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Toward the Identification of Selective Modulators of Protein Kinase C (PKC) Isozymes: Establishment of a Binding Assay for PKC Isozymes Using Synthetic C1 Peptide Receptors and Identification of the Critical Residues Involved in the Phorbol Ester Binding

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Abstract—Conventional and novel protein kinase C (PKC) isozymes contain two cysteine-rich C1 domains (C1A and C1B), both of which are candidate phorbol-12,13-dibutyrate (PDBu) binding sites. We previously synthesized C1 peptides (of approximately 50 residues) corresponding to all PKC isozymes and measured their PDBu binding affinity. While many of these peptide receptors exhibited PDBu affinities comparable to the respective complete isozyme, some of the C1A peptides could not be used because they undergo temperature dependent inactivation. This problem was however eliminated by 4 °C incubation or elongation of the 50-mer C1 peptides at both N- and C-termini to increase their folding efficiency and stability. These findings enabled us to determine the K_d's of PDBu for all PKC C1 peptides (except for θ-C1A) and establish the value of these peptides as readily available, stable, and easily handled surrogates of the individual isozymes. The resultant C1 peptide receptor library can be used to screen for new ligands with PKC isozyme and importantly C1 domain selectivity. Most of the C1 peptide receptors showed strong PDBu binding affinities with K_d's in the nanomolar range (0.45–7.4 nM). Two peptides (δ-C1A and θ-C1A) bound PDBu over 100-fold less tightly. To identify the residues that contribute to this affinity difference, several mutants of δ-C1A and θ-C1A were synthesized. Both the G9K mutant of δ-C1A and the P9K mutant of θ-C1A showed K_d's of 2–3 nM. This approach provides a useful procedure to determine the role of each C1 domain of the PKC isozymes by point mutation.

Introduction

Protein kinase C (PKC) isozymes are major receptors of tumor-promoting phorbol esters and also play a crucial role in cellular signal transduction via the second messenger diacylglycerol.[1–3] Since each PKC isozyme is involved in diverse biological events, these proteins serve as novel targets for chemotherapeutic intervention (for example, cancer, neuropathic pain[4] and vascular complications of diabetes[5]). PKC isozymes are subdivided into three classes (Fig. 1): calcium dependent or conventional PKCs (PKCα, βI/βII, γ), calcium independent or novel PKCs (PKCδ, ϵ, η, θ), and atypical PKCs (PKCζ, λ/ι) which do not bind to phorbol esters and diacylglycerols.[1–3]

The N-terminal regulatory region of conventional and novel PKC isozymes contains tandem cysteine-rich C1 domains designated C1A and C1B,[1] both of which are candidate phorbol ester and diacylglycerol binding domains.[6–8] Recent investigations revealed that each C1 domain plays differential roles in translocation and down-regulation of PKCδ,[9,10] which has a tumor suppressor role in vivo and in vitro.[11,12] The development of rapid screening strategies and procedures for the identification and evaluation (binding affinity) of PKC activating agents for each PKC C1 domain is thus

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indispensable for elucidation of the biochemical role of individual PKC isozymes and for the development of novel PKC-targeted therapeutic agents.

The C1 domains of PKC, C1A and C1B, are zinc fingers consisting of about 50 amino acid residues. We synthesized the fourteen C1A and C1B peptides of all conventional and novel PKC isozymes by an Fmoc solid-phase strategy (Fig. 2) and measured their dissociation constants (K_d) for [^{3}H]phorbol-12,13-dibutyrate (PDBu).[13,14] All C1B peptides folded upon zinc treatment, and exhibited potent PDBu binding affinities comparable to native PKC isozymes.[14] On the other hand, all C1A peptides except for PKCγ (γ-C1A) showed weak PDBu binding. We recently found that the incubation temperature (30 °C) of the binding assay significantly influences (decreases) the PDBu binding to the C1A peptide of protein kinase D (PKD,[15] also called as PKCμ[16]).[17] PKD like PKC contains a tandem repeat of cysteine-rich motifs, suggesting that some of the C1A peptides of PKC isozymes could also suffer from similar temperature dependent decreases of PDBu binding. Prompted by this novel temperature dependency, we examined all PKC C1 peptides with weak PDBu binding affinities and found that this temperature effect is also observed with γ-C1A, γ-C1B, ε-C1A, and η-C1A peptides. We also found that elongation of the 50-mer C1 peptides improves solubility and increases their folding efficiency. We report here the novel temperature dependent inactivation of PKC C1 peptides and the revised K_d values of PDBu for all PKC C1 peptides. The amino acid residues of the C1 peptides which play an important role in the PDBu binding were also identified.

