Molecular Basis for Protein Kinase C Isozyme-Selective Binding: The Synthesis, Folding, and Phorbol Ester Binding of the Cysteine-Rich Domains of All Protein Kinase C Isozymes

Kazuhiro Irie,*,† Kentaro Oie,† Akifumi Nakahara,† Yoshiaki Yanai,† Hajime Ohigashi,† Paul A. Wender,*,‡ Hiroyuki Fukuda,§ Hiroaki Konishi,⊥ and Ushio Kikkawa⊥

Contribution from Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan, Department of Chemistry, Stanford University, Stanford, California 94305, Nihon PerSeptive Ltd., Roppongi, Minato-ku, Tokyo 106-0032, Japan, and Biosignal Research Center, Kobe University, Kobe 657-8501, Japan

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Abstract: The protein kinase C (PKC) family of enzymes plays a crucial role in cellular signal transduction and tumor promotion. Conventional and novel PKC isozymes consist of a catalytic domain for protein phosphorylation and a regulatory domain which binds the endogenous messenger diacyl glycerol or exogenous agents such as phorbol esters. The N-terminal regulatory region of these isozymes contains two cysteine-rich domains (C1A and C1B, also known as CRD1 and CRD2), both of which are candidates for the phorbol ester-binding site. To determine the phorbol ester-binding sites of these isozymes and to elucidate the structural requirements for isozyme selective PKC modulation, *the C1 peptides, consisting of ca. 50 amino acids of all conventional and novel PKCs, along with those of atypical PKCs have been synthesized by a solid-phase Fmoc strategy*. Exceptionally high overall yields (10−20%) were achieved in the syntheses of most of the C1 peptides on a Pioneer Peptide Synthesizer (PerSeptive Biosystems) through the use of HATU as a coupling reagent. These peptides were successfully folded by zinc treatment, as monitored by CD spectroscopy. *Importantly, only the C1Bs of all conventional and novel PKCs, except for PKCγ, bound [^{3}H]phorbol-12,-13-dibutyrate (PDBu) with high affinities, comparable to those of the native isozymes*. Of special significance, *both* C1 peptides of PKCγ (*i.e.*, γ-C1A and γ-C1B) exhibited high-affinity binding, providing the structural basis for a novel approach to PKCγ-selective modulators, compounds of potential significance for the treatment of neuropathic pain. The effects of metal cations other than zinc on the binding of these isozymes were also investigated. Only the PKCγ surrogates (γ-C1A and γ-C1B), when treated with cadmium, exhibited no binding, while other similarly treated conventional and novel PKC surrogates strongly bound PDBu, as did the zinc-folded peptides. These results suggest that cadmium ion could serve as a new and effective tool for controlling the activation of PKCγ.

Introduction

Protein kinase C (PKC) isozymes are receptors of great current interest in the development of new medicinal leads and cancer prevention strategies because of their crucial role in cellular signal transduction and tumor promotion.[1] PKC isozymes are subdivided into three classes: conventional PKCs (PKCα, -βI/βII, -γ), which are calcium dependent, novel PKCs (PKCδ, -ϵ, -η, -θ), which are calcium independent, and atypical PKCs (PKCζ, -λ/ι), which lack the ability to bind phorbol ester-type tumor promoters (Figure 1).[2] Conventional and novel PKC isozymes consist of a catalytic domain for protein phosphorylation and a regulatory domain which binds the endogenous messenger diacyl glycerol or exogenous agents such as phorbol esters. The N-terminal regulatory region of conventional and novel PKCs contains tandem cysteine-rich domains (C1A and C1B, also known as CRD1 and CRD2),[3] consisting of *ca*. 50 amino acid residues. Initial studies[4] showed that both C1 domains of PKCγ bound phorbol-12,13-dibutyrate (PDBu) with high affinity, suggesting that C1A and C1B of PKCγ are functionally equivalent. In contrast to PKCγ, our latest study showed that only C1Bs of PKCδ and -η bound PDBu with high affinity, comparable to native PKCδ and -η, respectively.[5] Szallasi *et al*.[6] and Hunn and Quest[7] have also reported that

† Kyoto University. Tel.: +81-75-753-6282. Fax: +81-75-753-6284. E-mail: irie@kais.kyoto-u.ac.jp.
‡ Stanford University. Tel.: 650-723-0208. Fax: 650-725-0259. E-mail: Wenderp@Leland.Stanford.edu.
§ Nihon PerSeptive Ltd. Tel.: +81-3-5570-9050. Fax: +81-3-5570-9070.
⊥ Kobe University. Tel.: +81-78-803-1254. Fax: +81-78-803-0994.

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(3) “C1A and C1B” is a modern nomenclature advocated by Hurley *et al.*[1] in 1997. The conserved region of typical PKCs formerly known as “C1” becomes “C1A−C1B” in the new parlance. C1A and C1B are what we previously called CRD1 and CRD2, respectively.[5,16,28,41]

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Figure 1. Structure of conventional, novel, and atypical PKC along with PKCγ C1B and the classical zinc finger (Tramtrack).

C1A and C1B of PKCδ are not equivalent and that C1B plays the predominant role in translocation of PKCδ in response to phorbol esters. Collectively, these studies indicate that the PDBu-binding sites of the PKC isozymes studied to date do not follow a single pattern. Given the therapeutic significance of PKC signal modulation and the recent findings implicating PKCγ as a mediator of neuropathic pain,[8] PKCα as a target of antisense cancer chemotherapeutics,[9] and PKCβ as a key enzyme in vascular complications of diabetes,[10] the identification and analysis of the activator domains of all PKC isozymes are necessary for the development of rational approaches to novel PKC-targeted therapeutic agents.

The limited availability and cost of pure PKC isozymes have hampered both fundamental and applied research in this area. Thus far, PKC isozymes have been prepared almost exclusively by DNA recombination techniques.[11−14] However, these procedures are time-consuming and often produce isozymes of variable purity, a factor which undoubtedly contributes to some of the variability in the reported results. We have recently reported that the C1 peptides of PKCγ and -η can be synthesized in quantity and in high purity by automated solid-phase synthesis, and we have shown that these C1 peptides are efficiently folded upon zinc treatment, to produce PKC regulatory domain surrogates that bind PDBu with high affinities, *comparable to the native PKC isozymes themselves*.[15,16] This approach is especially useful for the preparation of large quantities of highly pure PKC subunits.

The purpose of this study is to determine the C1-binding site preferences of PDBu and to establish the utility of these C1 peptides as surrogates of native PKC isozymes. *As reported herein, we have synthesized and characterized the C1 peptides of all mouse PKC isozymes (Figure 2)*[17−25] *for the first time. Of special significance, all C1Bs bound PDBu with high affinity, comparable to native PKC isozymes, while all C1As except for that of PKCγ did not strongly bind PDBu.* The influence of metal ions on PKC binding was also examined. Only the C1 peptides of PKCγ, when treated with cadmium, lost binding, while other similarly treated C1Bs strongly bound PDBu. Of special importance, the amino acid residues which play a critical role in the PDBu binding of the C1 peptides were identified on the basis of sequence analysis and synthesis.

