that these proteins fulfill. This exploration will be facilitated by the isolation of cDNA clones encoding the individual isoforms of these proteins allowing transfection, overexpression, and gene knockout studies to be performed. The isolation of multiple cDNA clones has already been achieved for different isoforms of the 14-3-3 proteins and has just begun for the PKCI-1 proteins.

There are potential practical benefits to the characterization of these protein kinase C inhibitor proteins. The pure proteins as well as the cDNAs cloned into eukaryotic expression vectors may prove important in the examination of the involvement of protein kinase C in regulating specific cellular functions. Many of the current chemical and biological inhibitors of protein kinase C have non-specific actions unrelated to protein kinase C [78]. The development of specific inhibitors to these enzymes, therefore, could provide better research tools in the examination of protein kinase C actions within cells. It is also possible that specific inhibitors of protein kinase C may provide potential therapeutic tools in treating diseases where the inhibition of protein kinase C is useful (reviewed in Ref. 79). The potential for use of these tools will await their characterization in cell systems where well-defined functional endpoints can be assessed carefully.

## References


1. Nishizuka Y, Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* **258:** 607–614, 1992.
2. Dekker LV and Parker PJ, Protein kinase C—a question of specificity. *Trends Biochem Sci* **19:** 73–77, 1994.
3. Tanaka C and Nishizuka Y, The protein kinase C family for neuronal signaling. *Annu Rev Neurosci* **17:** 551–567, 1994.
4. Posner JB, Stern R and Krebs EG, Effects of electrical stimulation and epinephrine on muscle phosphorylase, phosphorylase *b* kinase, and adenosine 3′,5′-phosphate. *J Biol Chem* **240:** 982–985, 1965.
5. Walsh DA, Ashby CD, Gonzalez C, Calkins D, Fischer EH and Krebs EG, Purification and characterization of a protein inhibitor of adenosine 3′,5′-monophosphate-dependent protein kinases. *J Biol Chem* **246:** 1977–1985, 1971.
6. Whitehouse S and Walsh DA, Mg · $ATP^2$-dependent interaction of the inhibitor protein of the cAMP-dependent protein kinase with the catalytic subunit. *J Biol Chem* **258:** 3682–3692, 1983.
7. Scott JD, Fisher EH, Demaille JG and Krebs EG, Identification of an inhibitory region of the heat-stable protein inhibitor of the cAMP-dependent protein kinase. *Proc Natl Acad Sci USA* **82:** 4379–4383, 1985.
8. Olsen SR and Uhler MD, Inhibition of protein kinase-A by overexpression of the cloned human kinase inhibitor. *Mol Endocrinol* **5:** 1246–1256, 1991.
9. Hunter T and Pines J, Cyclins and cancer II: Cyclin D and CDK inhibitors come of age. *Cell* **79:** 573–582, 1994.
10. Pines J, Arresting developments in cell-cycle control. *Trends Biochem Sci* **19:** 143–145, 1994.
11. McDonald JR and Walsh MP, $Ca^{2+}$-binding proteins from bovine brain including a potent inhibitor of protein kinase C. *Biochem J* **232:** 559–567, 1985.
12. Walsh MP, Valentine KA, Ngai PK, Carruthers CA and Hollenberg MD, $Ca^{2+}$-dependent hydrophobic-interaction chromatography: Isolation of a novel $Ca^{2+}$-binding protein and protein kinase C from bovine brain. *Biochem J* **224:** 117–127, 1984.
13. Pearson JD, DeWald DB, Mathews WR, Mozier NM, Zurcher-Neely HA, Heinrikson RL, Morris MA, McCubbin WD, McDonald JR, Fraser ED, Vogel HJ, Kay CM and Walsh MP, Amino acid sequence and characterization of a potent inhibitor of protein kinase C. *J Biol Chem* **265:** 4583–4591, 1990.