Results and Discussion

Temperature effects on the [^{3}H]PDBu binding to PKCγ

C1 peptides (γ-C1A and γ-C1B), their glutathione *S*-transferase (GST) fusion proteins (GST-γ-C1A and GST-γ-C1A), and native PKCγ. We recently reported the K_d values at 4 °C of [^{3}H]PDBu for all PKC C1 peptides (Table 1).[14] These data were obtained by the method of Sharkey and Blumberg[18] who set the incubation temperature in the binding assay at 30 °C for 20 min. However, the K_d values for the PKCγ C1 peptides (65.8 nM for γ-C1A and 16.9 nM for γ-C1B) obtained in this way differed by 50–200 fold from those reported by Kazanietz et al. (0.37 nM for native PKCγ)[19] but agreed with those reported by Dimitrijevic et al.[20] (18.0 nM for native PKCγ). Since this discrepancy was thought to be ascribable to the binding assay conditions employed, we examined the effects of the incubation temperature (30 °C) and time (20 min) on the PDBu binding of γ-C1A and γ-C1B. As shown in Figure 3, the PDBu binding of both γ-C1A and γ-C1B decreased significantly at 30 °C while the binding was relatively constant at 4 °C over a 60 min incubation. Interestingly, no unusual temperature effect was observed for δ-C1B, ε-C1B, and η-C1B whose K_d values determined by incubation at 30 °C for 20 min (1.0, 1.5, and 0.91 nM) were quite similar to those reported by Kazanietz et al. (0.71, 0.63, and 0.58 nM),[19] indicating that this temperature effect is sequence-dependent.

To investigate whether this temperature dependent inactivation occurs only in the synthetic C1 peptide, we prepared the GST fusion proteins of each PKCγ C1 domain by the method of Quest and Bell.[21] The PDBu

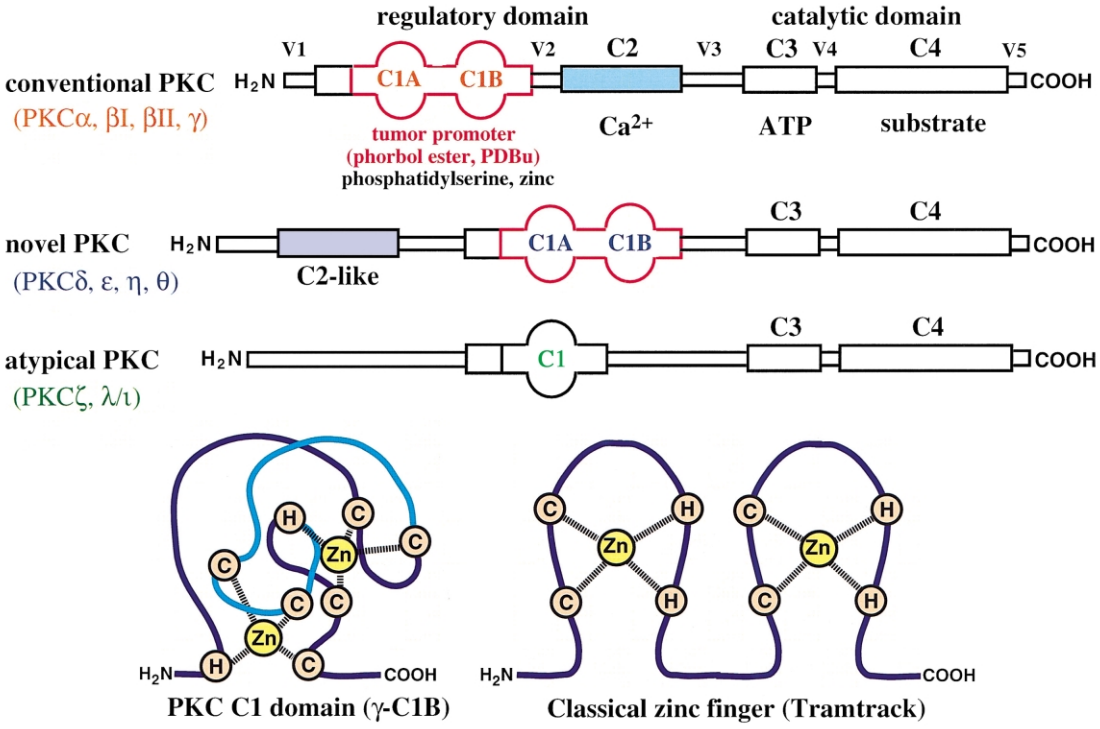

Figure 1. Structure of conventional, novel, and atypical PKC along with PKCγ C1B and the classical zinc finger (Tramtrack). The illustration of zinc-folded γ-C1B is based on the NMR solution structure of γ-C1B.[24]