Results and Discussion

Synthesis of the C1 Peptides of All PKC Isozymes. In our preliminary publications,[5,15] we have reported the solid-phase synthesis of the C1 peptides of mouse PKCη (η-C1A and η-C1B, Figure 2) by an Fmoc strategy.[26] Our initial studies were performed with a PerSeptive Biosystems model 9050 plus

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Figure 2. Sequence alignment of all C1 peptides synthesized in this study. All C1 sequences derive from mouse.[17−25] To prevent racemization and oxidation during synthesis, the carboxyl terminus was extended in each case from the final cysteine to a glycine. The consensus represents all of the conserved amino acids which are deduced to be necessary for the phorbol ester binding. The peptides are divided into three classes: those with potent PDBu-binding affinity (all C1Bs of conventional and novel PKCs, and C1A of PKCγ), those with very weak PDBu-binding affinity (δ-C1A and θ-C1A), and those without PDBu-binding affinity (α-C1A, β-C1A, ϵ-C1A, η-C1A, ζ-C1, and λ-C1).

automated peptide synthesizer, using HATU[27] as an activator for Fmoc chemistry. Fmoc amino acids (0.8 mmol), activated by HATU (0.8 mmol) in the presence of *N*,*N*-diisopropylethylamine (DIPEA, 0.95 mmol) in DMF, were coupled in a stepwise fashion on 0.2 mmol of preloaded Fmoc-Gly-PEG-PS resin. Piperidine (20%) in DMF was used for removal of Fmoc groups, and DMF was employed for flow washes throughout the entire synthesis. During the deprotection of each Fmoc group, the amount of Fmoc released was monitored by UV spectroscopy to confirm that the condensation occurred successfully. After completion of the chain assembly, each peptide resin was treated with trifluoroacetic acid (TFA) containing *m*-cresol, thioanisole, and ethanedithiol for final deprotection and cleavage from the resin. The resultant crude peptides were purified by gel filtration, followed by HPLC on a preparative C18 reversed-phase column. The main compound which eluted from the C18 column was collected and lyophilized. Molecular weights were determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Satisfactory data for both PKCη surrogate peptides were obtained (see the Experimental Section). However, the overall yields were low: 0.5% and 0.9% for η-C1A and η-C1B, respectively.[28]

Subsequent studies were performed with a new peptide synthesizer from PerSeptive Biosystems (Pioneer Peptide Synthesizer). This machine allows for an increased flow rate of each Fmoc amino acid and HATU solution in the coupling reaction. The flow rate of the reagent mixture (30 mL/min) was 5-fold higher than that in the previous method. The addition procedure for HATU in the coupling reaction was also modified. Since the coupling efficiency of dissolved HATU decreases with time, addition of the reagent mixture is set to begin within 2 min after addition of the base solution to the test tube containing each Fmoc amino acid and HATU. In the previous procedure, reagent addition began 10 min later. The overall yield of η-C1B using this new machine and procedure was a remarkably efficient 4.7%. Other C1 peptides in Figure 2 were similarly synthesized in excellent yields and characterized by MALDI-TOF-MS (see the Experimental Section and the Supporting Information). The yields of α-C1B, β-C1B, ϵ-C1B, θ-C1B, and ζ-C1 were especially high at 17.1%, 13.9%, 14.6%, 11.7%, and 11.3%, respectively, requiring average coupling yields of >97%. This indicates that *ca.* 100−200 mg of pure peptide can be obtained in a 0.2-mmol scale synthesis. Hitherto, several PKC fragments containing C1 domains have been synthesized by DNA recombination techniques.[4,7,29] The approach described herein is the first practical synthesis of PKC C1 domains by solid-phase synthesis.

Zinc Folding and PDBu Binding of the C1 Peptides of All Conventional and Novel PKCs. The C1 domain of PKC has six conserved cysteines and two histidines in the pattern $\mathbf{H}X_{12}\mathbf{C}X_2\mathbf{C}X_n\mathbf{C}X_2\mathbf{C}X_4\mathbf{H}X_2\mathbf{C}X_7\mathbf{C}$ ($n = 13$ or 14), where X is a variable amino acid residue. Each C1 domain coordinates two atoms of zinc with each metal bound by three sulfur atoms of cysteines and one nitrogen atom of histidine.[30,31] Differing from the classical zinc finger proteins, the C1 domain of PKC adopts a globular fold, allowing two nonconsecutive sets of zinc-

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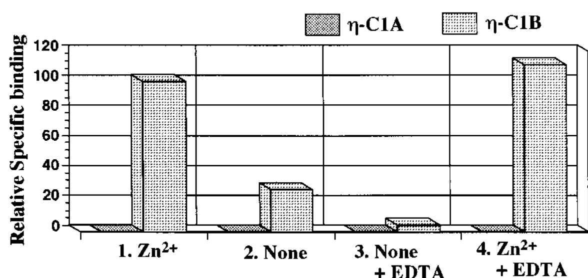

Figure 3. Effects of zinc ion on the PDBu binding of η-C1A and η-C1B. The binding was evaluated by the procedure of Sharkey and Blumberg.[38] Metal coordination was carried out in distilled water solution containing each model peptide (100 μg/mL) using 5 molar equiv of $ZnCl_2$ at 4 °C for 10 min. After 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 20 nM [^{3}H]PDBu (19.6 Ci/mmol). Final peptide concentration was 2 μM for η-C1A and 5 nM for η-C1B, respectively. The specific binding of zinc-treated η-C1B (14 300 dpm) was fixed at 100 (entry 1). Entry 2, the zinc-untreated η-C1s; entry 3, the zinc-untreated η-C1s were added to the reaction mixture containing 2 mM EDTA; entry 4, the zinc-treated η-C1s were added to the reaction mixture containing 2 mM EDTA. Each point represents the mean of three experimental values, with a standard deviation of less than 5%.

binding residues to form two separate metal-binding sites,[32-35] classified as a variant of a ring finger motif (Figure 1).[36] Since our synthetic PKC surrogates are produced in the absence of zinc[37] (unlike native PKC and several truncated mutants containing the C1 domain of PKC prepared by DNA recombination),[4,30-35] zinc coordination was carried out *before* the PKC surrogate peptides were added to the assay mixture.

