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Allosteric Regulation of Protein Kinase PKCζ by the N-Terminal C1 Domain and Small Compounds to the PIF-Pocket

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SUMMARY

Protein kinases are key mediators of cellular signaling, and therefore, their activities are tightly controlled. AGC kinases are regulated by phosphorylation and by N- and C-terminal regions. Here, we studied the molecular mechanism of inhibition of atypical PKCζ and found that the inhibition by the N-terminal region cannot be explained by a simple pseudosubstrate inhibitory mechanism. Notably, we found that the C1 domain allosterically inhibits PKCζ activity and verified an allosteric communication between the PIF-pocket of atypical PKCs and the binding site of the C1 domain. Finally, we developed low-molecular-weight compounds that bind to the PIF-pocket and allosterically inhibit PKCζ activity. This work establishes a central role for the PIF-pocket on the regulation of PKCζ and allows us to envisage development of drugs targeting the PIF-pocket that can either activate or inhibit AGC kinases.

INTRODUCTION

From bacteria to vertebrates, protein phosphorylation plays an important role in the transmission of intracellular signals (Pawson and Scott, 2005). Protein kinases, which catalyze phosphorylation events, have evolved stringent mechanisms for the regulation of their intrinsic activities and their ability to phosphorylate specific downstream substrates (Biondi and Nebreda, 2003; Huse and Kuriyan, 2002; Shi et al., 2006). Importantly, deregulation of intracellular phosphorylation can lead to human diseases such as diabetes, cancer or neurological disorders. Thus, there is significant interest in the development of drugs to regulate the activity of protein kinases (Cohen, 2002).

The kinase catalytic core consists of a small N-terminal lobe and a large C-terminal lobe. The ATP-binding site, which is common to all protein kinases and the target of most drug development programs against kinases, is located in between these two lobes (Knighton et al., 1991a) (Figure 1A). Protein kinases are themselves also regulated by phosphorylation, and members of the AGC kinase group share a mechanism of activation involving phosphorylation at three distinct sites: the activation loop (within the catalytic core) as well as the Z/turn-motif and the hydrophobic motif (HM) within a C-terminal extension that is only conserved in this family of protein kinases (Parker and Parkinson, 2001). In active AGC kinases, the HM binds to a specific pocket on the small lobe, which is defined by helices α-B and α-C and strands β-4 and β-5 (Knighton et al., 1991b; Yang et al., 2002a). We first characterized the regulatory role of the HM and its binding site in PDK1 and termed this site the "PIF-binding pocket" (Biondi et al., 2000). Notably, the binding of a polypeptide derived from the C-terminal HM of PRK2 (PIFtide) activated PDK1 in vitro, whereas mutations of residues at the PIF-pocket of PDK1 rendered mutant proteins with higher activity (Biondi et al., 2000), suggesting that the PIF-pocket was a key regulatory site for PDK1. It is now understood that many AGC kinases share a similar molecular mechanism of activation comprising HM phosphorylation and binding of the phospho-HM to the PIF-pocket, stabilizing the active structure of the kinase catalytic domain (Biondi et al., 2000; Frödin et al., 2002; Pearce et al., 2010; Yang et al., 2002b). Thus, the PIF-pocket is a key regulatory site for the molecular mechanism of activation of a large set of AGC kinases.

Interestingly, we and others were able to develop small compounds reversible activators of PDK1 (Engel et al., 2006; Hindie et al., 2009; Stockman et al., 2009; Stroba et al., 2009; Wei et al., 2010) and provided crystallography and solution data on the binding site and conformational change that prompted the activation (Hindie et al., 2009). A first hint that the PIF-pocket could also transduce the inhibition of PDK1 was the finding that a PDK1 protein mutated at the PIF-pocket residue Thr148 (PDK1 [Thr148Val]) was not activated but inhibited by PIFtide in vitro (Engel et al., 2006). More recently, mutation of PIF-pocket residues to Cys and interrogation with Cys-containing fragment library of compounds allowed the identification of activators and inhibitors of PDK1 via disulfide covalent trapping of the fragments to the

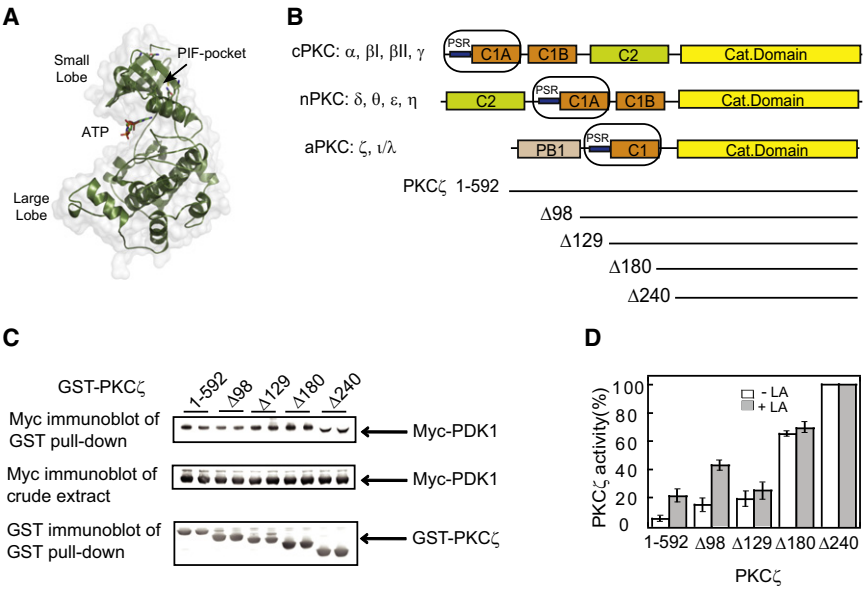

Figure 1. Molecular Mechanism of Regulation of PKCζ by N-Terminal Domains

(A) Structure of the catalytic core of PKCζ (model based on PKCι structure, PDB code 1ZRZ) (Messerschmidt et al., 2005).

(B) Schematic overview of PKC isoforms indicating the different domains present in the classical, novel and atypical PKCs (C2, C2 domain; PSR, pseudosubstrate region; C1, C1 domain; PB1, PB1 domain; Cat. Domain, protein kinase catalytic domain) and the PKCζ wild-type (1-592) and truncated versions used in this study.

(C) Interaction between PKCζ and PDK1. The N-terminal region of PKCζ does not regulate the ability of PKCζ to interact with its upstream kinase PDK1.

(D) Activity of the N-terminally truncated PKCζ constructs in the presence or absence of lipid activators (LA) using MBP as a substrate. A significant increase in the activity of PKCζ was observed after removal of the C1 domain. The average of two independent experiments using two different batches of purified deletion constructs is shown. The error bars indicate the standard deviation of the averages. The specific activity of PKCζ [Δ240] (100%) varied between 25 and 40 nmol/mg min in different purifications. Figure S1 shows the phosphorylation state of the enzymes shown in (D). Figure S2 shows that PKCζ and PKCι proteins without a GST-tag are also similarly activated by LA.

introduced Cys residues at the PIF-pocket (Sadowsky et al., 2011). Together, the findings open the possibility to pharmacologically target the PIF-pocket for the activation or inhibition of PDK1 and broaden the rather poorly represented field of development of allosteric activators of enzymes (Zorn and Wells, 2010).