binding of GST-γ-C1A (26–90) also decreased significantly at 30 °C although the binding was again relatively constant at 4 °C as shown in Figure 4. This result is quite similar to that found for the 52-mer peptide, γ-C1A (36–87). On the other hand, the temperature effect on GST-γ-C1B (91–172) was significantly less than that on the 51-mer peptide, γ-C1B (101–151). These results indicate that elongation of the C1 peptide and addition of the GST domain suppress the temperature-dependent inactivation. Furthermore, we examined the temperature effect on human recombinant PKCγ from a commercial source. No temperature dependent inactivation was observed on native PKCγ (Fig. 4). The temperature dependent inactivation is thus peculiar to the PKC C1 fragments even when they are prepared as GST fusion proteins by DNA recombination. It is thus strongly recommended that the binding assay using PKC C1 peptides and their GST fusion proteins should be done at 4 °C. An incubation time of 20 min is sufficient to reach equilibrium at 4 °C as shown in Figure 3. The reported K_d values of [^{3}H]PDBu for native PKC isozymes by Kazanietz et al.[19] were determined at 4 °C after PKC and [^{3}H]PDBu were incubated at 30 °C for 5 min to reach equilibrium. This assay condition is reasonable because the temperature dependent inactivation did not occur in native PKCγ (Fig. 4). The decrease in the PDBu binding of γ-C1A and γ-C1B is not attributable to degradation of these peptides since zinc-folded γ-C1A in Tris buffer (pH 7.4) did not decompose significantly at 30 °C for 60 min. Zinc-folded γ-C1A and γ-C1B might be labile to release zinc easily upon heat treatment since these peptides have the minimum length needed to coordinate two atoms of zinc as shown in Figure 1.[22–24]

Scatchard analysis of [^{3}H]PDBu binding of γ-C1A and γ-C1B at 4 °C gave dissociation constants (K_d) of 1.5 and 1.2 nM, respectively (Table 2). These values are in fairly good agreement with K_d of native PKCγ (0.37 nM).[19] The binding stoichiometries calculated from the B_{max} values of γ-C1A and γ-C1B were 14.5 and 19.3%, respectively. Similar binding stoichiometry (10–30%) was reported for the GST-fusion proteins of PKCγ C1 domains by Quest and Bell.[21] Scatchard

C1 peptides with strong PDBu binding ability (K_d : 0.45–1.5 nM)