The effects of zinc on the PDBu binding of η-C1A and η-C1B are summarized in Figure 3 as typical examples. A distilled water solution of each C1 peptide was *pretreated* with 5 molar equiv of $ZnCl_2$ to establish maximum binding. Maximum binding was also observed for the C1 peptides pretreated with 2.5 molar equiv of $ZnCl_2$, whereas an almost linear concentration dependence of the binding was observed with less than 2 molar equiv of $ZnCl_2$ (data not shown). 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 (20 nM). The PDBu binding was measured by the procedure of Sharkey and Blumberg.[38] η-C1A did not show any PDBu binding, even at 2 μM, while η-C1B bound PDBu with high affinity. The specific binding of $ZnCl_2$-treated η-C1B at 5 nM was, therefore, fixed at 100 as a reference (entry 1). Folding in distilled water (pH 5.2−5.7) gave *ca.* 2-fold higher specific binding than that in 0.01% TFA (pH 3.0) followed by neutralization, the standard folding condition used for the zinc finger peptides.[29,39] Careful examination of the pH dependence of PDBu binding indicated that the optimal pH for maximum binding is between 5.0 and 6.5 (data not shown). When folding was conducted at pH greater than 8, PDBu binding was not observed.

In accord with the importance of zinc in maintaining a proper fold of η-C1B, only modest PDBu binding was observed when η-C1B was not pretreated with $ZnCl_2$ (entry 2). Moreover, even this binding was abolished (entry 3) when $ZnCl_2$-untreated η-C1B was 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 η-C1B. It is especially noteworthy that the binding of $ZnCl_2$-treated η-C1B did not change after exposure to 2 mM EDTA (entry 4), even for periods up to 3 h, indicating that the zinc coordination is not readily reversed by EDTA. These results strongly suggest that zinc plays an important role in the folding and PDBu binding of η-C1B.

To investigate whether both η-C1A and η-C1B undergo metal-induced conformational changes, the CD spectra of the complexed and uncomplexed η-C1s were measured (Figure 4). A significant spectroscopic change was detected for both η-C1s when they were treated with $ZnCl_2$. In a control experiment, η-C1s treated with $MgCl_2$, which had no significant effect on the PDBu binding, gave a spectrum quite similar to that of untreated η-C1s. These results indicate that Zn^{2+} coordinates both η-C1A and η-C1B in a similar fashion, producing closely related peptide conformers, but only one binds PDBu with high affinity.

The zinc folding and PDBu binding of the C1 peptides of other PKC isozymes were examined in a similar fashion. Significantly, C1Bs of all other conventional and novel PKCs (PKCα, -βI/βII, -γ, -δ, -ϵ, -θ) showed strong PDBu binding, like that of η-C1B. In contrast, all C1As except for γ-C1A showed very weak or no PDBu-binding affinity, as did η-C1A, as shown in Figure 5. Since only γ-C1A showed potent PDBu binding affinity, similar to α-C1B and γ-C1B, the specific PDBu binding of γ-C1A (200 nM) was fixed at 100. Relative to this standard, δ-C1A, θ-C1A, β-C1A, and α-C1A showed very weak but detectable binding, while ϵ-C1A and η-C1A were completely inactive, *indicating that, with the exception of PKCγ, the major PDBu-binding site in conventional and novel PKC isozymes is C1B*. These results suggest a revision of the long-held view that both C1 domains of native PKC bind PDBu strongly.[4] After completion of this work, Szallasi *et al.*[6] and Hunn and Quest[7] reported studies on the C1 domains of PKCδ, finding that they, too, are not equivalent based on a DNA recombination technique. The groups of Nishizuka and Bell[4] conducted related studies on PKCγ, possibly because of the ease of making the truncated mutants of PKCγ, and found contrasting results. However, as demonstrated in this work, the binding characteristics of PKCγ C1 domains are exceptional among the PKC isozymes.

Scatchard analyses of all C1Bs along with γ-C1A, δ-C1A, and θ-C1A were carried out, and the resultant K_d values are summarized in Table 1. As control references, the K_d values of native PKC isozymes reported independently by several groups are also listed. All C1Bs of novel PKCs bound PDBu strongly with nanomolar K_d values: 1.0, 1.5, 0.91, and 3.4 nM for C1Bs of PKCδ, -ϵ, -η, and -θ, respectively. These values correspond closely to the values reported by Kazanietz *et al.*[11]

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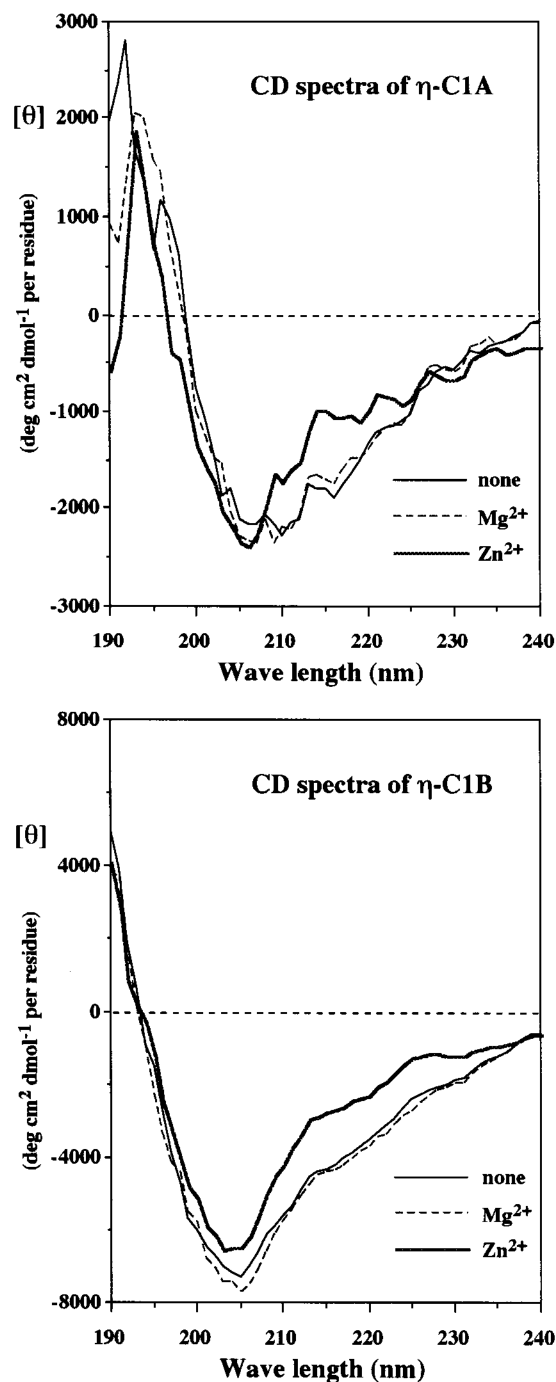

Figure 4. Top: CD spectra of η-C1A itself and η-C1A treated separately with 2.5 molar equiv of $ZnCl_2$ or $MgCl_2$ in helium-purged distilled water. Bottom: CD spectra of η-C1B itself and η-C1B treated separately with 2.5 molar equiv of $ZnCl_2$ or $MgCl_2$ in helium-purged distilled water. The spectra were obtained on a JASCO J-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.