In spite of the importance of protein kinases for cells, health, and disease, the molecular mechanistic detail of protein kinase regulation remains largely unknown, partly because very few full-length protein kinases have been crystallized. Recently, the full-length PKBα/Akt1 was crystallized in the presence of an allosteric inhibitor that requires the N-terminal pleckstrin-homology (PH) domain for its effective inhibition (Wu et al., 2010). The activity of protein kinase C (PKC) isoforms is also regulated by N-terminal domains (Hurley et al., 1997; Newton, 2010; Rosse et al., 2010). The pseudosubstrate region (PSR) blocks the substrate binding site and keeps the protein unable to phosphorylate substrates, whereas the binding of activating second messengers to other N-terminal domains release the pseudosubstrate inhibition exposing the substrate binding site and allowing the kinase to phosphorylate substrates. PKC isoforms differ in the identity of the domains present within the N-terminal region (Figure 1B). Although classical and novel PKCs possess a C2 domain and two C1 domains (C1A and C1B), atypical PKCs (ζ and ι/λ isoforms) do not have a C2 domain and have only one C1 domain. On the other hand, atypical PKCs possess a PB1 domain, which is absent in all other PKC isoforms.

All PKC isoforms contain a C1 domain preceded by the PSR. The binding of the PSR between the small and large lobes of the catalytic domain as if it were a substrate must position the C1A domain (in classical and novel PKCs) and the unique C1 domain (in atypical PKCs) directly adjacent to the peptide substrate binding site. A major step forward in the understanding of the molecular mechanism of regulation of PKCs was the publication of the crystal structure of full-length PKCβII (Leonard et al., 2011). The structure unveils the position of the C2 domain and the interactions of the C1B domain with the catalytic domain. The catalytic domain structure appears to be only partially active because the C1B domain sequesters a Phe residue (Phe629, part of a conserved NFD sequence) that frequently forms part of the ATP-binding site in active structures of AGC kinases. However, the role of the C1B domain-NFD interaction in the mechanism of regulation of the whole PKC family is not clear because atypical PKCs only have one C1 domain, which is equivalent to the C1A domain. It is also currently accepted that the PIF-pocket and the helix α-C do not play a role in the regulation of PKCs (Leonard et al., 2011).

Most of the studies on the regulation of PKCs have been based on classical PKCs. Atypical PKCs are expected to follow a similar general mechanism of inhibition of activity, where an N-terminal PSR binds to the active site and blocks protein substrate binding (Newton, 2010). However, in contrast to classical PKCs that are activated by the binding of diacylglycerol and Ca^{2+} to the C1 and C2 domains, atypical PKCs are regulated by a PB1 domain, which is known to be involved in modular protein-protein interactions (Ito et al., 2001), and by one atypical C1 domain (Colón-González and Kazanietz, 2006) whose function is not clear but that could mediate the binding to lipids and reverse the pseudosubstrate mediated inhibition (Hirai and Chida, 2003; Müller et al., 1995; Nakanishi and Exton, 1992). In either case, it is reasonable to expect that the common aspects of the molecular mechanism of regulation of all PKC isoforms are based on the common features present in the N-terminal region: the PSR and its contiguous C1 domain.

In the present work, we studied the molecular mechanism of regulation of the atypical PKCζ and found that the inhibition by the N-terminal region cannot be explained by a simple pseudosubstrate inhibitory mechanism. We also unexpectedly found that the inhibition of the kinase activity was directly mediated by the C1 domain. Moreover, we uncovered an unexpected role of the PIF-pocket in the regulation of atypical PKCs. Finally, we

confirmed the important role of the PIF-pocket by developing small compounds that, binding to the PIF-pocket of PKCζ, can allosterically inhibit its activity. Together, the present work provides evidence that the PSR and its adjacent C1 domain act in concert both for the allosteric inhibition and activation of atypical PKCs, whereas the PIF-pocket is central to the allosteric inhibition.

RESULTS

The Role of N-Terminal Domains in the Regulation of PKCζ

Substrates of PDK1 often regulate their activation-loop phosphorylation by modulating their ability to interact with the upstream kinase, PDK1 (Leslie et al., 2001). To study the mechanism of PKCζ regulation, we first tested whether the N-terminal regions mediate or regulate the interaction of PKCζ with PDK1. Deletion of the N-terminal domains (Figure 1B) did not significantly affect the interaction with PDK1, indicating that they do not play a major role in regulating the interaction (Figure 1C). We then measured the activity of these N-terminally truncated PKCζ constructs using ATP [γ^{32}P] and MBP as substrates. Because the surrogate substrate MBP is not a physiological substrate, it is not expected to induce specific conformational changes in PKCζ. Therefore, this assay should provide a direct measure of the proportion of PKCζ molecules that have an active conformation in solution. Full-length PKCζ (1-592) had low basal activity and was activated by the lipid activator (LA) phosphatidylserine (Figure 1D). GST-PKCζ [Δ98], which lacks the PB1 domain, had increased activity. However, this truncated construct also had increased phosphorylation in the activation-loop, suggesting that the increase in specific activity may be due, at least in part, to an indirect effect of the slightly higher level of phosphorylation (see Figure S1 available online). Interestingly, we observed that GST-PKCζ [Δ129], which also lacked the PSR, did not exhibit a major increase in basal activity as compared to GST-PKCζ [Δ98] (Figure 1D). This was surprising because a simple model of pseudosubstrate inhibition would predict a high increase in activity when the pseudosubstrate segment is removed. Finally, we observed that GST-PKCζ [Δ129] was not activated by LA, indicating that the pseudosubstrate region was important for the activation by lipids. This was also unexpected because the C1 domain (but not the pseudosubstrate region) was thought to be primarily responsible for lipid binding and activation. Notably, we found that a construct of PKCζ also lacking the C1 domain, GST-PKCζ [Δ180], had increased basal activity (Figure 1D), indicating that the C1 domain, in the absence of the PSR, participated in the inhibition of PKCζ basal activity. Altogether, the above data were not compatible with a simple model of pseudosubstrate-mediated inhibition of PKCζ activity. Rather, the results suggested that the C1 domain inhibited PKCζ basal activity independently of the PSR, and that the PSR was required for the LA-mediated activation of PKCζ. Furthermore, our data are consistent with the possibility that the C1 domain could allosterically inhibit PKCζ.