α-C1A: 27 H_2N-RQKNVHEVKD-
(long) HKFIARFFKQPTFCSHCTDFIWG-FGKQGFQCQVCCFVVHKRCHEFVTFSCPG-
ADKGPDTDDG-CO_2H

β-C1A: 27 H_2N-RQKNVHEVKN-
(long) HKFTARFFKQPTFCSHCTDFIWG-FGKQGFQCQVCCFVVHKRCHEFVTFSCPG-
ADKGPASDDG-CO_2H

β-C1B: 102 H_2N-HKFKIHTYSSPTFCDHCGSLLYG-LIHQGMKCDTCMMNVHKRCVMNVPSLCG-CO_2H

γ-C1A: 26 H_2N-RQKVVHEVKS-
(long) HKFTARFFKQPTFCSHCTDFIWG-IGKQGLQCQVCSFVVHRRCHEFVTFECPG-
AGKGPQTDDG-CO_2H

γ-C1A: 36 H_2N-HKFTARFFKQPTFCSHCTDFIWG-IGKQGLQCQVCSFVVHRRCHEFVTFECPG-CO_2H

γ-C1B: 91 H_2N-GPQTDDPRNK-
(long) HKFRLHSYSSPTFCDHCGSLLYG-LVHQGMKCSCCEMNVHRRCVRSVPSLCG-
VDHTERRGRLG-CO_2H

γ-C1B: 101 H_2N-HKFRLHSYSSPTFCDHCGSLLYG-LVHQGMKCSCCEMNVHRRCVRSVPSLCG-CO_2H

δ-C1B: 231 H_2N-HRFKVYNYMSPTFCDHCGSLLWG-LVKQGLKCEDCGMNVHHKCREKVANLCG-CO_2H

ε-C1B: 243 H_2N-HKFGIHNYKVPTFCDHCGSLLWG-LLRQGLQCKVCKMNVHRRCETNVAPNCG-CO_2H

η-C1B: 246 H_2N-HKFNVHNYKVPTFCDHCGSLLWG-IMRQGLQCKICKMNVHIRCQANVAPNCG-CO_2H

θ-C1B: 232 H_2N-HRFKVYNYKSPTFCEHCGTLLWG-LARQGLKCDACGMNVHHRCQTKVANLCG-CO_2H

C1 peptides with moderate PDBu binding ability (K_d : 4.3–7.4 nM)

9 16 24 36 42 45
α-C1B: 102 H_2N-HKFKIHTYGSPTFCDHCGSLLYG-LIHQGMKCDTCDMNVHKQCVINVPSLCG-CO_2H

α-C1B: 92 H_2N-GPDTDDPRSK-
(long) HKFKIHTYGSPTFCDHCGSLLYG-LIHQGMKCDTCDMNVHKQCVINVPSLCG-
MDHTEKRGRIG-CO_2H

ε-C1A: 170 H_2N-HKFMATYLRQPTYCSHCRDFIWGVIGKQGYQCQVCTCVVHKRCHELIITKCAG-CO_2H

η-C1A: 172 H_2N-HKFMATYLRQPTYCSHCREFIWGVFGKQGYQCQVCTCVVHKRCHHLIVTACG-CO_2H

C1 peptide with very weak PDBu binding ability (K_d > 50 nM)

δ-C1A: 159 H_2N-HEFIATFFGQPTFCSVCKEFVWG-LNKQGYKCRQCNAAIHKKCIDKIIGRCTG-CO_2H

θ-C1A: 160 H_2N-HEFTATFFPQPTFCSVCHEFVWG-LNKQGYQCRQCNAAIHKKCIDKVIAKCTG-CO_2H

Figure 2. Sequence of all C1 peptides synthesized in this study. They are divided into three classes: those with potent PDBu-binding affinity (K_d: 0.45–1.5 nM), those with moderate PDBu-binding affinity (K_d: 4.3–7.4 nM), and δ-C1A and θ-C1A with very weak PDBu-binding affinity (K_d > 50 nM). All C1 sequences drive from mouse and are available from the web page at: http://www.genome.ad.jp/dbget-bin/www_bfind?pir. To prevent racemization and oxidation during synthesis, the carboxyl terminus was extended in each peptide from the final residue to a glycine.

Table 1. K_d Values at 4 °C of the [^{3}H]PDBu binding for the PKC C1 peptides at 30 °C incubation[a]

Conventional PKC	K_d (nM)	Novel PKC	K_d (nM)
α-C1A	ND[f]	δ-C1A	300
α-C1B	46.7	δ-C1B	1.0
PKCα[b]	60.0	PKCδ[b]	4.0
PKCα[c]	0.15	PKCδ[c]	0.71
PKCα[d]	30.0		
		ε-C1A	ND[f]
β-C1A	ND[f]	ε-C1B	1.5
β-C1B	1.3	PKCε[b]	18.0
PKCβI[b]	3.9	PKCε[c]	0.63
PKCβII[b]	9.5		
PKCβ[c]	0.14	η-C1A	ND[f]
		η-C1B	0.91
γ-C1A	65.8	PKCη[c]	0.58
γ-C1B	16.9		
PKCγ[b]	18.0	θ-C1A	900
PKCγ[c]	0.37	θ-C1B	3.4
PKCγ[e]	2.4	PKCθ	NT[g]

[a]These data are cited from ref. 14.
[b]The K_d values in the absence of calcium reported by Dimitrijevic et al.[20]
[c]The K_d values in the absence of calcium reported by Kazanietz et al.[19]
[d]The K_d value in the absence of calcium reported by Zhu et al.[32]
[e]The K_d value in the absence of calcium reported by Quest et al.[33]
[f]Not determined. The K_d values could not be determined because of poor solubility or high temperature sensitivity of the C1 peptides.
[g]Not tested.

analysis at 4 °C of [^{3}H]PDBu binding to GST-γ-C1A (26–90) and GST-γ-C1B (91–172) prepared in this study gave K_d of 0.80 and 0.73 nM, respectively, which corresponded well with that of native PKCγ (0.37 nM). Quest and Bell[21] reported the K_d values for GST-γ-C1A and GST-γ-C1B as 19.7 and 9.6 nM, respectively, which differed significantly from our results. This difference could arise partly from the binding assay conditions since the PDBu binding of the PKCγ C1 fragments is sensitive to the incubation temperature as shown above.