14. Simpson GG, Clark G and Brown JS, Isolation of a maize cDNA encoding a protein with extensive similarity to an inhibitor of protein kinase C and a cyanobacterial open reading frame. *Biochim Biophys Acta* **1222:** 306–308, 1994.
15. Bustos SA, Schaefer MR and Golden SS, Different and rapid responses of four cyanobacterial psbA transcripts to changes in light intensity. *J Bacteriol* **172:** 1998–2004, 1990.
16. Robinson K and Aitken A, Identification of a new protein family which includes bovine protein kinase C inhibitor-1. *Biochem J* **304:** 661–664, 1994.
17. McDonald JR, Groschel-Stewart U and Walsh MP, Properties and distribution of the protein inhibitor ($M_r$ 17 000) of protein kinase C. *Biochem J* **242:** 695–705, 1987.
18. McDonald JR and Walsh MP, Inhibition of the $Ca^{2+}$- and phospholipid-dependent protein kinase by a novel $M_r$ 17,000 $Ca^{2+}$-binding protein. *Biochem Biophys Res Commun* **129:** 603–610, 1985.
19. McDonald JR and Walsh MP, Regulation of protein kinase C activity by natural inhibitors. *Biochem Soc Trans* **14:** 585–586, 1986.
20. Rane SG, Walsh MP, McDonald JR and Dunlap K, Specific inhibitors of protein kinase C block transmitter-induced modulation of sensory neuron calcium current. *Neuron* **3:** 239–245, 1989.
21. Mozier NM, Walsh MP and Pearson JD, Characterization of a novel zinc binding site of protein kinase C inhibitor-1. *FEBS Lett* **279:** 14–18, 1991.
22. Hug H and Sarre TF, Protein kinase C isoenzymes: Divergence in signal transduction? *Biochem J* **291:** 329–343, 1993.
23. Melner MH, Searles RP, Spindel ER, Kaynard AH and Nagalla SR, Cloning and expression of an inhibitor of protein kinase C in humans. *Endocrinology* (Suppl) **132:** 538, 1993.
24. Melner MH, Searles RP, Spindel ER, Simerly RB, Link BA, Kaynard AH, Sanders SL and Nagalla SR, Cloning of an inhibitor of protein kinase C in rats: High levels of expression in the ovary and testis. *Biol Reprod* **48**(Suppl 1): 189, 1993.
25. Mozier NM, Zurcher-Neely HA, Guido DM, Mathews WR, Henrikson RL, Fraser ED, Walsh MP and Pearson JD, Amino acid sequence of a 12-kDa inhibitor of protein kinase C. *Eur J Biochem* **94:** 19–23, 1990.
26. Walsh MP, Retraction concerning amino acid sequence of a 12-kDa inhibitor of protein kinase C: Mistaken identity of a protein kinase C inhibitor. *Eur J Biochem* **200:** 811, 1991.
27. Toker A, Ellis CA, Sellers LA and Aitken A, Protein kinase C inhibitor proteins: Purification from sheep brain and sequence similarity to lipocortins and 14-3-3 protein. *Eur J Biochem* **191:** 421–429, 1990.

28. Aitken A, Ellis CA, Harris A, Sellers LA and Toker A, Kinase and neurotransmitters. *Nature* **344:** 594, 1990.
29. Toker A, Sellers LA, Amess B, Patel Y, Harris A and Aitken A, Multiple isoforms of a protein kinase C inhibitor (KCIP-1/14-3-3) from sheep brain: Amino acid sequence of phosphorylated forms. *Eur J Biochem* **206:** 453–461, 1992.
30. Aitken A, Amess B, Howell S, Jones D, Martin H, Patel Y, Robinson K and Toker A, The role of specific isoforms of 14-3-3 protein in regulating protein kinase activity in the brain. *Biochem Soc Trans* **20:** 607–611, 1992.