The Effect of the Pseudosubstrate Region on the Stability of PKCζ

The results described above were puzzling because they did not reveal a central role for the PSR in the inhibition of PKCζ. Therefore, we next examined the role of the PSR in PKCζ. The pseudosubstrate region of PKCs comprises a high number of positively charged residues. To study the role of the PSR of PKCζ, we prepared a PKCζ construct (PKCζ [7Arg/Lys-Ala]) that had Arg116, Arg117, Arg120, Arg121, Arg123, Lys124, and Arg127 residues within the pseudosubstrate region mutated to Ala (KSIYRRGARRWRKLYRAN; mutated residues underlined). PKCζ [7Arg/Lys-Ala] was significantly less stable in temperature shift experiments than the wild-type protein (Figure 2A). The stability data indicated that the positively charged residues within the pseudosubstrate region interacted with other regions of the protein, providing stability. Interestingly, the wild-type protein was also less stable in the temperature shift assay when the incubation was performed in the presence of lipid activators (Figure 2B), suggesting that the binding of lipids to the wild-type protein reduced interactions that both inhibited and stabilized the protein. The loss of stability may be due to loss of interactions involving the different N-terminal regulatory regions of PKCζ (PB1 domain, pseudosubstrate or C1 domain). However, in parallel experiments, PKCζ [7Arg/Lys-Ala] did not lose any further stability in the presence of lipids (Figure 2C), indicating that the pseudosubstrate region, and not the PB1 domain or the C1 domain, mediated the LA-dependent loss of thermal stability. Together, the data suggested that specific interactions mediated by the positively charged residues within the pseudosubstrate segment were responsible for the decreased stability in the presence of lipid activators. The above data are consistent with the existence of an intramolecular interaction between the PSR and the catalytic domain, which is released upon the binding of lipids.

The Effect of PSRtide on the Activity of PKCζ

We also studied the specific activity of wild-type and N-terminally truncated mutants of PKCζ using a polypeptide corresponding to the pseudosubstrate region of PKCζ as a substrate, where Ala 119 is replaced by Ser (PSRtide) (House and Kemp, 1987). In contrast to MBP, this substrate is derived from a region of PKCζ that may prompt direct or indirect specific interactions with its catalytic core to inhibit PKCζ. The full-length PKCζ protein phosphorylated PSRtide very efficiently with a specific activity of 60–80 nmol/mg min (Figure 3A), supporting the idea that the pseudosubstrate region could indeed bind to the active site of PKCζ. Notably, the LA did not increase the activity of PKCζ toward this substrate, suggesting that the binding of PSRtide to PKCζ overcame its mechanism of inhibition. Moreover, in sharp contrast to the results using MBP as the substrate of the reaction, the truncated mutants did not have increased activity in comparison to full-length PKCζ when PSRtide was used (Figures 3A and 3B). A similar difference between MBP and PSRtide was observed using 1-587 atypical PKCι isoform and PKCζ (1-592) produced in insect cells with a 6xHis-tag and purified to homogeneity (Figure S2) indicating that the effect was not due to the GST fusion and that it was common to both atypical PKC isoforms. However, the GST-PKCζ [Δ129] construct, which included the C1 domain but not the pseudosubstrate region, had significantly lower specific activity than all other constructs tested (Figures 3A and 3C; Figure S2). This result indicated that in GST-PKCζ [Δ129], the inhibitory effect of the C1 domain could not be reversed by PSRtide. Because

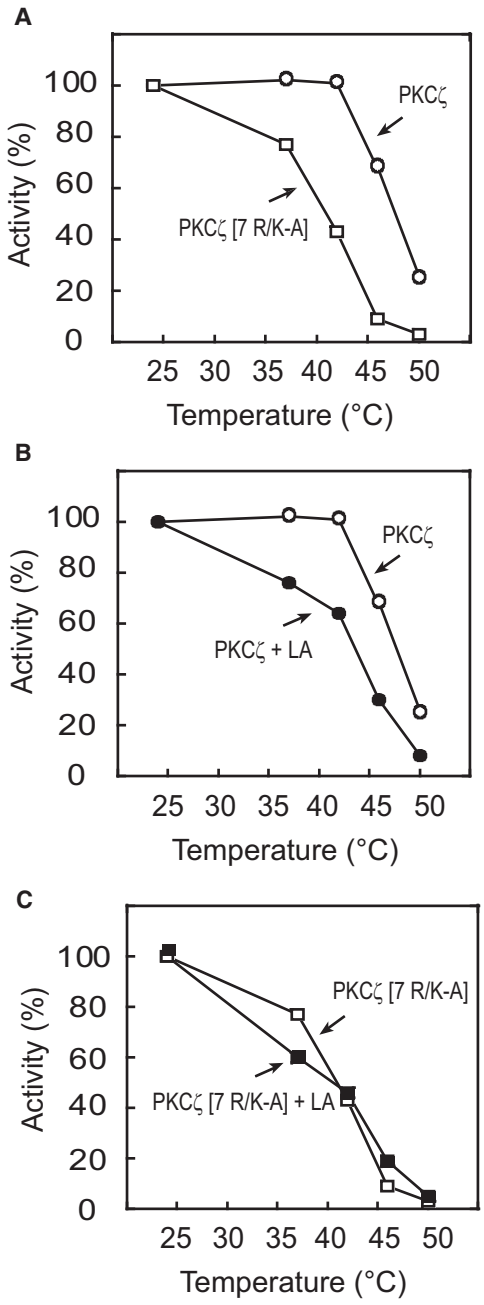

Figure 2. Thermal Stability of PKCζ and PKCζ [7R/K-A]

The wild-type (wt) GST-PKCζ (○) or the GST-PKCζ [7Arg/Lys-Ala] (7R/K-A) mutant (□) were incubated in the presence (closed symbols ●, ■) or absence (open symbols ○, □) of lipid activator (LA), incubated for 2 min at the indicated temperatures and assayed for the remaining protein kinase activity at 24°C using MBP as a substrate. The activity of PKCζ or PKCζ [7R/K-A] obtained by incubation at 24°C was set as 100%.

(A) PKCζ [7R/K-A] was significantly less stable during 2 min temperature shifts than PKCζ wt.

(B) The presence of LA destabilized PKCζ wt.

(C) PKCζ [7R/K-A] did not lose further protein stability in the presence of lipids, indicating that the pseudosubstrate region mediated the LA-dependent loss of thermal stability. The assay shown was performed in triplicate with similar results obtained in two separate experiments. The specific activity of the enzymes shown were 14–15 nmol/mg min.

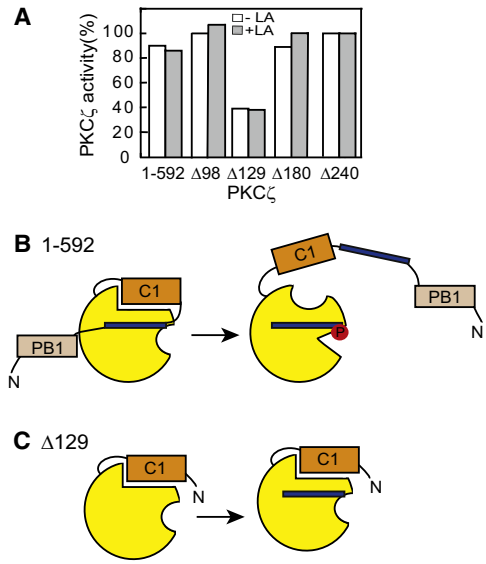

Figure 3. The Effect of PSRtide on the Activity of PKCζ

(A) The activity of GST-PKCζ wt and deletion constructs was measured using 100 μM of PSRtide as the substrate of the reaction in the presence (gray columns) or absence (white columns) of 100 ng phosphatidyl serine (LA).

