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Comparison of Chemical Characteristics of the First and the Second Cysteine-Rich Domains of Protein Kinase Cγ

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Abstract—Protein kinase C (PKC) is a key enzyme family involved in cellular signal transduction. The binding of endogenous diacyl glycerol (DAG) to the cysteine-rich domain (CRD) of PKC is associated with normal cell signaling and function. In contrast, the binding of exogenous phorbol esters to the CRD of PKC is considered to be a key initiating event in tumor promotion. Conventional PKC isozymes (PKCα, βI, βII, and γ) contain two CRDs, both of which are candidates for the phorbol ester binding site. In order to elucidate the binding requirements of phorbol esters and to obtain information on the phorbol ester binding site in native PKCγ, several key chemical characteristics of the first and the second CRDs consisting of ca. 50 amino acids of rat PKCγ (γ-CRD1 and γ-CRD2) were examined. In the presence of Zn^{2+} and phosphatidylserine (PS), both CRDs gave similar K_d values (65.3 nM for γ-CRD1, 44.1 nM for γ-CRD2) in phorbol 12,13-dibutyrate (PDBu) binding assays. In comparison, the binding affinity of PDBu for native rat PKCγ was found to be 6.8 nM. Zn^{2+} was shown to play an important role in the folding and PDBu binding of both CRDs. A Zn^{2+}-induced conformational change was observed for the first time by CD spectroscopic analysis of the complexed and uncomplexed CRDs. Relative to the pronounced Zn^{2+} effect, most divalent first row transition metal ions along with Ca^{2+}, Mg^{2+}, and Al^{3+} were ineffective in folding either CRD. Notably, however, Co^{2+} exhibited a γ-CRD1-selective effect, suggesting that metal ions, not unlike extensively used organic probes, might also become effective tools for controlling isozyme selective activation of PKC. Moreover, group Ib (Cu^{2+} and Ag^{+}) and group IIb element ions other than Zn^{2+} (Cd^{2+} and Hg^{2+}) were found to abolish PDBu binding of both CRDs. Importantly, these inhibitory effects of Cu^{2+}, Ag^{+}, Cd^{2+}, and Hg^{2+} were also observed with native PKCγ. These results indicate that recent reports on the modulation of conventional PKC by heavy metal ions could be explained by their coordination to the CRDs. While the similar affinities of γ-CRD1 and γ-CRD2 for PDBu suggest that either site qualifies as the PDBu binding site, new molecular probes of these CRDs have now been identified that provide information on the preferred site. These novel ligands (**5a** and **5b**) were synthesized by aza-Claisen rearrangement of (−)-N^{13}-desmethyl-N^{13}-allylindolactam-G (**4**). These compounds did not significantly affect the specific PDBu binding of γ-CRD1 but did inhibit that of γ-CRD2 with similar potency to (−)-indolactam-V. Moreover, these new probes did not significantly inhibit the PDBu binding of native PKCγ. (−)-Indolactam-V itself bound almost equally to γ-CRD1, γ-CRD2, and native PKCγ. These results suggest that the major PDBu binding site in native PKCγ is the first CRD, not the second CRD, unlike the novel PKCs. © 1997 Elsevier Science Ltd.

Introduction

The protein kinase C (PKC) isozymes play a central role in cellular signal transduction and are receptors of interest in the development of new medicinal leads.[1] While activated normally by the binding of diacyl glycerol, members of this family bind exogenous agents like the tumor promoting phorbol esters even more avidly. The PKC isozymes are subdivided by function into three classes: conventional PKCs (PKCα, βI, βII, and γ) which are calcium dependent, novel PKCs (PKCδ, ε, η/L, and θ) which are calcium independent, and atypical PKCs (PKCζ, λ/ι) which lack the ability to bind phorbol esters.[2] PKC isozymes possess a catalytic domain for protein phosphorylation and a regulatory domain which for conventional and novel PKCs incorporates a phorbol ester binding region (Fig. 1). In an N-terminal regulatory region, there are tandem cysteine-rich domains (CRDs) consisting of ca. 50 amino acid residues. A single CRD of PKCγ has been shown to be sufficient for phorbol ester binding.[3] In addition, truncated mutants containing the first or the second CRD of PKCγ have been found to bind phorbol 12,13-dibutyrate (PDBu) with similar K_d values of 31 and 59 nM, respectively.[4] Quite similar K_d values for PDBu (14.5 and 17.1 nM) have also been reported using carboxyl-terminal fusion protein of glutathione-*S*-transferase (GST) with the first or the second CRD of PKCγ.[5] These results indicate that both CRDs of native PKCγ are candidates for the phorbol ester binding site.

In contrast to the similar binding characteristics of the CRDs of PKCγ, our latest study on the CRDs of novel PKCs clearly showed that only the second CRDs of PKCη and PKCδ bound PDBu with high affinity, comparable to native PKCη and PKCδ.[6] Szallasi et al.[7] have also reported that the first and the second CRDs

Key words: cysteine-rich domain, indolactam, phorbol ester, protein kinase C, zinc finger.

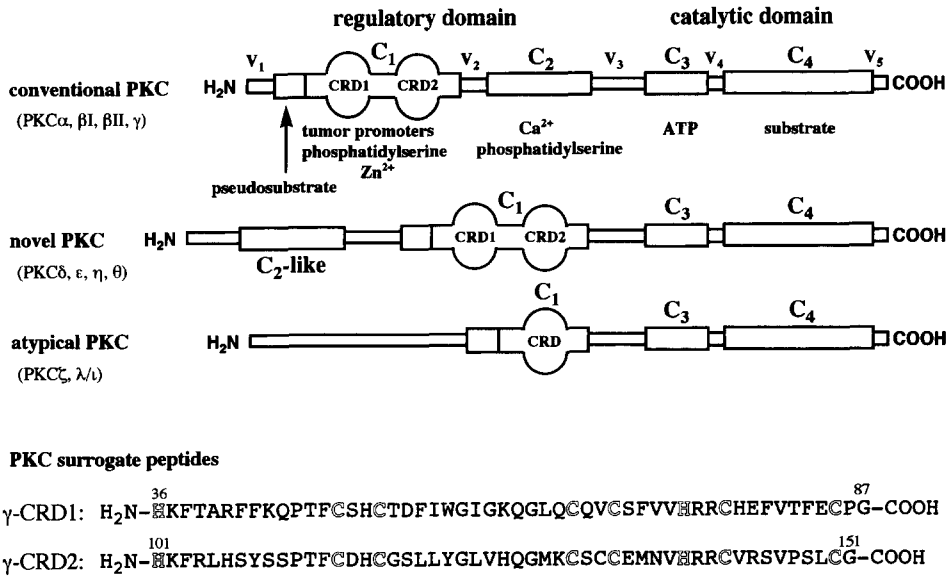

Figure 1. Structures of conventional, novel, and atypical PKC along with the rat PKCγ surrogate peptides, γ-CRD1 and γ-CRD2.

of PKCδ are not equivalent and that the second CRD plays the predominant role in translocation of PKCδ in response to 12-*O*-tetradecanoylphorbol-13-acetate (TPA). The evolutionary dendrogram showing phylogenetic relationships among PKC isotypes indicates that PKCη is closely related to PKCε, and PKCδ to PKCθ.[8] These observations underscore the importance of the second CRD peptide surrogates of novel PKC as useful molecular probes for the elucidation of the structural requirements for the recognition of phorbol esters. In fact, the second CRDs of PKCα, γ, and δ have exclusively been employed for research on the phorbol ester-PKC interaction by NMR, X-ray, and photoaffinity labeling.[9-13]

At present, there is no evidence that the second CRD is the only phorbol ester binding site of conventional PKC since both CRDs of PKCγ bind PDBu with similar affinities. Quest and Bell[5] have recently proposed that the major binding site of PDBu in PKCγ might be the first CRD since the calcium-binding domain (C_2 domain) reduced the PDBu binding ability of the second CRD. More recently, Slater et al.[14] have reported that PKCα contains two activator binding sites that bind phorbol esters and diacylglycerols with different affinities. Since it is known that conventional PKC binds up to only one equivalent of a phorbol ester,[15,16] one of the CRDs must serve as the exclusive or major phorbol ester binding site. In order to obtain information on the phorbol ester binding site in conventional PKCs and to understand the binding requirements of phorbol esters, an investigation of the factors that influence binding of both CRDs and native conventional PKC is essential. Toward this end, we have recently reported the synthesis and some biochemical properties of the first and the second CRD peptide surrogates of rat PKCγ (γ-CRD1 and γ-CRD2).[17] We now describe the initial study on the influence of zinc and other metal ions on the folding and PDBu binding of these CRDs, including the first spectroscopic evidence on their zinc-folding. In addition, new molecular probes of these CRDs have been identified. These novel γ-CRD2-selective ligands (**5a** and **5b**) have been synthesized by aza-Claisen rearrangement of (−)-N^{13}-desmethyl-N^{13}-allylindolactam-G (**4**). These compounds have been found to inhibit the specific PDBu binding of γ-CRD2 with potency similar to (−)-indolactam-V, but they do not significantly inhibit the PDBu binding of native PKCγ. (−)-Indolactam-V itself binds almost equally to γ-CRD1, γ-CRD2, and native PKCγ. These results suggest that the major PDBu binding site in native PKCγ is the first CRD, thereby differing from the novel PKCs in which the second CRD is the proposed PDBu binding site.