31. Yamauchi T, Nakata H and Fujisawa H, A new activator protein that activates tryptophan 5-monooxygenase and tyrosine 3-monooxygenase in the presence of $Ca^{2+}$-calmodulin-dependent protein kinase. Purification and characterization. *J Biol Chem* **256:** 5404–5409, 1981.
32. Martin H, Patel Y, Jones D, Howell S, Robinson K and Aitken A, Antibodies against the major brain isoforms of 14-3-3 protein. *FEBS Lett* **331:** 296–303, 1993.
33. Roseboom PH, Weller JL, Babila T, Aitken A, Sellers LA, Moffett JR, Namboodiri MAA and Klein DC, Cloning and characterization of the $\epsilon$ and $\zeta$ isoforms of the 14-3-3 proteins. *DNA Cell Biol* **13:** 629–640, 1994.
34. Aitken A, Collinge DB, van Heusden BPH, Isobe T, Roseboom PH, Rosenfeld G and Soll J, 14-3-3 Proteins: A highly conserved, widespread family of eukaryotic proteins. *Trends Biochem Sci* **17:** 498–501, 1992.
35. Swanson KD and Ganguly R, Characterization of a *Drosophila melanogaster* gene similar to the mammalian genes encoding the tyrosine/tryptophan hydroxylase activator and protein kinase C inhibitor proteins. *Gene* **113:** 183–190, 1992.
36. Hirsch S, Aitkin A, Bertsch U and Soll J, A plant homologue to mammalian brain 14-3-3 protein and protein kinase C inhibitor. *FEBS Lett* **296:** 222–224, 1992.
37. Lu G, DeLisle AJ, De Vetten NC and Ferl RJ, Brain proteins in plants: An *Arabidopsis* homolog to neurotransmitter pathway activators is part of a DNA binding complex. *Proc Natl Acad Sci USA* **89:** 11490–11494, 1992.
38. Tanji M, Horwitz R, Rosenfeld G and Waymire JC, Activation of protein kinase C by purified bovine brain 14-3-3: Comparison with tyrosine hydroxylase activation. *J Neurochem* **63:** 1908–1916, 1994.
39. Morgan A, Roth D, Martin H, Aitken A and Burgoyne RD, Identification of cytosolic protein regulators of exocytosis. *Biochem Soc Trans* **21:** 401–405, 1993.
40. Morgan A and Burgoyne RD, Exo 1 and Exo 2 proteins stimulate calcium-dependent exocytosis in permeabilized adrenal chromaffin cells. *Nature* **355:** 833–836, 1992.
41. Morgan A and Burgoyne RD, Interaction between protein kinase C and Exo 1 (14-3-3 protein) and its relevance to exocytosis in permeabilized adrenal chromaffin cells. *Biochem J* **286:** 807–811, 1992.
42. Roth D, Morgan A, Martin H, Jones D, Martens GJM, Aitken A and Burgoyne RD, Characterization of 14-3-3 proteins in adrenal chromaffin cells and demonstration of isoform-specific phospholipid binding. *Biochem J* **301:** 305–310, 1994.
43. van Heusden GPH, Wenzel TJ, Lagendijk EL, de Steensma HY and van den Berg JA, Characterization of the yeast BMH1 gene encoding a putative protein homologous to mammalian protein kinase II activators and protein kinase C inhibitors. *FEBS Lett* **302:** 145–150, 1992.
44. Martens GJM, Piosik PA and Danen EHJ, Evolutionary conservation of the 14-3-3 protein. *Biochem Biophys Res Commun* **184:** 1456–1459, 1992.
45. Prasad GL, Valverius EM, McDuffie E and Cooper HL, Complementary DNA cloning of a novel epithelial cell marker protein, HME1, that may be down-regulated in neoplastic mammary cells. *Cell Growth Differ* **3:** 507–513, 1992.