for the novel PKCs. The K_d value of PKCθ has not yet been reported. The K_d values of δ-C1B and ϵ-C1B are also in fairly good agreement with those reported by Dimitrijevic *et al.*[12]

In contrast to these results, the K_d values of C1Bs of conventional PKCs (PKCα, -βI/βII, and -γ) differ somewhat from those reported by Kazanietz *et al.*[11] but agree with those reported by Dimitrijevic *et al.*[12] or Zhu *et al.*[14] It is noteworthy that there is variation in the K_d values of PDBu in the absence of calcium for native PKCα (K_d = 60.0,[12] 30.0,[14] and 0.15 nM[11]) and for PKCγ (K_d = 18.0,[12] 6.8,[16] 2.4,[13] and 0.37 nM[11]). This

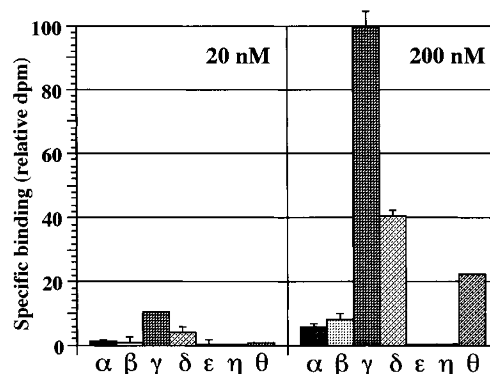

Figure 5. Specific PDBu binding of all C1A peptides. The binding was evaluated by the same procedure as described in Figure 3. The final concentration of each C1A peptide was 20 or 200 nM. The specific binding of 200 nM γ-C1A (55 900 dpm) was fixed at 100. The bars represent standard deviation.

Table 1. K_d Values from Scatchard Analyses of the Specific Binding of [^{3}H]PDBu to the PKC Surrogate Peptides

conventional PKC	K_d (nM)	novel PKC	K_d (nM)
α-C1A	>3000	δ-C1A	*ca.* 300
α-C1B	46.7 (2.1)[a]	δ-C1B	1.0 (0.1)
PKCα[b]	60.0	PKCδ[b]	4.0
PKCα[c]	0.15	PKCδ[c]	0.71
PKCα[d]	30.0		
β-C1A	>3000	ϵ-C1A	>10 000
β-C1B	1.3 (0.3)	ϵ-C1B	1.5 (0.2)
PKCβI[b]	3.9	PKCϵ[b]	18.0
PKCβII[b]	9.5	PKCϵ[c]	0.63
PKCβ[c]	0.14		
		η-C1A	>10 000
γ-C1A	65.8 (0.6)	η-C1B	0.91 (0.1)
γ-C1B	16.9 (3.0)	PKCη[c]	0.58
PKCγ[b]	18.0		
PKCγ[c]	0.37	θ-C1A	*ca.* 900
PKCγ[e]	2.4	θ-C1B	3.4 (1.0)
PKCγ[f]	6.8	PKCθ	nt[g]

[a] Standard deviation of at least two separate experiments. [b] The K_d values in the absence of calcium were reported by Dimitrijevic *et al.*[12] [c] The K_d values in the absence of calcium were reported by Kazanietz *et al.*[11] [d] The K_d value in the absence of calcium was reported by Zhu *et al.*[14] [e] The K_d value in the absence of calcium was reported by Quest *et al.*[13] [f] The K_d value in the absence of calcium was reported by Irie *et al.*[16] [g] Not tested.

variation is not unexpected because, in conventional PKCs, such as PKCα and -γ, the calcium- and phosphatidylserine-binding domain (C2 domain) is proximate to the phorbol ester-binding domain (C1 domain), and changes in the assay conditions would be expected to result in some variation in the K_d values. At present, while a range of K_d values for PDBu binding to native PKCα and -γ have been reported, the values determined for the C1 peptides are within this range.

Given that our binding data for the PKC surrogate peptides correspond well with the K_d values of native PKC isozymes, the C1B peptides serve as effective, readily available surrogates for native PKC isozymes and afford unique opportunities for the study of binding, affinity labeling, and solution structure related to the PKC regulatory domain. Only the C1 peptides of PKCγ (γ-C1A and γ-C1B) exhibited similar affinities for PDBu, indicating that both C1 peptides of PKCγ still qualify as PKCγ surrogates. Although recent investigations[16,40] suggested that the major binding site of PDBu in native PKCγ might be C1A rather than C1B, there is no evidence that C1B

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is not a PDBu-binding site. Our studies suggest that a peptide containing both C1 motifs would be an ideal surrogate of PKCγ. The synthesis and study of this system is in progress.

Effects of Metal Ions Other Than Zinc on the PDBu Binding of All Surrogate Peptides of Conventional and Novel PKCs. Although zinc coordination is required for folding and phorbol ester binding of the C1 peptides (and the corresponding PKCs), the effects of metal ions other than zinc on these processes have not been investigated. These effects are pertinent to the evaluation of assay data and, in addition, have significant biological and physiological ramifications. We have recently reported the influence of zinc and other metal ions on the folding and PDBu binding of γ-C1B and η-C1B, including the first observation of C1B-selective metal ion regulation of binding in a preliminary publication.[41] In this paper, we report the effects of metal ions other than zinc on the PDBu binding of all PKC surrogate peptides which exhibit potent PDBu-binding affinity (Figure 2).

The metal effects are summarized in Figure 6. The C1 peptides are classified into three groups according to the level of the background binding and their cadmium selectivity: (a) α-C1B, β-C1B, and δ-C1B; (b) ϵ-C1B, η-C1B, and θ-C1B; and (c) γ-C1A and γ-C1B. Group a peptides showed high levels of PDBu binding without zinc treatment (entry 2 in Figure 6a). However, this background binding was completely abolished in the presence of 2 mM EDTA in the assay mixture (entry 15). This indicates that chelatable ions in the assay mixture could account for the background folding of these peptides. Cr^{2+}-, Mn^{2+}-, Fe^{2+}-, Co^{2+}-, Ni^{2+}-, Mg^{2+}-, or Ca^{2+}-treated C1 peptides also showed high levels of binding (entries 3−9), which also disappeared when 2 mM EDTA was present in the assay mixture (data not shown); only the data for Co^{2+} are shown as a typical example (entry 17). In contrast to this observation, the binding of the Zn^{2+}- or Cd^{2+}-folded peptides did not change significantly upon exposure to 2 mM EDTA (entries 14 and 16). This suggests that the coordination of cadmium and zinc is strong and that the cadmium ion plays a role similar to that of the zinc ion in the folding of these C1 peptides. On the other hand, when the peptides were initially treated with Cu^{2+}, Ag^{+}, or Hg^{2+}, specific binding was completely abolished (entries 10, 11, and 13). This loss of specific binding was also found with these metal ions (2.5 molar equiv), even when the peptides were pretreated with zinc, indicating that these ions coordinate more strongly than zinc or otherwise interfere with binding and folding. In the classical zinc finger peptides, Cu^{2+} is sometimes found to oxidize the peptides, producing disulfide-linked species.[42] However, no significant difference in the PDBu binding of the $CuCl_2$-treated peptides was detected between aerobic and anaerobic conditions, suggesting that such oxidation did not occur to a significant extent. Group b peptides behaved quite similarly to group a peptides, except for the level of the background binding (Figure 6b). Zn^{2+} and Cd^{2+} were equally effective in the folding and PDBu binding of ϵ-C1B, η-C1B, and θ-C1B.