We examined the temperature effect on all PKC C1A peptides, almost all of which were considered to be weak PDBu binders.[14] Importantly, a significant inactivation by temperature similar to that found for γ-C1A and γ-C1B was observed in ε-C1A and η-C1A. No specific PDBu binding was detected even after 5 min incubation at 30 °C while the binding was constant at 4 °C incubation (data not shown), providing the basis for our previous observation that ε-C1A and η-C1A were inactive.[14] Scatchard analysis of [^{3}H]PDBu binding of ε-C1A and η-C1A at 4 °C incubation gave dissociation constants (K_d) of 5.6 and 4.3 nM, respectively (Table 2).

Synthesis and K_d values in the PDBu binding for the 72-mer C1 peptides. The C1A peptides for which K_d values could not be determined even at 4 °C incubation are α-C1A and β-C1A. A recent investigation by Bögi et al.[25] revealed that the C1A and C1B domains of PKCα play equivalent roles for translocation in response to tumor promoters, suggesting that the C1A of PKCα should potently bind to PDBu. In fact, we could detect specific PDBu binding of α-C1A and β-C1A previously, but their K_d values could not be determined because of the quite low level of the PDBu binding.[14] Since the solubilities of previous α-C1A and β-C1A peptides (52-mer) in water were fairly low compared with other C1A peptides, ten residues were added to both N- and C-termini of α-C1A and β-C1A to improve solubility. As shown in Figure 2, the 10 residues from N-terminus contain four basic and one or two acidic amino acids, and those from C-terminus three or four acidic and one basic amino acids. The 72-mer peptides (α-C1A-long and β-C1A-long) were synthesized by an Fmoc solid-phase strategy with a stepwise chain elongation. The resultant peptides showed good solubility as expected and bound to [^{3}H]PDBu with high affinity upon zinc treatment. No remarkable temperature-dependent inactivation was observed for either α-C1A-long or β-C1A-long (data not

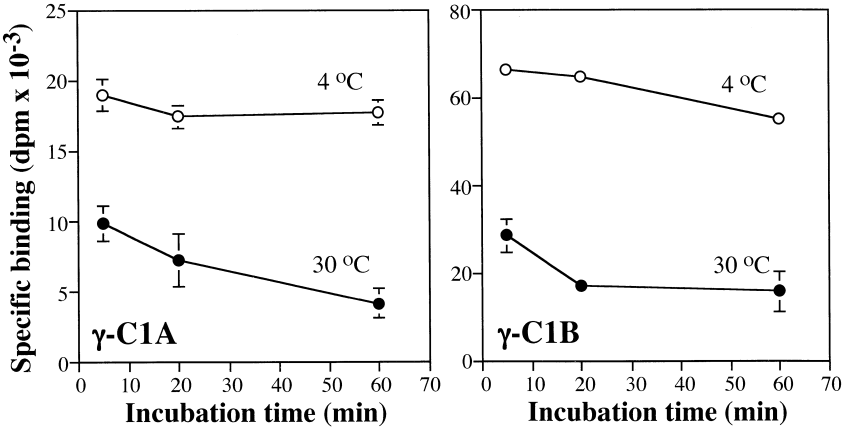

Figure 3. Temperature effects on the [^{3}H]PDBu binding of γ-C1A and γ-C1B. Zinc coordination was carried out in a distilled water solution of each C1 peptide using 5-molar equiv of $ZnCl_2$ at 4 °C for 10 min. After dilution with distilled water, an aliquot of the peptide solution (2.9 μL) was added to the reaction mixture (247.1 μL) consisting of 50 mM Tris-maleate (pH 7.4), 3 mg/mL γ-globulin, 50 μg/mL 1,2-di(*cis*-9-octadecenoyl)-*sn*-glycero-3-phospho-L-serine, and 20 nM [^{3}H]PDBu (20.0 Ci/mmol). The practical concentration of each C1 peptide was about 2.5 nM for γ-C1A and about 6 nM for γ-C1B which were calculated from the B_{max} value determined by Scatchard analysis. The reaction mixture was incubated at 4 or 30 °C for 5–60 min. The PDBu-peptide complex was precipitated at 4 °C by adding poly(ethyleneglycol) as reported previously.[14] ○, γ-C1A (left) and γ-C1B (right) at 4 °C incubation; ●, γ-C1A (left) and γ-C1B (right) at 30 °C incubation. The bars represent standard deviations of triplicate experiments.