Results and Discussion

Effects of zinc on the PDBu binding of the PKCγ surrogate peptides, γ-CRD1 and γ-CRD2

The CRDs of PKC have six conserved cysteines and two histidines in the following pattern $HX_{12}CX_2CX_nCX_2CX_4HX_2CX_7C$ (n = 13 or 14), where X is a variable amino acid residue. Our recent studies indicated that the CRD with n = 14 like the first CRD of PKCη had no PDBu binding ability.[6] Each CRD coordinates two atoms of zinc with each metal bound by three sulfur atoms of cysteines and one nitrogen atom of histidine.[18,19] Differing from the classical zinc finger proteins, the CRD of PKC adopts a globular fold allowing two nonconsecutive sets of zinc-binding residues to form two separate metal-binding sites,[9-11] classified as a variant of the *ring finger* motif.[20] Although this zinc coordination is believed to be essential for proper folding and phorbol ester binding, the effects of

zinc and other metal ions on these processes have not yet been investigated. Such studies are crucial for the proper use of these abundantly available surrogates of the difficult to access and purify natural isozymes. They also bear on the suggestion that PKCs might be implicated in metal poisoning. Since our synthetic γ-CRD1 and γ-CRD2 were shown to contain no zinc by atomic absorption spectroscopy[17] unlike several truncated mutants containing the first or the second CRD of PKC prepared by DNA recombination,[3-5,9-11] zinc coordination was carried out *before* adding these PKCγ surrogate peptides to the assay mixture.

The effects of zinc on the PDBu binding of γ-CRD1 and γ-CRD2 are summarized in Figure 2. A distilled water solution of each CRD was *pretreated* with 5 molar equiv of $ZnCl_2$. After incubation at 4 °C for 10 min, an aliquot of the resultant solution was then added to the assay mixture consisting of Tris–HCl (50 mM, pH 7.4), bovine γ-globulin (3 mg/mL), phosphatidylserine (PS, 50 μg/mL), and [^{3}H]PDBu (40 or 30 nM). The PDBu binding was measured by the procedure of Sharkey and Blumberg.[21] As reference standards, the specific binding of $ZnCl_2$-untreated γ-CRD1 (10 nM) and γ-CRD2 (10 nM) was fixed at 100 (entry 1). The binding increased four- to sixfold when each CRD was pretreated with $ZnCl_2$ in distilled water (entry 2). Folding in distilled water (pH 5.2–5.7) gave an approximately twofold higher specific binding than that in 0.01% trifluoroacetic acid (pH 3.0) followed by neutralization, a general folding condition for the zinc finger peptides.[22,23] Careful examination of the pH dependence of the PDBu binding indicated that the optimal pH for maximum binding is between 5.0 and 6.0 (data not shown). When folding was conducted at pH greater than 8, PDBu binding was not observed. This is consistent with the previous report that addition of $ZnCl_2$ to the assay mixture at pH 7.4 (50 mM Tris–HCl) did not increase binding.[17]

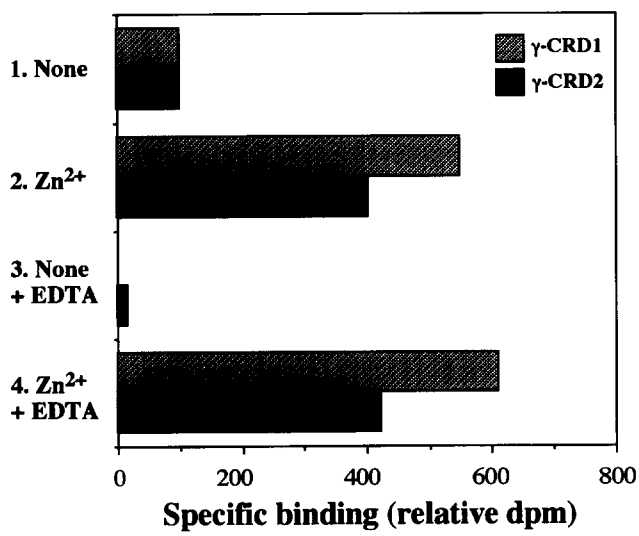

Figure 2. Effects of zinc ion on the PDBu binding of γ-CRD1 and γ-CRD2. The binding was evaluated by the procedure of Sharkey and Blumberg.[21] Metal coordination was carried out in a distilled water solution containing each model peptide (100 μg/100 μL) using 5-molar equiv of $ZnCl_2$ at 4 °C for 10 min. After dilution with the distilled water, an aliquot of the peptide solution (2.9 μL) was added to the reaction mixture (247.1 μL) consisting of 50 mM Tris–HCl (pH 7.4), 3 mg/mL bovine γ-globulin, 50 μg/mL phosphatidylserine, and [^{3}H]PDBu (19.6 Ci/mmol). For the γ-CRD1 binding assay, 40 nM [^{3}H]PDBu and 10 nM γ-CRD1 were used; for γ-CRD2, 30 nM [^{3}H]PDBu and 10 nM γ-CRD2 were employed. The specific binding of the $ZnCl_2$-untreated peptides was each fixed at 100 (entry 1). Entry 2: the $ZnCl_2$-treated peptides; entry 3: the peptides without $ZnCl_2$-treatment in the presence of 2 mM EDTA in the assay mixture; entry 4: the $ZnCl_2$-treated peptides in the presence of 2 mM EDTA in the assay mixture. Each point represents the mean of three experimental values with a standard deviation of less than 5%.

In accord with the importance of zinc in maintaining a proper fold of the peptides, only modest PDBu binding was observed when the peptides were not pretreated with $ZnCl_2$ (entry 1). Moreover, even this binding was abolished (entry 3) when the $ZnCl_2$-untreated CRDs were added to the assay mixture containing 2 mM EDTA, suggesting that chelatable ions in the assay mixture could account for the background folding of the untreated CRDs. It is especially noteworthy that the binding of $ZnCl_2$-treated γ-CRD1 and γ-CRD2 did not change by exposure to 2 mM EDTA (entry 4), even for periods up to 3 h, indicating that the zinc coordination is too strong to be readily reversed by EDTA.

Scatchard analyses of zinc-folded γ-CRD1 and γ-CRD2 gave dissociation constants (K_d) of 65.3 and 44.1 nM, respectively (Figure 3). In comparison, the binding affinity of PDBu for native rat PKCγ was found to be 6.8 nM under our conditions. Various K_d values of PDBu in the presence of calcium and PS have been reported for native PKCγ: K_d = 16,[24] 2.4,[25] and 0.33 nM.[26] The K_d values of γ-CRD1 and γ-CRD2 obtained in this study are comparable to the K_d of native PKCγ reported by Evans et al. (16 nM),[24] to that of the truncated mutants containing the first or the second CRD of PKCγ (31 and 59 nM),[4] and to that of GST-CRD1 and GST-CRD2 (carboxyl-terminal fusion protein of GST with the first or the second PKCγ cysteine-rich domain) reported by Quest and Bell (14.5 and 17.1 nM).[5] Since conventional PKCs such as PKCγ have the calcium- and PS-binding domain (C_2 domain) close to the phorbol ester-binding domain (C_1 domain), the PDBu binding affinity to native PKCγ might be affected by the C_2 domain as well as slight differences in the assay conditions employing calcium and PS. Overall, these results indicate that our PKCγ surrogate peptides are properly folded by addition of $ZnCl_2$, exhibit affinities comparable to the native isozyme, and consequently serve as effective models for native PKCγ.