46. Leffers H, Madsen P, Rasmussen HH, Honore B, Andersen AH, Walbum E, Vandekerckhove J and Celis JE, Molecular cloning and expression of the transformation sensitive epithelial marker stratifin: A member of a protein family that has been involved in the protein kinase C signalling pathway. *J Mol Biol* **231:** 982–998, 1993.
47. Nielsen PJ, Primary structure of a human protein kinase regulator protein. *Biochim Biophys Acta* **1088:** 425–428, 1991.
48. Zupan LA, Steffens DL, Berry CA, Landt M and Gross RW, Cloning and expression of a human 14-3-3 protein mediating phospholipolysis: Identification of an arachidonoyl-enzyme intermediate during catalysis. *J Biol Chem* **267:** 8707–8710, 1992.
49. Robinson K, Jones D, Patel Y, Martin H, Madrazo J, Martin S, Howell S, Elmore M, Finnen MJ and Aitkin A, Mechanism of inhibition of protein kinase C by 14-3-3 isoforms: 14-3-3 Isoforms do not have phospholipase $A_2$ activity. *Biochem J* **299:** 853–861, 1994.
50. Morrison D, 14-3-3: Modulators of signaling proteins? *Science* **266:** 56–57, 1994.
51. Fu H, Xia K, Pallas DC, Cui C, Conroy K, Narsimhan RP, Mamon H, Collier RJ and Roberts TM, Interaction of the protein kinase Raf-1 with 14-3-3 proteins. *Science* **266:** 126–129, 1994.
52. Fantl WJ, Muslin AJ, Kikuchi A, Martin JA, MacNicol AM, Gross RW and Williams LT, Activation of Raf-1 by 14-3-3 proteins. *Nature* **371:** 612–614, 1994.
53. Irie K, Gotoh Y, Yashar BM, Errede B, Nishida E and Matsumoto K, Stimulatory effects of yeast and mammalian 14-3-3 proteins on the Raf protein kinase. *Science* **265:** 1716–1719, 1994.
54. Freed E, Symons M, Macdonald SG, McCormick F and Ruggieri R, Binding of 14-3-3 proteins to the protein kinase Raf and effects on its activation. *Science* **265:** 1713–1716, 1994.
55. Reuther GW, Fu H, Cripe LD, Collier RJ and Pendergast AM, Association of the protein kinases c-Bcr and Bcr-Abl with proteins of the 14-3-3 family. *Science* **266:** 129–133, 1994.
56. Pallas DC, Fu H, Haehnel LC, Weller W, Collier RJ and Roberts TM, Association of polyomavirus middle tumor antigen with 14-3-3 proteins. *Science* **265:** 535–537, 1994.
57. Alam R, Hachiya N, Sakaguchi M, Kawabata S-i, Iwanaga S, Kitajima M, Mihara K and Omura T, cDNA cloning and characterization of mitochondrial import stimulation factor (MSF) purified from rat liver cytosol. *J Biochem (Tokyo)* **116:** 416–425, 1994.
58. Bazzi MD and Nelsestuen GL, Protein kinase C and annexins: Unusual calcium response elements in the cell. *Cell Signal* **5:** 357–365, 1993.
59. Schlaepfer DD, Jones J and Haigler HT, Inhibition of protein kinase C by annexin V. *Biochemistry* **31:** 1886–1891, 1992.
60. Mochly-Rosen D, Khaner H, Lopez J and Smith BL, Intracellular receptors for activated protein kinase C: Identification of a binding site for the enzyme. *J Biol Chem* **266:** 14866–14868, 1991.
61. Ron D and Mochly-Rosen D, Agonists and antagonists of protein kinase C function, derived from its binding proteins. *J Biol Chem* **269:** 21395–21398, 1994.
62. Ruegg CL and Strand M, Inhibition of protein kinase C and anti-CD3-induced $Ca^{2+}$ influx in Jurkat T cells by a synthetic peptide with sequence identity to HIV-1 gp41. *J Immunol* **144:** 3928–3935, 1990.