Remarkably, Cd^{2+} treatment of γ-C1A and γ-C1B abolished wholly the PDBu binding (entries 12 and 16 in Figure 6c), while Cd^{2+} treatment of the other PKC surrogate peptides resulted in significant binding, approaching the maximum level observed for the Zn^{2+}-treated peptides (entries 12 and 16 in Figure 6a and b). The inhibitory effect of Cd^{2+} on γ-C1A and γ-C1B

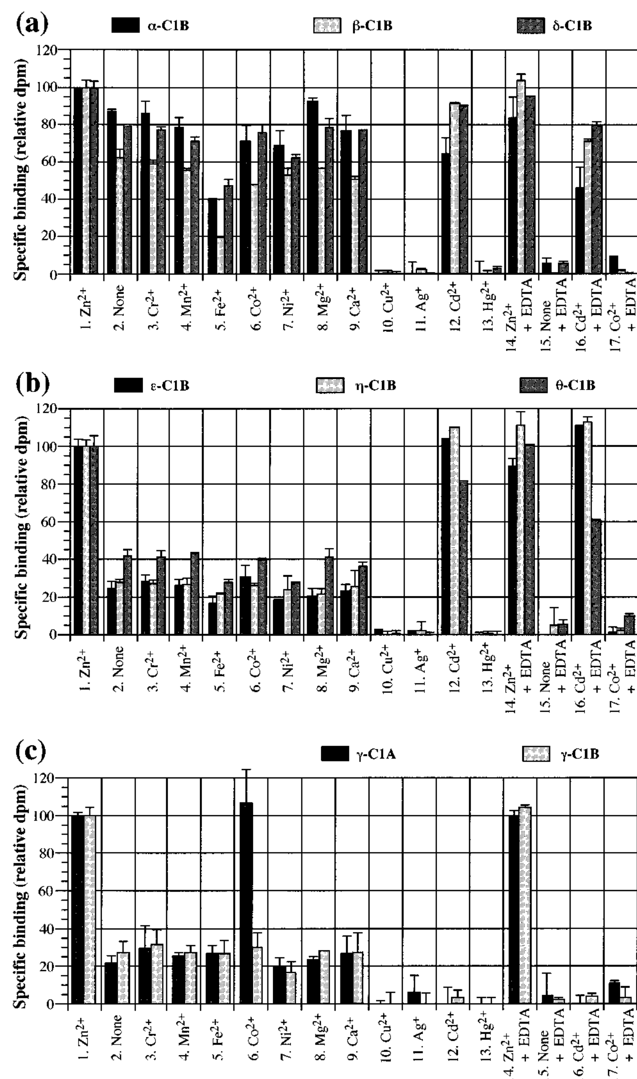

Figure 6. Effects of metal ions on the PDBu binding of all C1B peptides and γ-C1A. Metal coordination was carried out in a distilled water solution of each C1 peptide (100 μg/mL) using 5 molar equiv of each metal salt at 4 °C for 10 min. The PDBu binding was evaluated by the same procedure as described in Figure 3. The specific binding of the $ZnCl_2$-treated C1 peptides was each fixed at 100 (entry 1). Entry 2: the C1s without $ZnCl_2$ treatment; entry 3, the $CrCl_2$-treated C1s; entry 4, the $MnCl_2$-treated C1s; entry 5, the $FeSO_4(NH_4)_2SO_4$-treated C1s; entry 6, the $CoCl_2$-treated C1s; entry 7, the $NiCl_2$-treated C1s; entry 8, the $MgCl_2$-treated C1s; entry 9, the $CaCl_2$-treated C1s; entry 10, the $CuCl_2$-treated C1s; entry 11, the $AgNO_3$-treated C1s; entry 12, the $CdCl_2$-treated C1s; entry 13, the $HgCl_2$-treated C1s; entry 14, the $ZnCl_2$-treated C1s were added to the reaction mixture containing 2 mM EDTA; entry 15, the $ZnCl_2$-untreated C1s were added to the reaction mixture containing 2 mM EDTA; entry 16, the $CdCl_2$-treated C1s were added to the reaction mixture containing 2 mM EDTA; entry 17, the $CoCl_2$-treated C1s were added to the reaction mixture containing 2 mM EDTA. The bars represent standard deviation.

was very strong; addition of equimolar Cd^{2+} to Zn^{2+}-pretreated γ-C1B caused complete disappearance of binding (data not shown). Even addition of a 100-fold excess of Zn^{2+} to Cd^{2+}-pretreated γ-C1B did not restore binding, suggesting that binding of Cd^{2+} to γ-C1B is stronger than that of Zn^{2+}. Although Co^{2+} exhibited a γ-C1A-selective folding, cobalt coordination was not as strong as that of zinc since the binding of Co^{2+}-treated γ-C1A decreased drastically in the presence of 2 mM EDTA in the assay mixture (entry 17 in Figure 6c).

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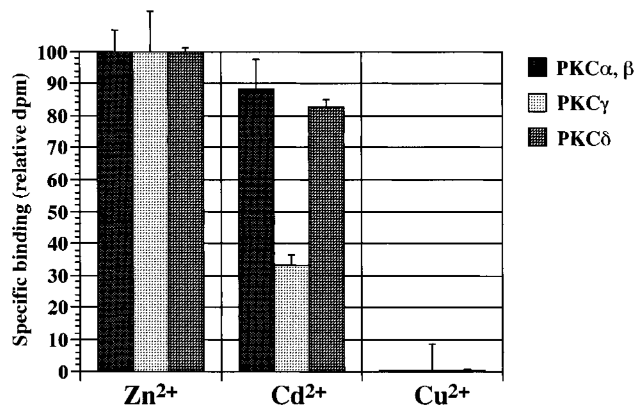

Figure 7. Effects of metal ions on the PDBu binding of native rat PKCα, -β, -γ, and -δ. The PDBu binding was evaluated by a procedure similar to that described in Figure 3, except for the concentrations of native PKC isozymes (*ca.* 1 nM), phosphatidylserine (100 μg/mL), and [^{3}H]PDBu (10 nM). The specific binding in the presence of 100 μM $ZnCl_2$ in the assay mixture was fixed at 100. The concentration of $CdCl_2$ and $CuCl_2$ was also 100 μM for PKCα-, β-, and γ-binding assay. For PKCδ-binding assay, PKCδ pretreated with each metal ion was added to the reaction mixture. The final metal concentration was 2 μM. The bars represent standard deviation.