Effects of metal ions other than zinc on the PDBu binding of γ-CRD1 and γ-CRD2

The effects of metal ions other than zinc on the PDBu binding are shown in Figure 4. Metal coordination was carried out in a distilled water solution of each surrogate peptide using 5 molar equiv of each metal salt as mentioned above. Five molar equiv of $ZnCl_2$ and

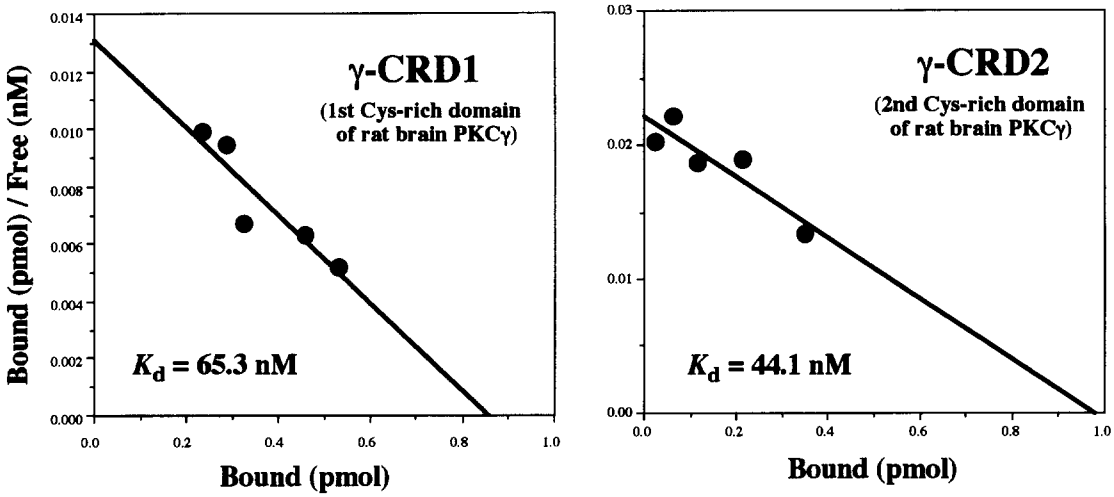

Figure 3. Scatchard analyses of the [^{3}H]PDBu binding to γ-CRD1 and γ-CRD2. Zinc-folded γ-CRD1 or γ-CRD2 was incubated with increasing concentrations of [^{3}H]PDBu in the presence of 50 μg/mL phosphatidylserine as described in Figure 2. Representative experiments for γ-CRD1 and γ-CRD2 are shown. Each point represents the mean of three experimental values with a standard deviation of less than 5%. Similar results were obtained in additional experiments.

other metal salts were used in the folding of these peptides to assure maximum binding. An almost linear concentration dependence on the binding was observed at less than 2 molar equiv of $ZnCl_2$ (data not shown). Metal salts of chloride ions were used due to their availability from commercial sources. It is noteworthy, however, that ZnI_2, $ZnSO_4$, and $Zn(CH_3COO)_2$ as well as $ZnCl_2$ showed quite similar levels of the PDBu binding (data not shown), suggesting that the counter anions do not play a significant role in the folding and binding experiments.

The specific binding of $ZnCl_2$-treated γ-CRD1 and γ-CRD2 was fixed at 100 (entry 1). Pretreatment of the CRDs with divalent first row transition metal ions such as Cr^{2+}, Mn^{2+}, Fe^{2+}, and Ni^{2+} in place of Zn^{2+} showed PDBu binding similar to that of the $ZnCl_2$-untreated peptides (entry 3–5, 7 versus entry 2), indicating that they had no significant effects on the folding. Mg^{2+}, Ca^{2+}, and Al^{3+} were also ineffective (entry 8–10). It is noteworthy however that $CoCl_2$-pretreatment of γ-CRD1 resulted in significant binding, approaching the maximum binding observed for the $ZnCl_2$-pretreated peptide (entry 1 versus 6) while $CoCl_2$-pretreatment of γ-CRD2 had no significant effect relative to the background binding (entry 2 versus 6). Of further importance, group Ib element ions such as Cu^{2+} and Ag^+, and Cd^{2+} and Hg^{2+} which belong to the same group (IIb) as Zn^{2+}, completely abolished binding (entry 11–14). Pb^{2+} slightly inhibited the binding of both CRDs (entry 15). Overall, the effects of metal ions on the PDBu binding of γ-CRD1 and γ-CRD2 were basically similar except for the remarkable effect of Co^{2+}.

The inhibitory effects of Cu^{2+}, Ag^+, Cd^{2+}, and Hg^{2+} were also observed with native rat PKCγ at 100 μM

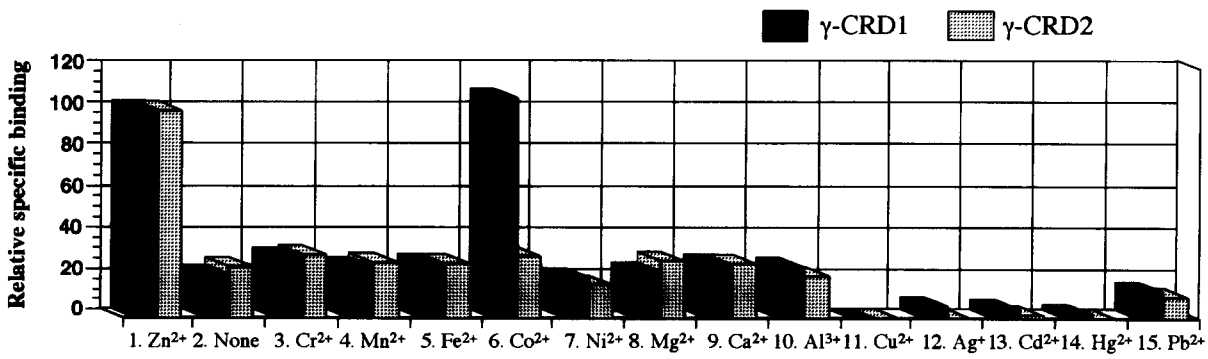

Figure 4. Effects of metal ions on the PDBu binding of γ-CRD1 and γ-CRD2. Metal coordination was carried out in a distilled water solution of each CRD (100 μg/mL) using 5-molar equiv of each metal salt at 4 °C for 10 min. The PDBu binding was evaluated by the same procedure described in Figure 2. The specific binding of the $ZnCl_2$-treated peptides was each fixed at 100 (entry 1). Entry 2: the peptides without $ZnCl_2$-treatment; entry 3: the $CrCl_2$-treated peptides; entry 4: the $MnCl_2$-treated peptides; entry 5: the $FeSO_4(NH_4)_2SO_4$-treated peptides; entry 6: the $CoCl_2$-treated peptides; entry 7: the $NiCl_2$-treated peptides; entry 8: the $MgCl_2$-treated peptides; entry 9: the $CaCl_2$-treated peptides; entry 10: the $AlCl_3$-treated peptides; entry 11: the $CuCl_2$-treated peptides; entry 12: the $AgNO_3$-treated peptides; entry 13: the $CdCl_2$-treated peptides; entry 14: the $HgCl_2$-treated peptides; entry 15: the $PbCl_2$-treated peptides. Each point represents the mean of three experimental values with a standard deviation of less than 5%.

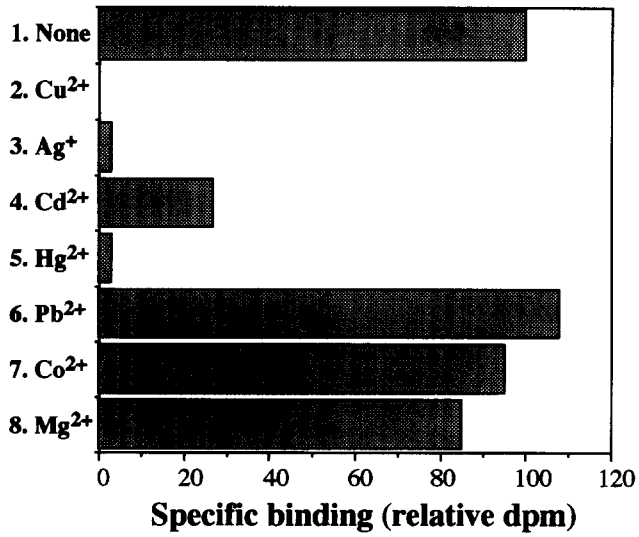

Figure 5. Effects of metal ions on the PDBu binding of native rat PKCγ. The PDBu binding was evaluated by the similar procedure described in Figure 2 except for the concentration of rat PKCγ (1 nM), phosphatidylserine (100 μg/mL), and [^{3}H]PDBu (10 nM). The specific binding without added metal salts was fixed at 100 (entry 1). The concentration of each metal salts in the assay mixture was 100 μM (entry 2–8). Each point represents the mean of three experimental values with a standard deviation of less than 10%.

(Figure 5). As control experiments, Co^{2+} and Mg^{2+} did not decrease the PDBu binding significantly. Fairly high concentrations of these metal ions were employed in order to compensate for the relatively high concentrations of EDTA and EGTA in our PKCγ sample; final chelator concentration of the reaction buffer was 20 μM. To further corroborate this metal ion effect, these inhibition experiments were carried out using commercially available native conventional PKC mixture from rat brain (PKCα, βI, βII, and γ). Final chelator concentrations in this case were 2 μM. The results indicated that 20 μM Cu^{2+}, Ag^{+}, Cd^{2+}, and Hg^{2+} ions inhibited almost completely the PDBu binding (data not shown).