63. Kadota J, Cianciolo GJ and Snyderman R, A synthetic peptide homologous to retroviral transmembrane envelope proteins depresses protein kinase C mediated lymphocyte proliferation and directly inactivated protein kinase C: A potential mechanism for immunosuppression. *Microbiol Immunol* **35:** 443–459, 1991.
64. Gottlieb RA, Kleinerman ES, O'Brian CA, Tsujimoto S,

Cianciolo GJ and Lennarz WJ, Inhibition of protein kinase C by a peptide conjugate homologous to a domain of the retroviral protein p15E. *J Immunol* **145:** 2566–2570, 1990.
65. Charp PA, Rice WG, Raynor RL, Reimond E, Kinkade JM, Ganz T, Selsted ME, Lehrer RI and Kuo JF, Inhibition of protein kinase C by defensins, antibiotic peptides from human neutrophils. *Biochem Pharmacol* **37:** 951–956, 1988.
66. Balazovich KJ, Smolen JE and Boxer LA, Endogenous inhibitor of protein kinase C: Association with human peripheral blood neutrophils but not with specific granule-deficient neutrophils or cytoplasts. *J Immunol* **137:** 1665–1673, 1986.
67. Balazovich KJ, McEwen EL, Lutze ML, Boxer LA and White T, Purification of PKC-I, an endogenous protein kinase C inhibitor, and types II and III protein kinase C isoenzymes from human neutrophils. *Biochem J* **284:** 399–405, 1992.
68. Huang C-K and Oshana SC, Partial characterization of protein kinase C and inhibitor activity of protein kinase C in rabbit peritoneal neutrophils. *J Leukoc Biol* **39:** 671–678, 1986.
69. Gandini E, Orlando P, Selvatici R, Balboni A, Boninsegna S and Rubini M, Identification of a novel protein kinase C inhibitor in microsomes from phytohaemagglutinin activated human peripheral blood mononuclear cells. *FEBS Lett* **329:** 324–328, 1993.
70. Pribilla I, Kruger H, Buchner K, Otto H, Schiebler W, Tripier D and Hucho F, Heat-resistant inhibitors of protein kinase C from bovine brain. *Eur J Biochem* **177:** 657–664, 1988.
71. Schwantke N and Le Peuch CJ, A protein kinase C inhibitory activity is present in rat brain homogenate. *FEBS Lett* **177:** 36–40, 1984.
72. Eyster KM, An endogenous inhibitor of protein kinase C in the pseudopregnant rat ovary. *Biochem Biophys Res Commun* **168:** 609–615, 1990.
73. Eyster KM, Waller MS, Miller TL, Miller CJ, Johnson MJ and Persing JS, The endogenous inhibitor of protein kinase-C in the rat ovary is a protein phosphatase. *Endocrinology* **133:** 1266–1273, 1993.
74. Fields S and Sternglanz R, The two-hybrid system: An assay for protein–protein interactions. *Trends Genet* **10:** 286–292, 1994.
75. Vojtek AB, Hollenberg SM and Cooper JA, Mammalian Ras interacts directly with the serine/threonine kinase Raf. *Cell* **74:** 205–214, 1993.
76. Olson EN, Burgess R and Staudinger J, Protein kinase C as a transducer of nuclear signals. *Cell Growth Differ* **4:** 699–705, 1993.
77. Young S, Parker PJ, Ullrich A and Stahel S, Down-regulation of protein kinase C is due to increased rate of degradation. *Biochem J* **244:** 775–779, 1987.
78. Wilkinson SE and Hallam TJ, Protein kinase C: Is its pivotal role in cellular activation over-stated? *Trends Pharmacol Sci* **15:** 53–57, 1994.
79. Bradshaw D, Hill CH, Nixon JS and Wilkinson SE, Therapeutic potential of protein kinase C inhibitors. *Agents Actions* **38:** 135–147, 1993.