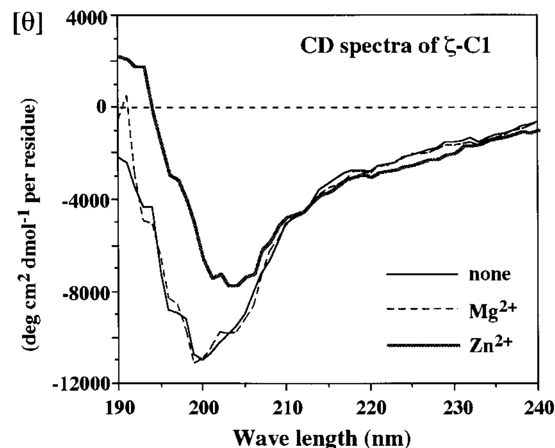

Figure 8. CD spectra of ζ-C1 itself and ζ-C1 treated separately with 2.5 molar equiv of $ZnCl_2$ or $MgCl_2$ in helium-purged distilled water. The spectra were obtained on a JASCO J-700 CD spectrophotometer in a 0.5-mm cell using 275 μg/mL solutions in 10 mM Tris-HCl buffer (pH 7.4) at 24 °C.

With the discovery of more than 10 PKC isozymes (PKCα, -βI/βII, -γ, -δ, -ϵ, -η, -θ, -ζ, -λ/ι),[2,43] increasing importance is placed on isozyme-specific analysis of function in order to elucidate the role of PKC in cellular signal transduction, tumor promotion, and therapeutic studies. Due to the limited information on the solution structure of the phorbol ester–PKC–phosphatidylserine aggregate,[32–35,44–46] most efforts to generate isozyme-selective agonists have focused thus far on variations in organic ligands. The present results strongly suggest that cadmium ion might serve as an effective tool for controlling isozyme-selective inhibition of PKCγ. To explore this possibility, the effects of Zn^{2+}, Cd^{2+}, and Cu^{2+} on the PDBu binding of native PKCα, -β, -γ, and -δ were examined (Figure 7). The specific PDBu binding in the presence of Zn^{2+} was each fixed at 100. Cu^{2+} completely inhibited the binding of all PKC isozymes, while Cd^{2+} inhibited that of only PKCγ, *suggesting that native PKCγ could be selectively regulated by Cd^{2+}.*

Modulation of PKC by neurotoxic heavy metals has recently been investigated, since PKC is involved in neurotransmitter release[47–49] and signal transduction.[50] For example, Speizer *et al.*[51] and Rajanna *et al.*[52] have reported that heavy metal ions such as Hg^{2+}, Cu^{2+}, and Cd^{2+} inhibited the enzyme activity of a conventional PKC mixture and its PDBu binding. Thus far, heavy metal ions have been proposed to interact with the catalytic domain of PKC in inhibiting PKC activation.[53,54] Our present results strongly indicate that one of the main targets of these heavy metal ions is the C1 domain itself in the regulatory domain.

PDBu Binding of the C1 Peptides of Atypical PKC and Several C1 Mutants. Atypical PKCs such as PKCζ and -λ/ι are the only isozymes which do not bind phorbol esters.[25,55,56] These isozymes possess a single copy of the C1 motif, unlike conventional and novel PKCs, as shown in Figure 1. Since zinc ion plays a critical role in the folding and PDBu binding of the C1 domains, as mentioned above, the C1 peptides of atypical PKCs (ζ-C1 and λ-C1) were synthesized, and their zinc-folding and PDBu-binding abilities were examined. Although a zinc-induced conformational change was detected in the CD spectra of ζ-C1 (Figure 8), the zinc-treated ζ-C1 and λ-C1 did not show any PDBu binding, even at 2 μM, as was found for ϵ-C1A and η-C1A, indicating that they are not the receptors for phorbol ester-type tumor promoters.

Sequence alignment of all C1 domains of PKC isozymes (Figure 2) clearly shows that six cysteines and two histidines are completely conserved. These residues play an important role in zinc folding and PDBu binding.[4,30,57] Kazanietz *et al.*[57] have recently reported that the mutations at 3-F, 8-Y, 11-P, 21-L, 25-L, 28-Q, and 39-V as well as at the above-mentioned six cysteines and two histidines of C1B of PKCδ drastically reduced the PDBu-binding affinity and indicated that these residues are also necessary for PDBu binding. However, these residues are conserved in all C1As, which showed only weak or no PDBu binding (Figure 2). Moreover, Kazanietz *et al.*[58] have also made the PKCζ mutant in which the 11th glycine was changed to proline. Even this mutant did not show any PDBu binding. These results indicate that amino acids other than the above 15 residues are necessary for PDBu binding.

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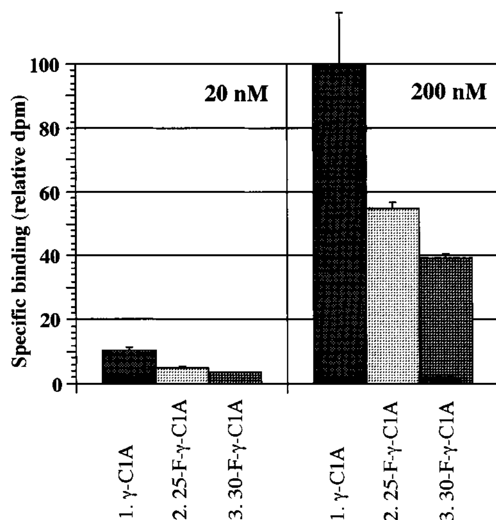

Figure 9. Specific PDBu binding of γ-C1A, 25-F-γ-C1A, and 30-F-γ-C1A. The binding was evaluated by the same procedure as described in Figure 3. The final concentration of each C1A peptide was 20 or 200 nM. The specific binding of 200 nM γ-C1A (73 000 dpm) was fixed at 100. All compounds were tested simultaneously, and the bars represent standard deviation. The K_d value from Scatchard analysis of the [^{3}H]PDBu binding to 30-F-γ-C1A was *ca.* 600 nM, 10-fold larger than that of γ-C1A (65.8 nM).