Modulation of PKC by neurotoxic heavy metals as Cd^{2+}, Cu^{2+}, Hg^{2+}, or Pb^{2+} has recently been investigated[27-34] since PKC is involved in neurotransmitter release[35-37] and signal transduction.[38] For example, Rajanna et al.[34] have reported that Hg^{2+} and Pb^{2+} inhibited the enzyme activity of PKC and that Hg^{2+} was a potent inhibitor of the PDBu binding to PKC. Thus far, it has been mainly assumed that Hg^{2+} and Pb^{2+} interact with the catalytic domain of PKC to inhibit the PKC activation.[29,32] However, our present results strongly indicate that another or sole target site for these heavy metal ions could be the CRD of the regulatory region. Our experiments suggest that the effects of Cu^{2+}, Ag^{+}, Cd^{2+}, and Hg^{2+} on native PKC activation in the presence and absence of PDBu merits further examination in order to understand the possible role of heavy metals in the modulation of PKC. Inhibition of the PDBu binding by Ag^{+} is also noteworthy especially in Europe and USA where leaching from silverware could provide a possible silver source.

CD spectra of the complexed and uncomplexed CRDs of rat PKCγ

In order to investigate the metal induced conformational change of γ-CRD1 and γ-CRD2, CD spectra of the complexed and uncomplexed CRDs were measured using conditions employed in the binding assays (Figures 6 and 7). In accord with the binding data in Figure 4 (entry 2 versus entry 3, 4, 8, 9), each CRD treated with $CrCl_2$, $MnCl_2$, $MgCl_2$, or $CaCl_2$, gave a spectrum quite similar to the CRD itself; only spectra for the $MgCl_2$-treated CRDs are shown in the figures. However, a significant spectroscopic change was detected for both CRDs on treatment with $ZnCl_2$. This is the first spectroscopic evidence bearing on the zinc-folding of CRDs of PKC. The two CRDs exhibited opposite changes; $ZnCl_2$-treatment of γ-CRD1 *increased* negative rotation at 205 nm while similar treatment of γ-CRD2 *decreased* negative rotation. A quite similar spectroscopic change (to the $ZnCl_2$-treated peptides) was observed for each CRD treated with $CdCl_2$ (data not shown). Treatment with $CoCl_2$ or $CuCl_2$ also resulted in similar changes, suggesting that these metal ions coordinate each CRD in a similar fashion to Zn^{2+} and Cd^{2+} to produce closely related peptide conformers. The lack of PDBu binding of Cd^{2+} or Cu^{2+} treated peptides would reflect the finely different conformation from that of the Zn^{2+} treated peptides though it is unclear at present why such selectivity arises. In the classical zinc finger peptides, Cu^{2+} is sometimes found to oxidize the peptides, producing disulfide-linked species.[39] However, no significant difference in the PDBu binding and CD spectra of the $CuCl_2$-treated peptides was detected between aerobic and anaerobic conditions, suggesting that such oxidation did not occur to a significant extent. Furthermore, Fe^{2+} which is reported to coordinate zinc fingers[39] was tested. A quite similar CD change to that observed with Cu^{2+} and Co^{2+} treatment was found for each CRD (data not shown).

Chelation of metal ions to the CRDs is classified into four categories. Metal ions which coordinate the CRD and enhance the PDBu binding (Zn^{2+} and Co^{2+} for γ-CRD1), those which coordinate but do not change the binding (Fe^{2+} and Co^{2+} for γ-CRD2), those which coordinate and decrease the binding (Cu^{2+}, Ag^{+}, Cd^{2+}, and Hg^{2+}), and those which do not coordinate (Cr^{2+}, Mn^{2+}, Mg^{2+}, and Ca^{2+}). The coordination by Fe^{2+} and Co^{2+} is not as strong as Zn^{2+} since addition of 2 mM EDTA to the reaction buffer completely abolished the PDBu binding of the CRDs pretreated with $FeCl_2$ or $CoCl_2$ (data not shown) in contrast to the zinc-folded CRDs (Figure 2, entry 4). Addition of 2.5 molar equiv of $CuCl_2$, AgCl, or $CdCl_2$ to the distilled water solution of the zinc-folded peptides caused complete disappearance of binding (data not shown). On the other hand, addition of a 100-fold excess of $ZnCl_2$ to $CuCl_2$, AgCl,

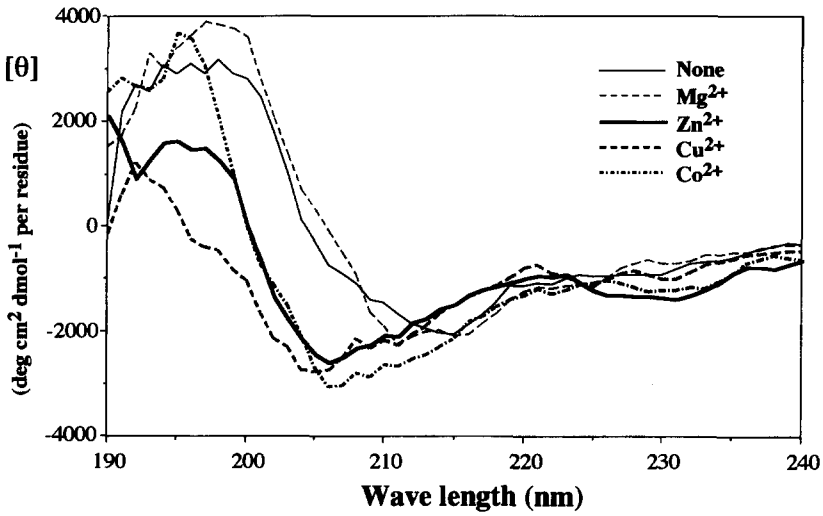

Figure 6. CD spectra of γ-CRD1 itself and γ-CRD1 treated separately with 2.5-molar equiv of $ZnCl_2$, $MgCl_2$, $CuCl_2$, or $CoCl_2$ in helium-purged distilled water. The spectra were obtained on a JASCO 700 CD spectrophotometer in a 0.5 mm cell using 275 μg/mL solutions in 10 mM Tris–HCl buffer (pH 7.4) at 24 °C.

or $CdCl_2$-pretreated peptides did not restore binding, suggesting that coordination of Cu^{2+}, Ag^+, or Cd^{2+} to the CRDs is stronger than that of Zn^{2+}. The strength of coordination to γ-CRD1 and γ-CRD2 is thus roughly estimated as follows: Cu^{2+}, Ag^+, Cd^{2+} > Zn^{2+} > Co^{2+}, Fe^{2+} > Cr^{2+}, Mn^{2+}, Mg^{2+}, Ca^{2+}. CD spectra of the peptides treated with other ions (Al^{3+}, Hg^{2+}, and Pb^{2+}) have not yet been measured.

It is noteworthy that γ-CRD1 treated with $CoCl_2$ showed the maximum level of the PDBu binding. Since each CRD of PKCγ treated with $ZnCl_2$, $CuCl_2$, $CdCl_2$, or $CoCl_2$ showed a very similar CD spectrum, $CuCl_2$, $CdCl_2$, or $CoCl_2$-treated CRDs of PKC isozymes other than PKCγ might also be expected to show modified PDBu binding. In agreement with this analysis, $CdCl_2$-treatment of η-CRD2[40] (the second CRD of mouse PKCη amino acids 246–296) resulted in significant binding, approaching the maximum level observed for $ZnCl_2$-treated η-CRD2,[41] while $CdCl_2$-treatment of γ-CRD2 completely abolished binding (Figure 4, entry 13). With the discovery of at least eleven PKC isozymes,[2] increasing importance is placed on isozyme specific analysis of function in order to elucidate the role of PKC in cellular signal transduction and tumor promotion. Due to the limited information on the solution structure of the phorbol ester–PKC–phosphatidylserine aggregate,[9–12] most efforts to generate isozyme selective agonists have focused thus far on variations in organic ligands. The above results indicate that metal ions, not unlike the more extensively explored organic ligands for PKC, might also become effective tools for controlling isozyme selective activation and inhibition of PKC.

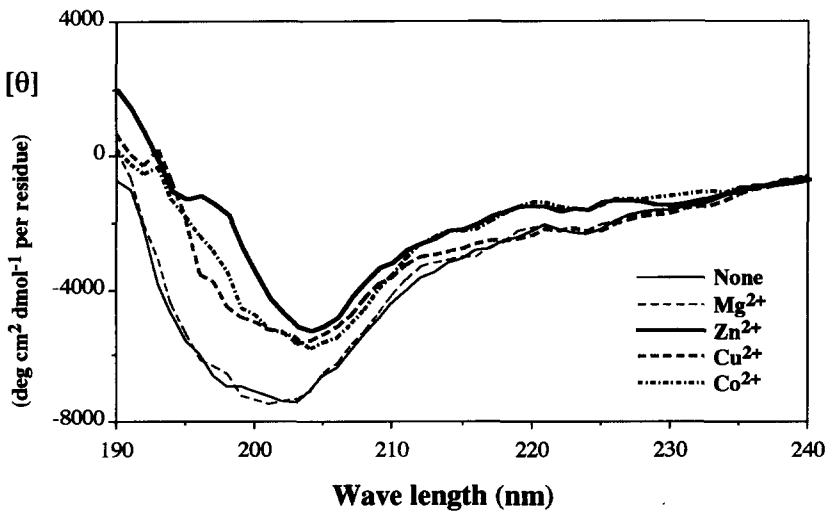

Figure 7. CD spectra of γ-CRD2 itself and γ-CRD2 treated separately with 2.5-molar equiv of $ZnCl_2$, $MgCl_2$, $CuCl_2$, or $CoCl_2$ in helium purged distilled water. The spectra were obtained on a JASCO 700 CD spectrophotometer in a 0.5 mm cell using 275 μg/mL solutions in 10 mM Tris–HCl buffer (pH 7.4) at 24 °C.