(B and C) Schematic models representing how PSRtide overcomes the allosteric inhibition of PKCζ (1-592) and not PKCζ [Δ129]. The model represents the C1 domain interacting with the catalytic domain (yellow) and allosterically affecting the active site (round shape). Release of the allosteric inhibition by the C1 domain allows the active site to stabilize in an active conformation (angular shape). (B) PKCζ (1-592) in the presence of PSRtide had high basal activity and was not further activated by LA, similarly to PKCζ [Δ240], which is comprised of only the catalytic domain of PKCζ. The model represents that when PSRtide competes and displaces the PSR from the PKCζ active site, it also displaces all inhibition by N-terminal domains. (C) A model explaining the effect of PSRtide on PKCζ [Δ129]. PKCζ [Δ129], which lacks the PSR, had much lower activity than the other constructs, indicating that even in the presence of PSRtide, PKCζ [Δ129] remained significantly in an inactive conformation. Together, the resulting model suggests that the PSR and C1 domain act in concert to inhibit PKCζ and that the displacement of PSR is also coupled to the release of the inhibition by its contiguous C1 domain. Figure S3 represents models consistent with the results obtained with the different constructs of PKCζ.

our data indicated that the PSR indeed occupied the substrate binding site, this result further confirmed that the C1 domain inhibition was allosteric and not the result of simple inhibition by competition for the substrate binding site. In all PKC isoforms, the PSR is directly linked to a C1 domain (C1A domain in classical PKCs). Therefore, the position of the C1 domain and its allosteric regulatory site must be located along a region that comprises the helix α-C, which limits the PIF-pocket in AGC kinases.

Allosteric Communication between the PIF-Pocket and the C1 Interaction Site on the Catalytic Domain of Atypical PKCs

We set up a novel assay to investigate the possible interaction between the C1 domain constructs fused to GST and the catalytic domain of atypical PKCs containing a 6xHis-tag. The assay is based on the alphascreen technology using a donor bead coupled to anti-GST antibodies (to bind the GST-PSR-C1 or GST-C1 constructs derived from PKCι) and Ni-NTA acceptor

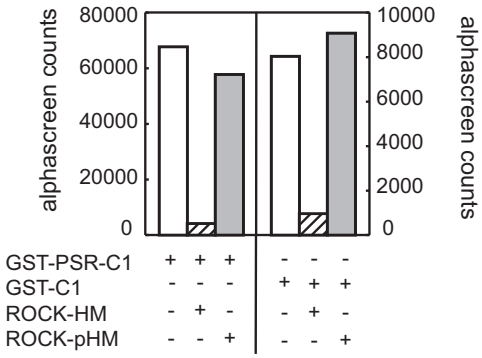

Figure 4. Interaction of C1 domain constructs of PKCι with Its Catalytic Domain

AlphaScreen interaction assay showing the binding of GST-PSR-C1 (left y axis) or GST-C1 (right y axis) to His-PKCι Δ223. The interaction of both, GST-PSR-C1 and GST-C1, with His-PKCι Δ223 was strongly diminished upon addition of the HM-peptide derived from the AGC-kinase ROCK (ROCK-HM). In contrast, the corresponding peptide phosphorylated at the HM phosphorylation site (ROCK-pHM) was not able to displace the binding, indicating a high degree of selectivity. Figure S4 shows structural models of PSR and C1 domain interaction with the catalytic core of PKCζ. Figure S6 represents a scheme on the activation of PKCζ by LA.

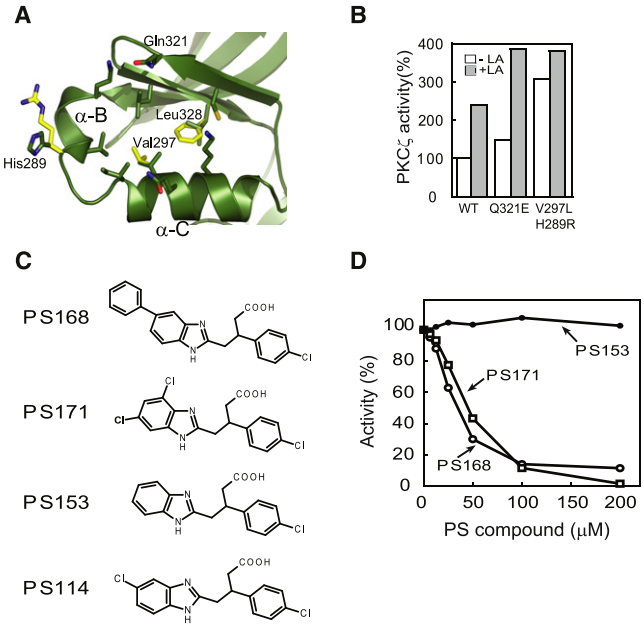

Figure 5. The PIF-Pocket Mediates the Inhibition of PKCζ

(A) A detailed view of the PIF-pocket in the small lobe of the PKCζ model as shown in Figure 1A. Key residues Gln321, Leu328, His289, and Val297 are depicted.

(B) Activity assay of PKCζ wild-type (wt) and mutants using MBP as a substrate. Mutation of Val297 to Leu and His289 to Arg resulted in a protein with higher basal activity than PKCζ wild-type, suggesting that this mutant protein partially bypassed the inhibition.

(C) Structures of the low molecular weight compounds described in this study.

(D) PKCζ (1-592) is inhibited by PS168 (○) and PS171 (□) but not by compound PS153 (●). The comparison between PS168, PS171, and PS153 indicates that the different substituents on the benzimidazole ring play an important role in the mode of action of the PS168 and PS171 inhibitory compounds and provides evidence for the specificity of their inhibition. Figure S5 shows the inhibition of full length and catalytic domain of PKCζ by PS168 and PS171 using PSRtide as a substrate of the reaction. The selectivity for the inhibition of PKCζ is shown in Table S1.

beads that bind to His-PKCι Δ223 (catalytic domain). The interaction is measured by the emission of light from the acceptor beads that happens when the two beads are in close proximity. GST-PSR-C1 readily interacted with His-PKCι Δ223. Interestingly, PIFtide and the HM polypeptide derived from another AGC kinase, ROCK (ROCK-HM, VGNQLPFIGFTYFRENL), but not the phosphorylated peptide derived from the HM of ROCK (ROCK-pHM, VGNQLPFIGFTYFRENL), displaced the interaction (Figure 4). Notably, the construct lacking the PSR (GST-C1) had low but measurable affinity for His-PKCι Δ223 and this interaction was also displaced by the HM polypeptide from ROCK but not by the phosphorylated HM from ROCK (Figure 4) or by an unrelated polypeptide (RTWALCGTPEYLAPEIILKK) derived from the activation loop of PKA (not shown), indicating that the interaction between the C1 domain and the catalytic domain was highly selective. The results show that the C1 domain of an atypical PKC directly interacts with the catalytic domain and suggest an allosteric communication between the PIF-pocket and the C1 domain interaction site. The C1 domain does not possess the classical Phe-Xaa-Xaa-Phe HM sequence and modeling does not predict that it could occupy the hydrophobic PIF-pocket as the HM. Indeed modeling of the pseudosubstrate into the substrate binding site, allows the interaction of the C1 domain with the external part of the helix α-C that is a main component of the PIF-pocket (Figure S4).