To identify the additional amino acid residues required for PDBu recognition, we focused on the two C1 domains, β-C1A and γ-C1A. Although these peptides differ only in the five amino acid residues at positions 25, 30, 36, 41, and 50, the former is almost inactive, while the latter is a potent PDBu binder. Careful sequence comparison led to the hypothesis that the 25-F and 30-F residues are responsible for the drastic decrease of the PDBu binding of β-C1A (and α-C1A), since these residues are close to the putative PDBu-binding domain.[34,35] Residue 30-F is especially noteworthy because all C1As with weak or no PDBu-binding ability have aromatic amino acids such as tyrosine and phenylalanine in the 30th position. In contrast, all C1Bs and γ-C1A with strong PDBu-binding affinity have aliphatic amino acids such as leucine and methionine in this position.

Two mutants, 25-F-γ-C1A and 30-F-γ-C1A, were synthesized and assayed for PDBu binding to test this idea. As shown in Figure 9, the PDBu-binding affinity of both mutants significantly decreased. Scatchard analysis gave a K_d of *ca.* 600 nM for 30-F-γ-C1A, 10-fold greater than that of γ-C1A (65.8 nM). These results indicate that the 25th isoleucine and 30th leucine in γ-C1A are necessary for strong PDBu binding. This result also provides an explanation for the lack of PDBu-binding affinity of atypical PKCs along with Kazanietz's 11-P mutant of PKCζ,[58] in which the 30th amino acid residue is tyrosine. Therefore, 11-P-30-L-ζ-C1 was synthesized and its PDBu-binding affinity determined. However, 11-P-30-L-ζ-C1 did not show any binding, even at 2 μM, suggesting that further amino acid residues are responsible for lack of the PDBu binding of PKCζ.

It is also noteworthy that ϵ-C1A and η-C1A contain one excess valine at the 24th position, unlike the other C1 peptides. This additional valine might interfere with the proper folding and PDBu binding of these C1 peptides, since recent investigations[34,35] suggest that phorbol esters fit into a narrow groove of the C1 created by the 20−27th amino acid residues.

Conclusion

In summary, we have synthesized the cysteine-rich domains of all known PKC isozymes and determined their chemical characteristics for the first time. Extremely high yields were achieved on a Pioneer Peptide Synthesizer using Fmoc chemistry with HATU as a coupling reagent. The zinc-folded C1B peptides of all conventional and novel PKCs bound strongly to PDBu, with K_d values comparable to those corresponding native PKC isozymes. The zinc-folded peptides in buffer solution were stable for at least 1 month at 4 °C, suggesting that they can serve as effective tools for PKC isozyme binding assay of tumor promoters, for identifying new medicinal leads related to isozyme selective PKC modulation, and for characterization of the regulatory domain structure under physiologically relevant conditions.

Only the C1A peptide of PKCγ (γ-C1A) showed potent PDBu binding comparable to that of the C1B peptides of PKCα and -γ (α-C1B and γ-C1B). This is an unexpected result, since it has been long believed that the two C1 domains of PKC isozymes are functionally equivalent.[4,16] What is the major role of C1A, then? Do they bind only phosphatidylserine to make the membrane-bound PKC stable? Might they function as weak pre-coordination sites to increase the effective local concentration of diacylglycerols destined for C1B? Alternatively, there might be ligands other than phorbol esters which bind to C1A. In fact, Slater *et al.*[59] have recently reported that PKCα contains two domains that bind phorbol esters and diacylglycerols with opposite affinities. Identification of compounds with potent C1A-binding affinity is a challenging problem.

The fact that PDBu is a γ-C1A-selective ligand and that PKCγ has two high-affinity phorbol ester-binding domains provides a structural blueprint for the rational design of PKCγ-selective activators or inhibitors. The synthesis and study of such systems is currently in progress. The effects of metal ions other than zinc have also shown that PKCγ can be selectively regulated by cadmium ion. PKCγ is a unique isozyme which exists exclusively in the brain and the nervous system, unlike the other PKCs. Recently, Malmberg *et al.*,[8] drawing on studies of a PKCγ knockout mouse, suggested that PKCγ could play a significant role in pathological pain. This indicates that it may be possible to alleviate nerve injury-induced neuropathic pain states without profound side effects if selective inhibitors of PKCγ can be developed. The present results clearly show that PKCγ differs from the other PKC isozymes both in the PDBu binding of C1A and in the inhibitory effect by cadmium ion. These results provide the basis for the rational design of new medicinal agents with PKCγ selectivity.

Experimental Section

General Methods. The following spectroscopic and analytical instruments were used: MALDI-TOF-MS, PerSeptive Biosystems Voyager-DE STR (20 kV); CD, Jasco J-700; peptide synthesizer, PerSeptive Biosystems model 9050 plus and model 9030 (Pioneer Peptide Synthesizer); HPLC, Waters model 600E with model 484 UV detector and Waters model 625LC with model 486 UV detector. MALDI-TOF-MS was measured as follows: each C1 peptide dissolved in 0.1% TFA aqueous solution (50 pmol/μL) was mixed with saturated α-cyano-4-hydroxycinnamic acid in 50% CH_3CN containing 0.1% TFA in the ratio of 1:1. One microliter of the resultant solution was subjected to the measurement. Angiotensin I and ACTH (7−38) were used as external references. HPLC was carried out on YMC-packed SH-342-5 (ODS, 20 mm i.d. × 150 mm) and YMC-packed A-311 (ODS, 6 mm i.d. × 100 mm) columns (Yamamura Chemical Laboratory). [^{3}H]PDBu

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(19.6 or 21.0 Ci/mmol) was purchased from NEN Research Products. Unless otherwise noted, reagents were obtained from Sigma or Wako Pure Chemical Industries. Native rat PKCα, -β, -γ, and -δ were prepared by the method reported previously.[60]

Synthesis of the C1 Peptides of All PKC Isozymes. Almost all of the C1 peptides were synthesized in a stepwise fashion on Fmoc-Gly-PEG-PS resin (PerSeptive Biosystems) by the Pioneer Peptide Synthesizer using Fmoc chemistry.[26] Fmoc amino acids (PerSeptive Biosystems) were used with the following side-chain protection: Cys(Trt), Asp(OtBu), Glu(OtBu), His(Trt), Lys(Boc), Asn(Trt), Gln(Trt), Arg(Pbf), Ser(tBu), Thr(tBu), Tyr(tBu). The Fmoc group was deblocked with 20% piperidine in DMF for 5 min (flow rate, 8.8 mL/min). The coupling reaction was carried out using each Fmoc amino acid (0.8 mmol), HATU (0.8 mmol), and DIPEA (0.95 mmol) in DMF for 60 min (flow rate, 30 mL/min). Each Fmoc amino acid (0.8 mmol) and HATU (0.8 mmol) weighed in a test tube was dissolved in 3.8 mL of the base solution (0.25 M DIPEA in DMF). The mixture was purged with a N_2 stream and added within 2 min to the column containing the resin with a flow rate of 30 mL/min. Final amino acid and HATU concentrations in the coupling reaction were *ca.* 0.2 M. In the synthesis using the model 9050 plus peptide synthesizer, the mixture was added 10 min after addition of the base solution. Final amino acid and HATU concentrations were *ca.* 0.25 M, and the flow rate was 6 mL/min, respectively.