Table 1. Calculation of the CD spectra of complexed and uncomplexed CRDs of PKCγ[a]

	γ-CRD1		γ-CRD2	
Metal ion	**α-helix (%)**	**β-sheet (%)**	**α-helix (%)**	**β-sheet (%)**
None/Mg^{2+}	20.2	32.5	0.5	58.3
Zn^{2+}	15.5	45.8	9.2	56.3
Cu^{2+}	11.3	54.0	5.2	58.5
Co^{2+}	16.0	49.5	5.2	60.5

[a]This calculation was done by the method of Yang et al.[42]

The CD spectra of γ-CRD1 and γ-CRD2 were analyzed by the method of Yang et al.[42] (Table 1). This calculation indicates that 20.2% and 32.5% of the residues of untreated γ-CRD1 are in α-helix and β-sheet, respectively. In $ZnCl_2$-treated γ-CRD1, these estimates are 15.5% α-helix and 45.8% β-sheet while in $CuCl_2$-treated and $CoCl_2$-treated γ-CRD1, the values are 11.3% and 16.0% α-helix, and 54.0% and 49.5% β-sheet, respectively. These results suggest that coordination of Zn^{2+}, Cu^{2+}, or Co^{2+} to γ-CRD1 increases significantly the β-sheet content. In contrast, coordination of these metal ions to γ-CRD2 increases drastically the α-helix content while the β-sheet content does not change significantly as shown in Table 1. These results suggest that the solution structure of each CRD without zinc-treatment differ significantly from each other. However, regardless of this large difference in the structures of the zinc-untreated CRDs, treatment of these peptides with $ZnCl_2$ resulted in a similar conformation: 15.5% α-helix and 45.8% β-sheet for γ-CRD1; 9.2% α-helix and 56.3% β-sheet for γ-CRD2.

Inhibition of the specific [^{3}H]PDBu binding to γ-CRD1 and γ-CRD2

Since there are few differences between γ-CRD1 and γ-CRD2 in the binding constants for PDBu and the effects of metal ions on the folding and PDBu binding, the availability of molecular probes which could distinguish between these two CRDs is important for determining the PDBu binding site of native PKCγ. The binding abilities of other compounds known to interact with the phorbol ester binding site on PKC and consequently to competitively inhibit the binding of [^{3}H]PDBu to these CRDs were, therefore, determined by varying the concentration of the compound in question. Dose–response curves were plotted for each compound, and the concentration at which 50% of the specific [^{3}H]PDBu binding was inhibited (IC_{50}) was determined. The binding constant, K_i, was calculated by the method of Sharkey and Blumberg.[21] Table 2 gives the results of this analysis for PDBu, 1,2-dioctanoyl-*sn*-glycerol, (−)-indolactam-V, teleocidin B-4, mezerein, 4α-phorbol 12,13-didecanoate, and resiniferatoxin. These structure–binding relationships are similar to those previously observed for PKC itself.[43] Unfortunately, not unlike the similar effects observed for metal ions, none of these compounds exhibited differential CRD binding. Recently, mezerein regulation of bovine PKCα was reported to occur predominantly *via* the first CRD.[44] However, mezerein showed no significant selectivity in the PDBu binding to each CRD of rat PKCγ.

Synthesis of new conformationally restricted analogues of (−)-indolactam-G with high CRD selectivity

Due to the absence of CRD-selective compounds among known PKC activators, efforts have been directed at the identification of new ligands with high CRD selectivity. Conformationally restricted analogues of indole alkaloid-type tumor promoters were selected as potential candidates due to their tunability. We have recently reported that bridge formation between positions 5 and 13 of such indolactam derivatives serves as a particularly effective analogue design strategy, allowing

Table 2. Inhibition of the specific [^{3}H]PDBu binding to γ-CRD1 and γ-CRD2[a]

Compound	**K_i (nM) for γ-CRD1**	**K_i (nM) for γ-CRD2**
Phorbol 12,13-dibutyrate (PDBu)	111	54
1,2-Dioctanoyl-*sn*-glycerol	2919	2930
(−)-Indolactam-V	1082	1030
Teleocidin B-4	3.8	2.0
Mezerein	2.4	8.4
4α-Phorbol 12,13-didecanoate	Inactive	Inactive
Resiniferatoxin	Inactive	Inactive

[a]This assay was carried out by the procedure of Blumberg.[21] The assay solution (250 μL) consisted of 50 mM Tris–HCl (pH 7.4), 3 mg/mL bovine γ-globulin, 40 nM [^{3}H]PDBu, 50 μg/mL PS, 10 nM γ-CRD1, and various concentrations of an inhibitor. For γ-CRD2 binding assay, 30 nM [^{3}H]PDBu and 10 nM γ-CRD2 were used. Both CRDs were folded by 5-molar equivalents of $ZnCl_2$ in helium-purged distilled water.

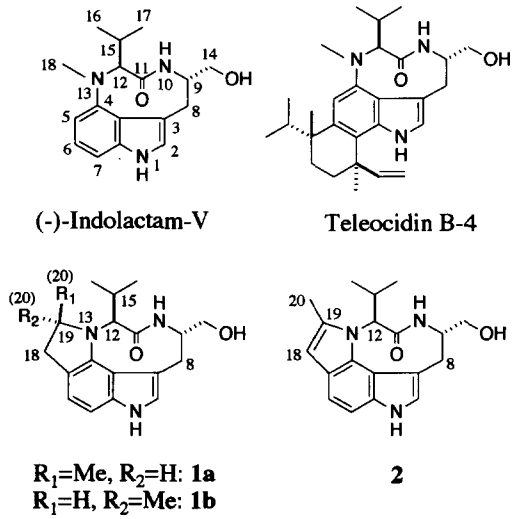

Figure 8. Structures of teleocidin B-4, (−)-indolactam-V and its conformationally restricted analogues (**1a**, **1b**, and **2**).

for the ***remote control*** of the conformation of the recognition units and for the introduction of a wide range of structural variations, as required for the development of new PKC activators with high isozyme selectivity.[13] Since CRD-selective compounds could not been found among conformationally restricted analogues of (−)-indolactam-V (**1a**, **1b**, and **2**; Figure 8),[13] we have synthesized bridged analogues of (−)-indolactam-G for this study. (−)-Indolactam-G was selected because it exists mainly in a fold conformer in solution at room temperature[45] which is different from the two conformers of (−)-indolactam-V, with the *cis* amide in a twist conformation and the *trans* amide in a sofa form (Figure 9).[46] This conformational difference is ascribable to the lack of the isopropyl group at position 12 and provides the basis for fine-tuning twist restricted analogues with high CRD selectivity.

(−)-N^{13}-Desmethyl-N^{13}-allylindolactam-G (**4**)[47] was derived from allylation of (−)-N^{13}-desmethylindolactam-G (**3**), which in turn was synthesized from L-tryptophan methyl ester by the method of Nakatsuka et al.[48] and Endo et al.[49] Allylation was achieved by treatment of **3** with allyl bromide in methanol containing $NaHCO_3$, which gave the *N*-allyl product (**4**) in 52% yield.[50] Compound **4** was treated with 0.7 equiv of $AlCl_3$ under reaction conditions similar to those employed in the reaction of (−)-N^{13}-desmethyl-N^{13}-allylindolactam-V.[13] This reaction gave three products (**5a**, **5b**, and **6**, Figure 10) in 1.9, 6.6, and 1.1% yields, respectively. Unlike the aza-Claisen rearrangement of (−)-N^{13}-desmethyl-N^{13}-allylindolactam-V, the yield of the [3,3]-rearrangement product (**6**) was especially low (36% versus 1.1%) while that of the cyclization products was similar.

The ^{1}H NMR spectrum of **5a** in deuterochloroform indicated that **5a** existed only as a single conformer in solution at room temperature. This conformer was assigned the twist structure by comparison with several characteristic signals and coupling constants exhibited by the two conformers of (−)-indolactam-V (Table 3). Significant NOEs between H-8α (δ 3.00) and H-12α (δ 4.43) of **5a** supported this conformation. The absolute configuration at position 16 was deduced to be *R* by the NOESY spectra; no NOE was observed between H-12β (δ 3.86) and H_3-17 (δ 1.44). Quite similar ^{1}H NMR signals and coupling constants for **1a** and **5a** (Table 3) also support this assignment.