The Role of the PIF-Pocket of PKCζ in the Regulation of Its Activity

Our previous work showed that AGC kinases can be activated by conformational changes induced in the allosteric PIF-pocket and transmitted to the active site. After we obtained evidence that PKCζ inhibition involved an allosteric inhibition by the C1 domain, we investigated whether the PIF-pocket also participated in the mechanism of inhibition of PKCζ. Mutation of Gln321 to Glu only minimally affected the basal activity and was activated normally by LA. However, we found that mutation of two residues within the PIF-pocket, Val297, which is located at the bottom of the hydrophobic pocket and His289, located on helix α-B (Figure 5A), to Leu or Arg respectively, resulted in a protein with consistently higher basal activity and lower activation by LA (Figure 5B). This result was compatible with a model in which the PIF-pocket of PKCζ indeed participates in the allosteric inhibition of the activity by the N-terminal regions and where the PIF-pocket mutations release most of this inhibition.

Allosteric Inhibition of PKCζ with Low Molecular Weight Compounds Targeting the PIF-Pocket

We and others previously showed that the PIF-binding pocket of PDK1 can be targeted by low molecular weight compounds that activate the kinase (Engel et al., 2006; Hindie et al., 2009; Stockman et al., 2009; Stroba et al., 2009; Wei et al., 2010). The identification of small compounds that activated PDK1 upon binding to the PIF-binding pocket was conclusive evidence

that the PIF-pocket, on its own, was able to transduce the conformational changes that activate PDK1. As part of our follow-up study, we found that the compounds PS171 and PS168 inhibited PKCζ activity with <50 μM IC_{50} (Figures 5C and 5D). In contrast, the related compound PS153, lacking the phenyl ring or the Cl atoms on the benzimidazole ring, did not inhibit PKCζ, suggesting that the observed inhibition was specific. PS171 and PS168 inhibited the basal activity of PKCζ when MBP was used as a substrate (Figure 5D), and PKCζ and PKCζ[Δ240] were inhibited when the peptide PSRtide was used as a substrate (Figure S5). Surprisingly, the compounds were considerably more potent inhibitors of PKCζ than of the closely related atypical PKCι (Table S1). Selectivity profiling also revealed that PS171 weakly activated PDK1 and that the activation of PDK1 was specific due to the binding of PS171 to the PIF-binding pocket. Indeed, PS171 and other compounds from this series also led to specific activators of PDK1. For instance, PS114 (Figure 5C) (the 5-chloro-benzimidazolyl analog of PS171) activated PDK1 3–4-fold with AC_{50} = 32 μM (Figure 6A). Moreover, the crystal structures of PDK1 in complex with PS171 and PS114 confirmed that these compounds bound to the PIF-binding pocket (Figures 6B and 6C; Table S2). These results indicated that compounds of the 4-benzimidazolyl-3-(4-chlorophenyl) butanoic acid scaffold activated PDK1 but inhibited PKCζ by binding to the corresponding PIF-pockets of these AGC kinases, respectively acting as "agonists" or "antagonists" of the protein kinase activity.

To confirm that PS168 and PS171 were indeed binding to the PIF-pocket of PKCζ, we examined whether they could also inhibit PKCζ proteins that were mutated within this pocket. Notably, PKCζ [Val297Leu; His289Arg] was resistant to inhibition by PS168 and PS171 (Figure 5D), suggesting that the PIF-pocket mediated the inhibition by these compounds. In addition, because PKCζ [Val297Leu; His289Arg] also bypassed the inhibition by the N-terminal domains, the results further suggested that the small compounds and the N-terminal domains used the same PIF-pocket-mediated mechanism of inhibition. We noted above (Table S1) that atypical PKCι isoform was not inhibited by PS168 and PS171 to the same degree as PKCζ. The two isoforms have a relatively conserved PIF-pocket but differ in one residue at the bottom of the PIF-pocket that is Leu328 in PKCζ and Phe in PKCι. Replacement of PKCζ Leu328 for Phe, the equivalent residue in PKCι, rendered a mutant protein that, similar to PKCι, was not inhibited by the small compounds (Figure 6D). To obtain more conclusive evidence for the binding site of PS168 and PS171, we mutated the PIF-binding pocket of PDK1 by replacing eight residues with the equivalent PKCζ amino acids (Leu113Val, Ile118Val, Ile119His, Val124Ile, Thr128Gln, Arg131Lys, Thr148Cys, and Phe157Leu), rendering a chimera protein, PDK1-[PIF-pocket-PKCζ] (Figure 6E). Whereas PDK1 was weakly activated by PS168 and PS171, the PDK1-[PIF-pocket-PKCζ] mutant was now inhibited by PS168, resembling the effect observed on PKCζ (Figure 6F). Thus, our data indicated that the PIF-pocket of PKCζ was the target for the small compounds that allosterically inhibited PKCζ. Because the PIF-pocket also mediated the inhibition by N-terminal domains, we conclude that the PIF-pocket is a key site participating in the molecular mechanism of inhibition of PKCζ.

The Effect of PS Compounds on PKCζ-Dependent NFκB Signaling in U937 Cells

Inhibition of PKCζ was postulated to be therapeutically useful for treatment of immune and inflammatory diseases (Trujillo et al., 2009). In this context, it was previously reported that PKCζ plays an important role in the activation of NFκB (Levy et al., 2011; Müller et al., 1995). Preincubation of U937 cells with PS171 and PS168, but not with their inactive counterpart PS153, blocked TNFα-induced NFκB activation with an IC_{50} < 50 μM (Figure 7). A larger set of 4-benzimidazolyl-3-phenylbutanoic acids compounds variants of PS168 and PS171 showed a good correlation between the in vitro inhibition of PKCζ activity and the inhibition of NFκB signaling in cells (Fröhner et al., 2011), providing further evidence that the cellular target is likely PKCζ. This result indicates that the PIF-pocket of PKCζ is accessible in vivo under physiological conditions and suggests that equivalent allosteric effects that inhibit PKCζ in vitro also inhibit its physiological function in cells. The results therefore support the idea that allosteric PIF-pocket drugs could act as inhibitors of signaling pathways where AGC kinases are involved.

DISCUSSION

To catalyze specific and timely phosphorylation of substrates, the activity of protein kinases is tightly regulated. In spite of the importance of protein kinases in the signaling of physiological and pathological responses, the molecular mechanisms that operate the regulation of protein kinases remain largely unknown. The mechanism of regulation of PKC isoforms has been widely investigated over the years, and a general model was established. The common aspects of the molecular mechanism of regulation of PKCs by N-terminal domains must be based on common features present in all isoforms. Because all PKC isoforms possess a PSR and an adjacent C1 domain, it is reasonable to expect that the common mechanism of regulation by the N-terminal domains must involve these two regions and their interaction sites on the catalytic domain. However, the recent crystal structure of PKCβII does not reveal the molecular details of the interaction between the PSR or its contiguous C1 domain and the catalytic core. The present work provides new data to support a model for the molecular mechanism of inhibition by the PSR and its contiguous C1 domain: (1), the C1 domain makes specific interactions with the catalytic domain; (2), the C1 domain allosterically inhibits PKCζ activity; (3), mutations in the PIF-pocket bypass the inhibition by the N-terminal domains; (4), HM-polypeptides displace the interaction between the C1 domain and the catalytic domain; and (5), small compounds that specifically bind to the PIF-pocket of PKCζ transduce conformational changes that inhibit its activity. The data allows us to revise the model of the mechanism of inhibition of atypical PKCζ, assigning an allosteric inhibitory role to the C1 domain and a central regulatory role to the PIF-pocket. Because the PSR and its adjacent C1 domain are common to all PKC isoforms, we suggest that the mechanisms described here may be shared by the whole family.