After completion of the chain assembly, each peptide resin (*ca.* 1.5 g) was treated with a cocktail containing TFA, *m*-cresol, thioanisole, and ethanedithiol (24, 0.6, 3.6, and 1.8 mL, respectively). After 2 h of shaking at room temperature, the resin was filtered and washed with a small amount of TFA. The filtrate was then distributed in four tubes (*ca.* 10 mL each). Ether (35 mL) was added to each tube to precipitate the crude peptide. The mixture remained at 4 °C for 10 min and was then centrifuged (3000 rpm × 5 min). The precipitate was washed with ether five times and dried under an argon stream.

The crude peptide was dissolved in 10% acetic acid (*ca.* 10 mL) and applied to the gel filtration column (Sephadex G-15, Pharmacia, 200 g), equilibrated with 10% acetic acid. Elution with 10% acetic acid gave several ninhydrin-positive fractions, which were pooled and lyophilized. The gel-filtered peptide was purified by HPLC using the SH-342-5 column with elution at 8 mL/min by a 160-min linear gradient of 10−60% CH_3CN in 0.1% TFA. The peak of each C1 peptide was collected and concentrated *in vacuo* below 30 °C to remove CH_3CN. Lyophilization of each residue gave a corresponding pure C1 peptide whose purity was confirmed by HPLC (>98%) using the A311 column. Each purified peptide exhibited satisfactory mass spectrometric data (see the Supporting Information). The yields and mass data of these C1 peptides are summarized in Table 2.

Metal Coordination. Metal coordination was carried out in a helium-purged distilled water solution (pH 5.5−6.0) of each C1 peptide (5 or 10 μg/50 μL). Five molar equivalents (2 μL) of each metal salt in helium-purged distilled water (5 mM) were added to the peptide solution, and the solution was allowed to stand at 4 °C for 10 min. After 950 μL of helium-purged distilled water was added, 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 peptide was 55 μg/154 μL. To the peptide solution were added 2.5 molar equiv (6 μL) of each metal salt in helium-purged distilled water (5 mM), and the solution was allowed to stand at 4 °C for 10 min. After addition of 40 μL of 50 mM Tris-HCl (pH 7.4), the solution was subjected to the CD measurements.

[^{3}H]PDBu-Binding Assay of the PKC Surrogate Peptides. The PDBu binding to the PKC surrogate peptides was evaluated using the procedure of Sharkey and Blumberg.[38] The standard assay mixture (250 μL) in a 1.5-mL Eppendorf tube contained 50 mM Tris-HCl (pH 7.4 at 25 °C), 50 μg/mL 1,2-di-(*cis*-9-octadecenoyl)-*sn*-glycero-3-phospho-L-serine, 3 mg/mL bovine γ-globulin, [^{3}H]PDBu (19.6 or 21.0 Ci/mmol), and each PKC surrogate peptide. For determination of PDBu saturation curves for Scatchard analysis, concentrations of free [^{3}H]PDBu between 2 and 100 nM were used. In the standard binding assay, 5 nM C1 peptide and 20 nM [^{3}H]PDBu were used. For the α-C1B, γ-C1A, and γ-C1B binding assays, 10 nM C1 peptide and 40 nM [^{3}H]PDBu were used. Phosphatidylserine was suspended in 50 mM Tris-HCl (pH 7.4) by sonication (1 min) and added to the above reaction mixture.

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Table 2. Yields and MALDI-TOF-MS Data of All C1 Peptides[a]

C1 peptides	yield (%)	obsd mass	cald mass (MH$^+$)	molecular formula
α-C1A	4.4	6122.83	6123.23	$C_{282}H_{402}N_{75}O_{66}S_7$
α-C1B	17.1	5682.08	5682.69	$C_{246}H_{382}N_{69}O_{70}S_8$
β-C1A	7.9	6111.75	6111.18	$C_{280}H_{398}N_{75}O_{67}S_7$
β-C1B	13.9	5775.30	5774.93	$C_{248}H_{390}N_{71}O_{68}S_{10}$
γ-C1A	4.3	6096.71	6097.13	$C_{276}H_{404}N_{77}O_{69}S_6$
γ-C1B	4.2	5772.43	5772.81	$C_{244}H_{384}N_{77}O_{68}S_9$
δ-C1A	0.5	5928.46	5928.02	$C_{265}H_{409}N_{74}O_{69}S_6$
δ-C1B	2.7	5858.39	5858.92	$C_{254}H_{394}N_{75}O_{69}S_8$
ϵ-C1A	3.4	6162.59	6163.36	$C_{271}H_{422}N_{79}O_{70}S_8$
ϵ-C1B	14.6	5815.67	5814.89	$C_{252}H_{398}N_{79}O_{66}S_7$
η-C1A	0.5	6077.25	6077.23	$C_{269}H_{408}N_{79}O_{67}S_8$
η-C1B	4.7	5816.22	5815.94	$C_{252}H_{397}N_{78}O_{65}S_8$
θ-C1A	9.0	5937.03	5936.95	$C_{265}H_{400}N_{73}O_{71}S_6$
θ-C1B	11.7	5798.22	5797.82	$C_{251}H_{391}N_{78}O_{67}S_7$
ζ-C1	11.3	5922.70	5922.08	$C_{259}H_{415}N_{84}O_{64}S_6$
λ-C1	9.8	5956.97	5957.17	$C_{260}H_{424}N_{85}O_{64}S_6$
25-F-γ-C1A	5.9	6131.02	6131.14	$C_{279}H_{402}N_{77}O_{69}S_6$
30-F-γ-C1A	4.6	6131.87	6131.14	$C_{279}H_{402}N_{77}O_{69}S_6$
11-P-30-L-ζ-C1	15.7	5911.94	5912.13	$C_{259}H_{421}N_{84}O_{63}S_6$

[a] These peptides were synthesized by a PerSeptive Biosystems model 9030 (Pioneer Peptide Synthesizer), except for η-C1A, δ-C1A, and δ-C1B, which were synthesized by a PerSeptive Biosystems model 9050 plus.

The above-mentioned C1 peptide solution (2.9 μL), pretreated with $ZnCl_2$ or other metal salt, was added to the standard assay mixture (247.1 μL), and the solution was incubated at 30 °C for 20 min. After the mixture was 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 mixture was 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 of each tube was removed by aspiration. The tips of the tubes were cut off, and the radioactivity in the pellets was measured to determine the bound [^{3}H]PDBu. 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 its partition coefficient. In each experiment, each point represents the average of at least triplicate determinations.

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Supporting Information Available: MALDI-TOF-MS spectra of all C1 peptides along with a HPLC chromatogram of α-C1B (20 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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