Compound **5b** exists as two conformers (10:1) in deuteromethanol at room temperature. The major conformer was assigned the twist structure on the basis of the low-field shift of H-12α (δ 4.68) and H_2-14 (δ 3.75), and the NOESY spectra; a significant NOE between H-12α (δ 4.68) and H-8α (δ 3.36) was observed. The absolute configuration was established as *S* by the NOESY spectra; a significant NOE was observed between H-12β (δ 3.64) and H_3–17 (δ 1.41). The above results strongly suggest that **5a** and **5b** exist mainly in the twist conformation which is the active conformation of the teleocidin-related compounds.[13,51,52] Bridge formation between position 5 and 13 of indolactam skeleton is an effective analogue design strategy as exemplified in the conversion of inactive folded conformation of (−)-indolactam-G into the biologically active twist conformation.

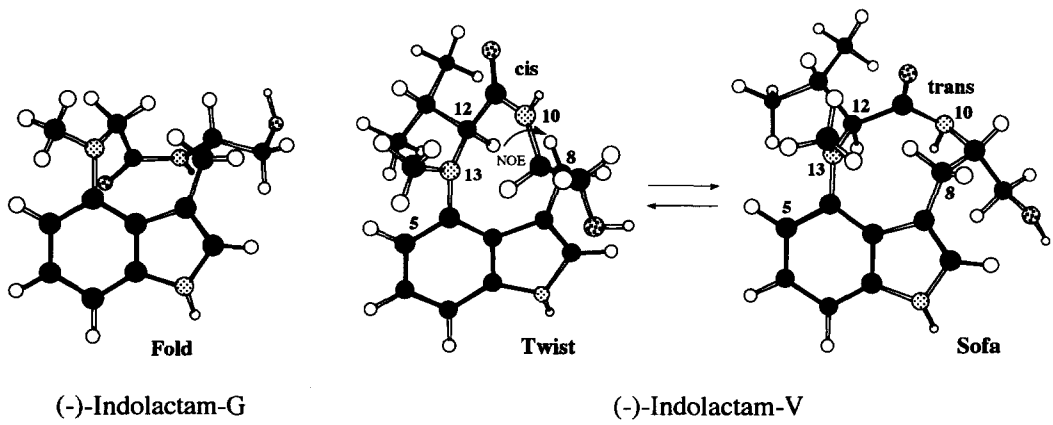

Figure 9. Conformation of (−)-indolactam-G and (−)-indolactam-V.[45,46]

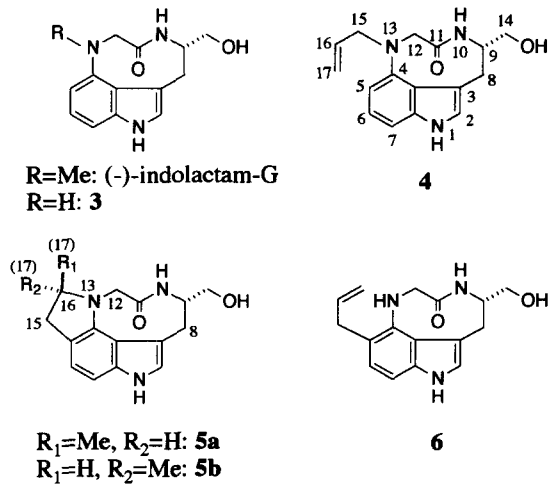

Figure 10. Structure of (−)-indolactam-G and its *ortho* rearrangement products (**5a**, **5b**, and **6**).

The binding affinity of these conformationally restricted analogues (**5a** and **5b**) to γ-CRD1 and γ-CRD2 was evaluated by inhibition of the specific binding of [^{3}H]PDBu. The dose–response curves are shown in Figure 11a, b. Both analogues (**5a** and **5b**) inhibited specific PDBu binding to γ-CRD2 with potencies similar to (−)-indolactam-V. Importantly, these analogues did not inhibit the binding of γ-CRD1 which is inhibited significantly by (−)-indolactam-V. The K_i values to γ-CRD2 of **5a**, **5b**, and (−)-indolactam-V were calculated to be 1407, 925, and 1030 nM, respectively, while those to γ-CRD1 were >50,000, >50,000, and 1082 nM, respectively. These results indicate that **5a** and **5b** are γ-CRD2-selective ligands.

The binding ability of these new ligands to native rat PKCγ was also determined. Figure 11c clearly shows that γ-CRD2-selective **5a** and **5b** did not bind to PKCγ although (−)-indolactam-V showed significant binding (K_i = 88.1 nM). These results indicate that the major binding site of PDBu in rat PKCγ is the first CRD, unlike novel PKC for which the second CRD is the dominant binding domain. This conclusion is in agreement with the results of the Bell group obtained by using a CRD2 and C_2 domain fusion protein.[5] Hitherto, the second CRDs of PKCα, γ, and δ have been exclusively employed for NMR, X-ray, and photoaffinity labeling studies.[9–13] However, the present results indicate that the first CRD is the more important phorbol ester-binding site in PKCγ and that the tertiary structure of the first CRD of conventional PKC could be of special relevance to studies on the molecular mechanism of tumor promotion. Further studies on the CRDs of other conventional PKC isozymes (PKCα, βI, and βII) are also of interest in view of these findings. In addition, direct identification of the phorbol ester-binding site of native PKCγ by photoaffinity labeling is indispensable to confirm this conclusion since the calcium binding domain (C_2 domain) is thought to affect the PDBu binding ability of the second CRD in native PKCγ as Bell et al. proposed.[5]

The binding affinities of **5a**, **5b**, and (−)-indolactam-V to η-CRD2 were also measured (Figure 11d). Our recent study has revealed that η-CRD2 is the major phorbol ester binding site of PKCη.[6] Although **5b** did not bind to η-CRD2, **5a** showed significant binding (K_i = 130 nM). Since **5a** did not bind to native PKCγ significantly, it is concluded that **5a** can also discriminate PKCη and PKCγ significantly. This indicates that **5a** might be the first example of an isozyme selective ligand of PKC which exhibits high selectivity. Our results also strongly suggest that PKC isozyme-selective compounds can be prepared by fine-tuning the active conformation of such tumor promoters.

Conclusion

In summary, we have examined a series of chemical characteristics of the first and the second CRDs of rat PKCγ (γ-CRD1 and γ-CRD2 consisting of ca. 50 amino acids) in order to investigate the binding requirements of phorbol esters and to obtain the information on the phorbol ester binding site in native PKCγ. Of significance, this study shows that the synthetic PKC surrogate peptides can be effectively folded by $ZnCl_2$-treatment in neutral pH, and it provides the first demonstration that this zinc-induced conformational change can be clearly detected by CD spectroscopy. In addition, this study shows that metal ions other than Zn^{2+} can play an

Table 3. Several characteristic signals of the ^{1}H NMR spectra of (−)-indolactam-V, the *ortho* cyclization products (**1a** and **5a**) in deuterochloroform (500 MHz)

	δ (multiplicity, *J* in Hz)			
	(−)-Indolactam-V			
Position	Twist conformer[a]	Sofa conformer[a]	Compound 1a Twist conformer[b]	Compound 5a Twist conformer[c]
2	6.89 (s)	7.05 (d, *J* = 1.7)	6.85 (s)	6.83 (s)
10	6.59 (br s)	4.72 (d, *J* = 10.8)	6.48 (br s)	6.41 (br s)
12α	4.39 (d, *J* = 10.2)	2.99 (d, *J* = 10.8)	4.33 (d, *J* = 10.1)	4.43 (d, *J* = 15.8)
14	3.54, 3.74 (m)	3.44 (m)	3.55, 3.71 (m)	3.56, 3.74 (m)
14-OH	2.01 (br s)	1.32 (br s)	2.05 (br s)	1.99 (br s)

[a]sofa:twist = 1.0:2.6 (0.004 M, 27 °C). [b]Twist only (0.01 M, 27 °C). [c]Twist only (0.004 M, 27 °C).