Our data clearly disprove the existence of a simple pseudosubstrate mechanism of inhibition in PKCζ, because deletion of the PSR did not increase the specific activity of the kinase. Nevertheless, consistent with the accepted model, our studies

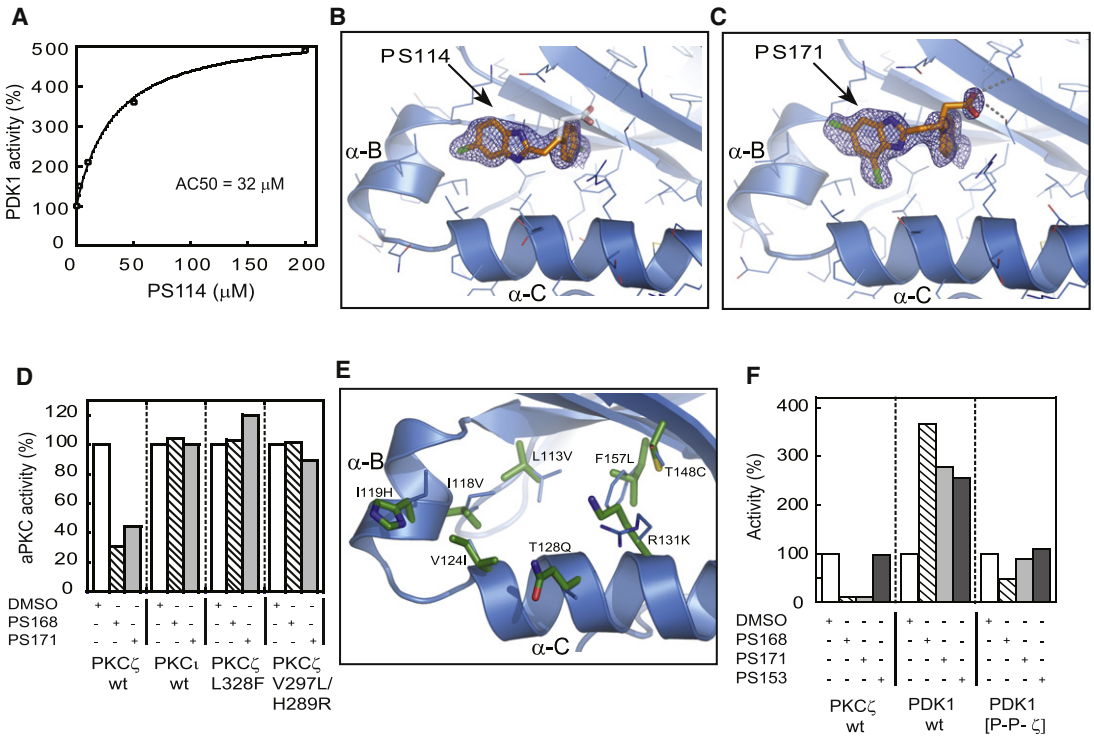

Figure 6. Binding of the Benzimidazole Compounds to the PIF-Pocket of Different AGC kinases can allosterically activate or inhibit the kinases, acting as agonists or antagonists of the activity.

(A) Activation of PDK1 by PS114.

(B and C) Crystal structures of PDK1 in complex with the allosteric activators PS114 (B) and PS171 (C). The ring systems of both compounds are positioned similarly in the PIF-binding pocket of PDK1. The carboxylate moiety of PS114 is unresolved and is shown in transparent white. The depicted $2F_o$-F_c electron density maps are contoured at 1σ.

(D) Activity assays with PKCζ mutants identify the PIF-pocket as the target site of PS168 and PS171. PS168 and PS171 (50 μM) inhibit PKCζ wt but not PKCι or PKCζ proteins mutated within the PIF-pocket (PKCζ [Leu328Phe] and PKCζ [Val297Leu]; His289Arg).

(E) The PIF-binding pocket of PDK1 showing that mutants that mimic PKCζ PIF-pocket (PDK1-[PIF-pocket-PKCζ], in the figure named as PDK1[P-P-ζ]).

(F) PDK1[P-P-ζ] can no longer be activated by PS168 and PS171 (100 μM). PS168 activates PDK1 wt but inhibits PDK1[P-P-ζ], indicating that the replacement of the PIF-binding pocket amino acids with those of PKCζ changes the conformational transition from activation to inhibition by compounds targeting the same site. Table S2 shows the data collection and refinement statistics for the structures shown in (B) and (C).

support the notion that the PSR interacts with the catalytic domain as a pseudosubstrate and that this interaction is lost in the presence of lipid activators. Our data further indicate that in the absence of the PSR, the C1 domain allosterically inhibits PKCζ. Because both the PSR and the C1 domain are contiguous in the sequence, our results suggest that the PSR and the C1 domain acted in concert at two neighboring sites, blocking the active site and affecting the helix α-C at the PIF-pocket, to accomplish the inhibition of PKCζ (Figure S4). This result was surprising because the current accepted models for the regulation of PKCs do not describe a major role for either the C1A domain or the PIF-pocket in the autoinhibition. However, there is one report that shows only a small increase in specific activity in a PKCζ protein where the PSR is deleted (Le Good and Brindley, 2004) and there is at least one report showing that the C1A domain of PKCα allosterically inhibits its activity (Kirwan et al., 2003). Therefore, it is likely that the mechanism that we have described here is not only valid for atypical PKCs, but also for classical PKCs and possibly for novel PKC isoforms. In protein kinases, the protein substrate binding site faces the ATP-binding site between the small and the large lobe (Knighton et al., 1991a; Yang et al., 2002a). The binding of the PSR at the peptide binding site as a pseudosubstrate determines the location of the adjacent C1 domain (C1A domain in classical PKCs) directly in the proximity of the helix α-C and the activation loop. This assumption is confirmed by the SAXS structure of PKCβII (Leonard et al., 2011). The allosteric inhibition by the C1 domain is therefore consistent with the idea that the C1 domain could directly interact with residues from the helix α-C, mediating the effects on the PIF-pocket of PKCζ. The PIF-pocket can potentially mediate its effect on the activity by altering the helix α-B and indirectly affecting the Gly-rich loop, or by altering the helix α-C and affecting the Glu-Lys pair interaction that helps position the phosphates from ATP for catalysis. Alternatively, it is possible that the C1 domain of PKCζ and the C1A domain of classical PKCs may directly disturb the formation of the helix α-C in a manner that resembles the interaction between the PH domain of PKB/Akt and its catalytic core (Wu et al., 2010).