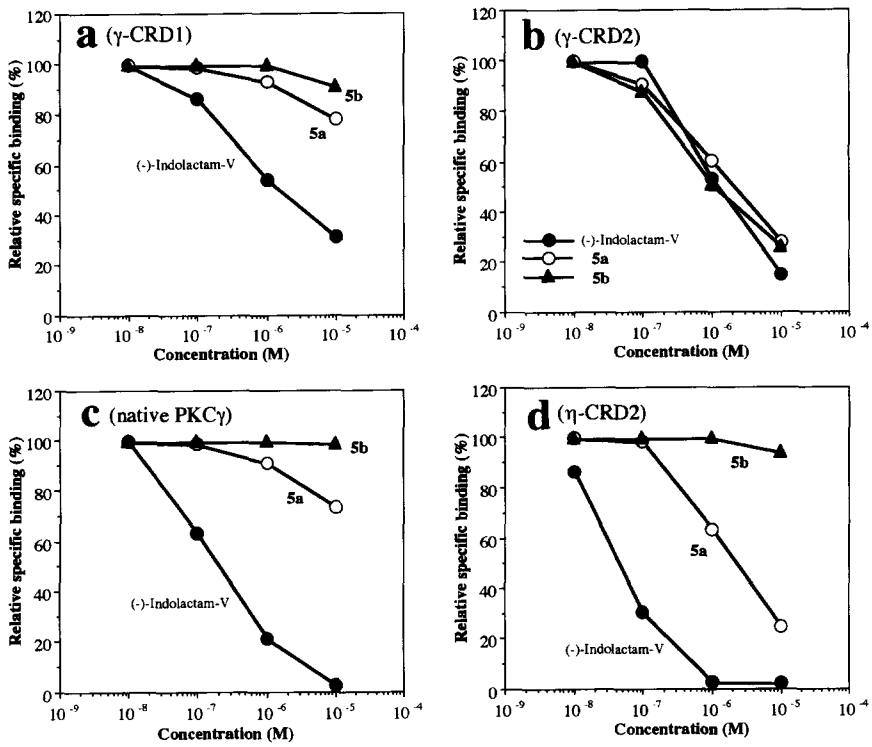

Figure 11. γ-CRD1, γ-CRD2, η-CRD2, and native PKCγ binding of the conformationally restricted analogues of (−)-indolactam-G (**5a** and **5b**) along with (−)-indolactam-V. The PDBu binding was evaluated by the same procedure described in Table 2 for γ-CRD1 and γ-CRD2. In the native rat PKCγ binding assay, 100 μg/mL PS, 10 nM [^{3}H]PDBu, and 1 nM PKCγ were used; in the η-CRD2 binding assay, 5 nM η-CRD2 and 20 nM [^{3}H]PDBu were employed. Each point represents the mean of three experimental values with a standard deviation of <5%.

important role in isozyme folding and PDBu binding. It is noteworthy that Cu^{2+}, Ag^{+}, Cd^{2+}, and Hg^{2+} showed strong inhibition of the binding of both PKCγ surrogate peptides as well as native rat PKCγ. While the significance of this novel inhibition in signal transduction and neurotransmitter release is under investigation, it is certain that trace metal ions can dramatically affect binding and functional assays, and as such could more generally serve as modulators of PKC mediated signaling, providing a molecular basis for the toxic effect of heavy metal ions like Hg^{2+} and Pb^{2+}. The high selectivity of Co^{2+} on the PDBu binding of γ-CRD1 suggests that metal ions could also serve as effective tools for controlling isozyme selective activation and inhibition of PKC as shown in the case of $CdCl_2$-treated η-CRD2.[41] Further studies on this novel approach to isozyme control are focused on the elucidation of the NMR solution structure of metal coordinated γ-CRD1 and γ-CRD2 and on the role of these ions in PKC function.

In view of the finding that γ-CRD1 and γ-CRD2 exhibit little difference in affinity for PDBu and are not differentially affected by most metal ions, the identification of new ligands (**5a** and **5b**) with high CRD selectivity is especially noteworthy. These new ligands (**5a** and **5b**), based on our recently reported strategy for the design of conformationally restricted indolactam derivatives, inhibited the specific PDBu binding of γ-CRD2 with similar potency to (−)-indolactam-V, but did not significantly inhibit the specific PDBu binding of native PKCγ. Taken together, these results indicate that the major PDBu binding site in native PKCγ is the first CRD, not the second CRD as is proposed for novel PKC. Since there are no reports on the structure of the first CRD of any PKC isozymes, the assignment of the tertiary structure of the first CRD of conventional PKC is expected to be of importance in efforts to understand the molecular basis for activation of conventional PKCs by tumor promoters and in the role of conventional PKCs in signal transduction. Furthermore, direct identification of the phorbol ester-binding site of native PKCγ by photoaffinity labeling is also indispensable to confirm this conclusion.

Experimental

General

The following spectroscopic and analytical instruments were used: UV, Shimadzu UV-200; $[\alpha]_D$, Jasco DIP-1000; ^{1}H NMR, Bruker ARX500 (ref. TMS); HPLC, Waters Model 600E with Model 484 UV detector; (HR) EI-MS, JEOL JMS-DX300. HPLC was carried out on a YMC packed AQ-323 (ODS, 10 mm ID × 250 mm) and SH-342-5 (ODS, 20 mm ID × 150 mm) (Yamamura Chemical Laboratory). Wakogel C-100 and C-200 (silica gel, Wako Pure Chemical Industries) were used for column chromatography.

Unless otherwise noted, reagents were obtained from Sigma and used without further purification. [^{3}H]PDBu

(19.6 Ci/mmol) was from NEN Research Products; 4α-phorbol 12,13-didecanoate, resiniferatoxin, and mezerein were obtained from LC Laboratories. Teleocidin B-4 and (−)-indolactam-V were isolated from *Streptoverticillium blastmyceticum* NA34-17.[53] Native rat PKCγ was prepared by the method reported previously.[54] The native conventional PKC mixture from rat brain was purchased from Boehringer Mannheim.

γ-CRD1 (rat PKCγ amino acids 36–87) and γ-CRD2 (rat PKCγ amino acids 101–151) were synthesized by the previous method[17] with a PerSeptive Biosystems 9050 plus automated peptide synthesizer using Fmoc amino acids. To prevent racemization and oxidation during synthesis, the carboxyl-terminus was extended in each case from the final cysteine to a glycine. The crude peptides, which were obtained by cleavage and deprotection by trifluoroacetic acid (TFA) containing *m*-cresol, ethanedithiol, and thioanisole, were purified by gel filtration, followed by HPLC using SH-342-5 (ODS, 20 mm ID × 150 mm, Yamamura Chemical Laboratory) with elution at 8 mL/min by a 160-min linear gradient of 10–50% acetonitrile in 0.1% TFA. The peaks of γ-CRD1 (t_R = 107 min) and γ-CRD2 (t_R = 110 min) were collected, and each fraction was concentrated *in vacuo* below 30 °C to remove acetonitrile. Lyophilization of each residue gave pure γ-CRD1 and γ-CRD2 in 1.5% and 4.2% yield, respectively, which exhibited satisfactory mass spectral data [matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS)]: for γ-CRD1 (MH^+, average molecular mass: obsd 6097.07, calcd for $C_{276}H_{404}N_{77}O_{69}S_6$, 6097.13), and for γ-CRD2 (MH^+, average molecular mass: obsd 5772.76, calcd for $C_{244}H_{384}N_{77}O_{68}S_9$, 5772.81).

Metal coordination

Metal coordination was carried out in a helium-purged distilled water solution (pH 5.5) of each CRD (10 μg/98 μL). Five molar equivalents (2 μL) of each metal salt in helium-purged distilled water (5 mM) were added to the CRD solution, and the solution was allowed to stand at 4 °C for 10 min. After adding 900 μL helium-purged distilled water, an aliquot of the peptide solution (2.9 μL) was used in the [^{3}H]PDBu binding assay described below.

For CD measurements, the initial concentration of each CRD was 55 μg/154 μL. A quantity of 2.5 molar equivalents (6 μL) of each metal salt in helium-purged distilled water (5 mM) were added to the CRD solution, and the solution was allowed to stand at 4 °C for 10 min. After adding 40 μL of 50 mM Tris–HCl (pH 7.4), the solution was subjected to the CD measurements.

[^{3}H]PDBu binding to γ-CRD1 and γ-CRD2

The PDBu binding to γ-CRD1 and γ-CRD2 was evaluated using the procedure of Sharkey and Blumberg.[21] The standard assay mixture (250 μL) in a 1.5 mL of Eppendorf tube contained 50 mM Tris–HCl (pH 7.4 at 25 °C), 50 μg/mL phosphatidylserine (PS), 3 mg/mL bovine γ-globulin, [^{3}H]PDBu (19.6 Ci/mmol), and γ-CRD1 or γ-CRD2. For determination of PDBu saturation curves, concentrations of free [^{3}H]PDBu between 5 and 80 nM were used. In the standard γ-CRD1 binding assay, 10 nM γ-CRD1 and 40 nM [^{3}H]PDBu were used; in the standard γ-CRD2 assay, 10 nM γ-CRD2 and 30 nM [^{3}H]PDBu were employed. PS was suspended in 50 mM Tris–HCl (pH 7.4) by sonication (1 min) and added to the above reaction mixture.

The above $ZnCl_2$ (or other metal salt) pretreated CRD solution (2.9 μL) was added to the standard assay mixture (247.1 μL), and the solutions were incubated at 30 °C for 20 min. After the mixtures were cooled at 0 °C for 5 min, 187 μL of 35% (w/w) poly(ethyleneglycol) (average molecular weight, 8000) was added to the tubes and the mixtures were vigorously stirred. The tubes were incubated at 0 °C for 15 min and centrifuged for 20 min at 12,000 rpm in an Eppendorf microcentrifuge at 4 °C. A 50 μL aliquot of the supernatant of each tube was removed, and its radioactivity was measured to determine the free [^{3}H]PDBu concentration. The remainder of the supernatant was removed by aspiration, and the tubes were blotted with a Kimwipe. The tips of the tubes were cut off, and the radioactivity in the pellets was measured to determine the bound [^{3}H]PDBu. Nonspecific binding was measured in the presence of 500-fold nonradioactive PDBu, and the partition coefficient for [^{3}H]PDBu between supernatant and pellet was determined. Specific binding represents the difference between the total and nonspecific binding, where the nonspecific binding for each tube was calculated from its measured free [^{3}H]PDBu concentration and the partition coefficient of [^{3}H]PDBu. In each experiment, each point represents the average of at least triplicate determinations. In competition experiments, various concentrations of an inhibitor in DMSO solution were added to the reaction mixture. The final DMSO concentration of the mixture was less than 2%. Binding affinity was evaluated by the concentration required to cause 50% inhibition of the specific [^{3}H]PDBu binding, IC_{50}, which was calculated by a computer program (Statistical Analysis System) with a probit (probability unit) procedure.[55] The binding constant, K_i, was calculated from IC_{50} and the dissociation constants (K_d) of PDBu to each PKCγ surrogate peptide by the method of Sharkey and Blumberg.[21]

Synthesis of the conformationally restricted analogues of (−)-indolactam-G (5a, 5b, and 6)

(−)-N^{13}-Desmethylindolactam-G (**3**, 65 mg, 0.265 mmol) was treated with allyl bromide (127 μL, 1.44 mmol) and $NaHCO_3$ (186 mg, 2.21 mmol) in MeOH (1.3 mL) at room temperature for 19 h. After the reaction mixture was partitioned between EtOAc and water, the EtOAc layer was separated, dried over Na_2SO_4, and concentrated *in vacuo* to dryness. The

residue was purified by column chromatography on Wakogel C-100 using toluene and increasing amounts of acetone, followed by HPLC on YMC SH-342-5 using 55% MeOH to give **4** (39.1 mg, 0.137 mmole) in 52% yield: $[\alpha]_D$ −232.4° (*c* = 0.85, MeOH, 24 °C); UV λ_{max} (MeOH) nm (ε) 293 (9500), 286 (10,700), 226 (42,600); ^{1}H NMR (300 MHz, CD_3OD, 0.018 M, 52 °C, fold conformer only): δ 2.94 (dd, *J* = 14.8, 7.1 Hz, 1H, H-8a), 3.23 (dd, *J* = 14.8, 7.2 Hz, 1H, H-8b), 3.36 (d, *J* = 13.2 Hz, 1H, H-12a), 3.62 (dd, *J* = 11.2, 7.0 Hz, 1H, H-14a), 3.70 (dd, *J* = 11.2, 4.5 Hz, 1H, H-14b), 3.74 (dd, *J* = 13.8, 6.9 Hz, 1H, H-15a), 3.88 (ddt, *J* = 13.8, 5.7, 1.4 Hz, 1H, H-15b), 4.11 (d, *J* = 13.2 Hz, 1H, H-12b), 4.83 (br s, 1H, H-9), 5.08 (dd, *J* = 10.2, 1.6 Hz, 1H, H-17a), 5.23 (dd, *J* = 17.2, 1.6 Hz, 1H, H-17b), 5.93 (m, 1H, H-16), 6.91 (dd, *J* = 7.5, 1.0 Hz, 1H, H-5), 6.92 (s, 1H, H-2), 7.04 (t, *J* = 7.7 Hz, 1H, H-6), 7.13 (dd, *J* = 8.0, 1.0 Hz, 1H, H-7); HR-EI-MS *m/z* 285.1473 (M^+, calcd for $C_{16}H_{19}N_3O_2$, 285.1477).

Compound **4** (36.2 mg, 127 μmol) was distributed in two vials. After the sample was dried over P_2O_5, $AlCl_3$ (5.9 mg, 44 μmol) and xylene (127 μl) was added to each vial containing **4** (18.1 mg, 63.5 μmol). Each vial was sealed tightly and heated at 140 °C for 20 min. The reaction was quenched by addition of 2 N NaOH methanol–water (1:1) solution, and the mixture was extracted with EtOAc. After the EtOAc layer was dried over Na_2SO_4, the EtOAc extracts were purified by column chromatography on Wakogel C-100 using toluene and increasing amounts of acetone, followed by HPLC on YMC SH-342-5 using 50% MeOH and on YMC AQ-323 using 45% MeOH, to give **5a** (0.7 mg, 2.5 μmol, 1.9%), **5b** (2.4 mg, 8.4 μmol, 6.6%), and **6** (0.4 mg, 1.4 μmol, 1.1%).

Compound **5a**: UV λ_{max} (MeOH) nm (ε) 313 (6400), 284 (5500), 232 (24,400); ^{1}H NMR (500 MHz, $CDCl_3$, 0.004 M, 27 °C, twist conformer only): δ 1.44 (d, *J* = 6.2 Hz, 3H, H_3-17), 1.99 (br s, 1H, OH-14), 2.64 (dd, *J* = 14.6, 10.2 Hz, 1H, H-15a), 3.00 (br d, *J* = 16.6 Hz, 1H, H-8b), 3.18 (dd, *J* = 16.6, 5.9 Hz, 1H, H-8b), 3.38 (dd, *J* = 14.6, 9.0 Hz, 1H, H-15b), 3.56 (m, 1H, H-14a), 3.74 (m, 1H, H-14b), 3.84 (m, 1H, H-16), 3.86 (d, *J* = 15.8Hz, 1H, H-12a), 4.31 (br s, 1H, H-9), 4.43 (d, *J* = 15.8Hz, 1H, H-12b), 6.41 (br s, 1H, NH-10), 6.79 (d, *J* = 8.0Hz, 1H, H-7), 6.83 (s, 1H, H-2), 6.94 (d, *J* = 8.0Hz, 1H, H-6), 7.86 (br s, 1H, NH-1); HR-EI-MS *m/z* 285.1476 (M^+, calcd for $C_{16}H_{19}N_3O_2$, 285.1477).

Compound **5b**: UV λ_{max} (MeOH) nm (ε) 313 (6300), 284 (5900), 232 (24,700); ^{1}H NMR (500 MHz, CD_3OD, 0.017 M, 27 °C, major:minor = 10:1): major conformer, δ 1.41 (d, *J* = 6.1 Hz, 3H, H_3-17), 2.62 (dd, *J* = 13.9, 11.8 Hz, 1H, H-15a), 3.20 (m, 2H, H-8a, H-15b), 3.36 (m, 2H, H-8b, H-9), 3.64 (d, *J* = 15.6 Hz, 1H, H-12a), 3.75 (m, 2H, H_2-14), 4.36 (m, 1H, H-16), 4.68 (d, *J* = 15.6Hz, 1H, H-12b), 6.75 (d, *J* = 8.0 Hz, 1H, H-7), 6.81 (d, *J* = 8.0 Hz, 1H, H-6), 6.98 (s, 1H, H-2); minor conformer, δ 1.20 (d, *J* = 6.6 Hz, 3H, H_3-17), 7.03 (d, *J* = 8.1 Hz, 1H, H-7), 7.04 (s, 1H, H-2), 7.13 (d, *J* = 8.1 Hz, 1H, H-6); other peaks of the minor conformer had weak intensities and/or overlapped the peaks of the major conformer; HR-EI-MS *m/z* 285.1480 (M^+, calcd for $C_{16}H_{19}N_3O_2$, 285.1477).

Compound **6**: $[\alpha]_D$ −176.7° (*c* = 0.11, MeOH, 24 °C); UV λ_{max} (MeOH) nm (ε) 298 (5000), 282 (6000), 229 (30 100); ^{1}H NMR (300 MHz, CD_3OD, 0.014 M, 52 °C, fold conformer only): δ 3.07 (dd, *J* = 15.3, 5.8 Hz, 1H, H-8a), 3.15 (dd, *J* = 15.3, 6.3 Hz, 1H, H-8b), 3.51 (m, 2H, H_2-14), 3.63 (dd, *J* = 11.1, 7.0 Hz, 1H, H-15a), 3.72 (dd, *J* = 11.1, 4.6 Hz, 1H, H-15b), 3.79 (d, *J* = 14.0 Hz, 1H, H-12a), 4.05 (d, *J* = 14.0 Hz, 1H, H-12b), 4.60 (br s, 1H, H-9), 5.03 (m, 2H, H_2-17), 6.05 (m, 1H, H-16), 6.89 (d, *J* = 8.2 Hz, 1H, H-7), 6.91 (s, 1H, H-2), 7.03 (d, *J* = 8.2 Hz, 1H, H-6); HR-EI-MS *m/z* 285.1489 (M^+, calcd for $C_{16}H_{19}N_3O_2$, 285.1477).

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