Our data also provide information about the molecular mechanism of activation of PKCζ. We found that PKCζ [Δ129], which lacks the pseudosubstrate region but still possesses the C1 domain, could not be activated by lipids, indicating that the

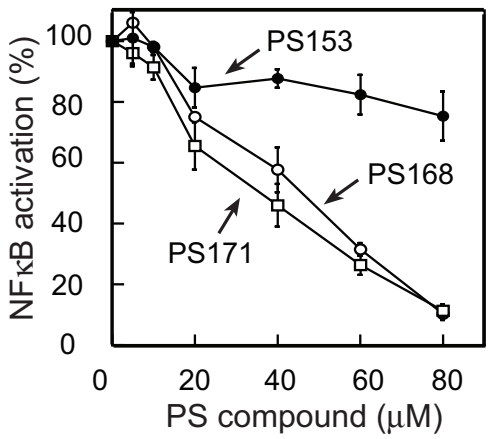

Figure 7. The Effect of Compounds on PKCζ-Dependent NFκB Activation

Preincubation with PS168 and PS171 inhibits PKCζ-dependent NFκB activation in U937 cells (IC_{50} < 50 μM). In contrast, PS153, an analog compound that is inactive in vitro, had no effect on NFκB activation by TNFα. Incubation of the cells at each concentration of the compounds tested was performed in triplicate. A representative experiment out of three is shown. The error bars represent the standard deviation of the triplicates.

binding of LA to the C1 domain was not sufficient to release the allosteric inhibition of PKCζ. This result further indicated that the PSR was required for the activation of PKCζ by LA. These data are compatible with a model where the PSR and the C1 domain are both required and act in concert to bind LA and to release the interactions with the catalytic core that inhibit PKCζ activity. Because polypeptides derived from the PSR of PKC isoforms can also bind to lipids (Mosior and McLaughlin, 1991), it is possible that the concerted displacement of the interaction may be due to the synergistic binding of phosphatidylserine to the PSR and the C1 domain.

It was previously suggested that the helix α-C may not play an important role in the regulation of PKC isoforms because it was always observed as a stable helix in all PKC crystal structures solved to date. However, we have previously also observed the "stable" helix α-C in several crystal packings of PDK1, even when hydrogen/deuterium exchange experiments revealed that the helix α-C of PDK1 was very flexible in solution (Hindie et al., 2009). In agreement with the hypothesis that the PIF-pocket plays a major role in the regulation by the N-terminal domains, hydrogen/deuterium exchange experiments on the catalytic domain construct of atypical PKCι isoform revealed that a polypeptide comprising the helix α-C had very high hydrogen/deuterium exchange rates, whereas the equivalent polypeptide of the full-length construct was highly protected (not shown). Therefore, all our data suggest that the α-C helix of PKCs may not be as rigid in solution as had been expected from crystallography data alone, and that it indeed undergoes conformational changes upon regulation by the N-terminal domains or small compounds.

In the process of activation of AGC kinases by HM phosphorylation, the HM binds to the hydrophobic PIF-pocket whereas the phosphate binds to a neighboring phosphate-binding site (Biondi et al., 2002). In this manner, residues of the helix α-C and the phosphate binding site are stabilized in an active conformation, supporting increased specific activity (Hindie et al., 2009). Phosphorylation at the Z/turn-motif site, which is also within the C-terminal extension, supports the activity of AGC kinases acting as a zipper, facilitating the binding of the HM to the PIF-pocket (Hauge et al., 2007). The NFD motif that interacts with the C1B domain of PKCβII (Leonard et al., 2011) is also part of the C-terminal extension to the catalytic core that holds the Z/turn-motif and HM phosphorylation sites. It is tempting to speculate that the interaction of the C1B domain with the NFD motif may affect the whole C-terminal region and have allosteric effects on the PIF-pocket. However, it is not clear if such a mechanism of regulation happens in atypical PKCs because they lack the second C1 domain.

In the present work we defined the role of the C1 domain and the PIF-pocket on the regulation of atypical PKCs using isolated components. For in vitro activation of the kinase we employed phosphatidylserine. However, this procedure did not lead to complete activation of the full-length kinase. This could be explained by the finding that the conditions that activated atypical PKCs in vitro (e.g., phosphatidylserine) also destabilized the kinase. Alternatively, it is possible that in a cellular environment other cellular factors may participate in the activation of atypical PKCs and stabilize the active forms of the kinases. In previous work aimed to understand the mechanism of regulation of PKCζ in cells, overexpression of different PKCζ mutants led to a complicated pattern of results where degradation of the overexpressed proteins prompted the appearance of PKCζ forms lacking all N-terminal domains (presumably constitutively active and lacking proper localization) whereas the specific activity of the constructs did not always correlate with their ability to promote cellular transformation (Le Good and Brindley, 2004). Together, we believe that the molecular events that regulate atypical PKCs in vivo remain to be fully established and may be best addressed with knock-in technologies.

We previously confirmed the importance of the PIF-binding pocket for the activation of PDK1 by developing low molecular weight compounds that activated the kinase by binding specifically to the PIF-binding pocket (Engel et al., 2006; Hindie et al., 2009). The results of these experiments provided evidence that the PIF-pocket on its own could mediate the conformational changes that activate an AGC kinase by phosphorylation. In the present work, we provide the first evidence, to the best of our knowledge, that the PIF-pocket can also participate in the molecular mechanism of inhibition of an AGC kinase. Notably, we confirm that the PIF-pocket can transduce the inhibition of PKCζ through the use of low molecular weight compounds that, binding to the PIF-pocket of PKCζ, allosterically inhibit the kinase in vitro and inhibit a PKCζ-dependent pathway in cells in culture.

Herein, we have thoroughly evaluated the molecular mechanism of inhibition of PKCζ and shown that the C1 domain allosterically inhibits its activity and that the PIF-pocket mediates the allosteric inhibition of PKCζ. These data complement the understanding of the molecular mechanism of regulation of the PKC protein kinase family as a whole. Finally, as a means to confirm the central role for the PIF-pocket in vitro and in cells, we describe the first low molecular weight compounds that target the PIF-pocket and inhibit the activity of PKCζ. The exact molecular events that occur locally at the PIF-pocket and allosterically at the ATP-binding site upon the interaction with the C1

domain or the allosteric inhibitory compounds remain unknown. However, our current work allows us to envisage the future development of drugs that can either activate or inhibit AGC kinases by pharmacological intervention hijacking the mechanisms that nature designed as natural "ON-OFF" switches located at the PIF-pocket.

SIGNIFICANCE

It was broadly accepted that PKC isoforms, like PKA, would not be regulated by the PIF-pocket. Our work establishes that PKCζ is allosterically inhibited by the C1 domain and that it can be allosterically inhibited by compounds that bind to the PIF-pocket. The identification of allosteric inhibition in a PKC isoform is not a minor change in the model of regulation of the kinase because it has very profound effects on the potential for drug discovery to the whole family of PKC isoforms. The regulation of the activity of protein kinases is one example of the mechanisms used by nature to modulate the conformation of proteins. In AGC kinases, a widespread regulatory step is given by the folding of the C-terminal phosphorylated HM onto the PIF-pocket, whereas other AGC kinase-specific mechanisms of regulation involve the folding of other domains (e.g., PH or C1 domains) onto the catalytic core. A key for the development of pharmacological ON-OFF switches for AGC kinases was the identification and characterization of a major regulatory site, the PIF-pocket and the in-depth evaluation of the molecular mechanism of regulation of each protein of the group. It is tempting to speculate that the conformational changes in a large array of proteins may also be regulated by the folding of N- or C-terminal domains onto the regulated domain. Similarly to AGC kinases, the identification of the key pockets and regulatory sites on proteins and protein families may then allow the more extensive development of pharmacological ON-OFF switches. Such approach will open the field of drug discovery to a wide range of proteins, for example, to proteins that undergo conformational changes upon post-translational modifications in regions outside the regulated domain. Of interest, once a regulatory site is identified, our experience here presented shows that focused libraries of compounds to the site may lead to allosteric activators and allosteric inhibitors (agonists and antagonists) of the conformational change. Altogether, the current work completes the first example of pharmacological ON-OFF switch to the family of protein kinases and supports the novel field of drug development targeting key regulatory sites for the development of modulators of conformational changes for research and future innovative therapies for human diseases.

EXPERIMENTAL PROCEDURES

General materials and methods, the expression and purification of protein kinases, and the synthesis and characterization of compounds are described in the Supplemental Information.

Protein Kinase Activity Assay

The protein kinase activity assays were performed essentially as previously described (Engel et al., 2006). The assays were done in a 96-well format and 4 μl aliquots were spotted on P81 phosphocellulose papers (Whatman) using an epMotion 5070 (Eppendorf). The papers were washed in 0.01% phosphoric acid, dried, and then exposed and analyzed using PhosphorImager technology (FLA-9000 Starion, Fujifilm). Atypical PKC activity assays were performed in a total volume of 20 μl containing 50 mM Tris-HCl pH 7.5, 0.05 mg/ml BSA, 0.1% (v/v) 2-mercaptoethanol, 10 mM $MgCl_2$, 100 μM [γ^{32}P]ATP (5–50 cpm/pmol), 0.003% Brij, 30–50 ng PKC, and MBP (10 μM) or PSRtide (biotin-KSIYRRGSRRWRKLYRA-COOH, 100 μM) as the substrate. After 15 min preincubation, the kinase reaction was started by addition of 6 μl of an ATP-Mg mix. When required, lipid activator (LA) phosphatidylserine (100 ng) or PKC lipid activator mix (Millipore, 1×) was included in the preincubation. Low basal activity and consistent activation of 1-592 PKCζ and Δ98 PKCζ by LA were observed when the preincubation time was started by addition of the whole mix on the enzyme.

The activity assays were performed in duplicate or triplicate (in the case of the thermal stability assay) with <10% difference between the duplicate pairs. The activity assays shown were repeated at least twice with similar results. Most of the assays shown, however, were repeated multiple times with enzymes from independent purifications with similar results. Representative experiments are shown.

PKCζ Thermal Stability Assay

To measure the thermal stability of PKCζ, the activity of PKCζ toward MBP in the presence or absence of lipid activator was measured after incubation of the enzyme for 2 min at different temperatures (24°C, 37°C, 42°C, 46°C, and 50°C) prior to the activity assay. The samples were then left on ice for 2 min, and 9 μl aliquots were transferred to different tubes containing 11 μl of a solution giving a final concentration of 50 mM Tris (pH 7.5), 0.2 mg/ml MBP, 0.003% Brij, 10 mM $MgCl_2$, and 100 μM [γ-^{32}P]ATP (5–50 cpm/pmol). The reaction was stopped after 30 min by adding 5 μl of 200 mM phosphoric acid. Aliquots of 4 μl of each sample were spotted on P81 phosphocellulose papers (Whatman). The papers were washed in 0.01% phosphoric acid, dried, exposed, and analyzed using PhosphorImager technology (FLA-9000 Starion, Fujifilm).

PKCζ-PDK1 Interaction Assay

The protein-protein interaction experiments shown in Figure 1 were performed by cotransfection of HEK293 cells in 10 cm Petri dishes as previously described (Dettori et al., 2009) with 5 μg of a pEBG-2T-PKCζ plasmid that encodes GST-PKCζ (wild-type or truncated mutants) together with 5 μg of a pCMV5-PDK1 plasmid that encodes myc-tagged PDK1. Forty-eight hours posttransfection, the cells were lysed in 0.6 ml of lysis buffer. The lysates were cleared by centrifugation at 13,000 × g for 10 min at 4°C, and 0.5 ml of supernatant was incubated for 2 hr at 4°C with 30 μl of glutathione Sepharose. The beads were washed twice with lysis buffer containing 0.5 M NaCl followed by two further washes with a buffer containing 50 mM Tris-HCl, 0.1 mM EGTA and 0.1% β-mercaptoethanol. The beads were resuspended in 30 μl of buffer containing 100 mM Tris/HCl (pH 6.8), 4% (w/v) SDS, 20% (v/v) glycerol, and 200 mM dithiothreitol, and the duplicates for each condition were subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblotting. Analysis and quantification of the interaction were performed with a fluorescence infrared imager system (Fujifilm FLA 9000 Starion). Duplicates of independent transfections and independent pull-down experiments performed in parallel are presented.

Alphascreen Interaction-Displacement Assay

The AlphaScreen assay was performed according to the manufacturer's general protocol (Perkin-Elmer). Reactions were performed in a 25 μl final volume in white 384-well microtiter plates (Greiner). The reaction buffer contained 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM dithiothreitol, 0.01% (v/v) Tween-20, and 0.1% (w/v) BSA. A total of 50 nM of His6-tagged PKCι Δ223 were mixed with 100 nM GST-C1 (PKCι 131-186) or 25 nM GST-PSR-C1 (PKCι 100-185) in the absence or presence of unlabeled PIFtide or peptides derived from the HM of ROCK (ROCK-HM, VGNQLPFIGFTYFRENL or ROCK-pHM, VGNQLPFIGFT(P)YFRENL) or the activation loop of PKA (RTWALCGTPEYLAPEIILKK). Subsequently, 5 μl of beads solution containing nickel chelate-coated acceptor beads and glutathione-coated donor beads was added to the reaction mix in a final concentration of 40 μg/ml for His-PKCι Δ223 and GST-C1 or 20 μg/ml for His-PKCι Δ223 and GST-PSR-C1,

respectively. Proteins and beads were incubated in the dark for 1 hr 30 min at room temperature and the emission of light from the acceptor beads was measured in the EnVision reader (Perkin-Elmer) and analyzed using the EnVision manager software.

PKCζ-Dependent NFκB Signaling in U937 Cells

In U937 lymphoma cells, tumor necrosis factor alpha (TNFα)-dependent activation of NFκB is dependent on PKCζ activity (Folgueira et al., 1996; Müller et al., 1995).

U937cells were transiently transfected with a plasmid encoding luciferase under the control of NFκB-response elements (pGL4.32 [luc2P/NF-κB-RE/Hygro]; Promega). After serum starvation overnight, the cells were incubated in 96-well plates with the compounds or DMSO (0.25%) for 3 hr and stimulated with TNFα (50 ng/ml; PeproTech) for 90 min. Bright-Glo Luciferase Assay reagent (Promega) was added, and the luciferase activity was measured using the multilabel reader station EnVision (Perkin-Elmer).

ACCESSION NUMBERS

Coordinates have been deposited in the Protein Data Bank with accession codes 4a06 and 4a07.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.chembiol.2011.08.010.

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