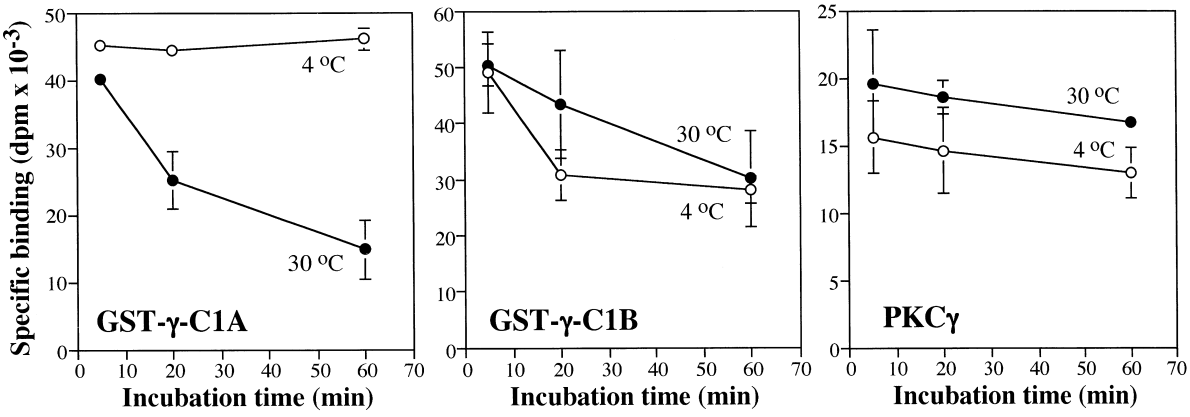

Figure 4. Temperature effects on the [^{3}H]PDBu binding of GST-γ-C1A (26–90), GST-γ-C1B (91–172), and human recombinant PKCγ from a commercial source. The experimental procedure was same as that of Figure 3. ○: GST-γ-C1A (left), GST-γ-C1B (middle), and native PKCγ (right) at 4 °C incubation; ●, GST-γ-C1A (left), GST-γ-C1B (middle), and native PKCγ (right) at 30 °C incubation. The bars represent standard deviations of triplicate experiments.

shown). Scatchard analysis of [^{3}H]PDBu binding of α-C1A-long and β-C1A-long at 4 °C incubation gave K_d of 1.1 and 1.3 nM, respectively. These values are a little bit larger than those of native PKCα and β (0.15 and 0.14 nM) reported by Kazanietz et al.[19] The binding stoichiometries calculated from the B_{max} values of α-C1A-long and β-C1A-long were 6.2 and 7.5%, respectively, indicating that the folding efficiency is not as high as that for most C1B peptides (Table 2).

To examine the effects of the 20 residue elongations on the K_d values for C1 peptides, similarly elongated 72-mer peptides of γ-C1A and γ-C1B (γ-C1A-long and γ-C1A-long) were synthesized (Fig. 2). The K_d values for these peptides determined at 4 °C incubation were 0.97 and 1.2 nM, respectively, indicating that such elongation barely changes K_d's for C1 peptides. However, the B_{max} values of the long version of γ-C1A and γ-C1B (19.3 and 26.2%) were higher than the corresponding 51- or 52-mer peptides (14.5 and 17.7%), suggesting that elongation increases the folding efficiency of the C1 peptides. Since the B_{max} value of α-C1B was low, a similarly elongated peptide (α-C1B-long) was synthesized. The K_d value did not change significantly by elongation, and the B_{max} value increased significantly (from 7.6 to 23.5%).

Table 2. K_d Values of the [^{3}H]PDBu binding for the PKC C1 peptides at 4 °C incubation

cPKC	K_d (nM)	B_{max} (%)	nPKC	K_d (nM)	B_{max} (%)
α-C1A-long[a]	1.1 (0.1)[c]	6.2 (1.2)	δ-C1A	51.9 (15.6)	38.5 (2.5)
α-C1B	7.4 (0.3)	7.6 (2.0)	δ-C1B	0.53 (0.14)	25.0 (7.0)
α-C1B-long[a]	5.3 (1.6)	26.5 (2.7)	PKCδ[b]	0.71	
PKCα[b]	0.15				
			ε-C1A	5.6 (0.6)	10.0 (4.0)
β-C1A-long[a]	1.3 (0.3)	7.5 (2.0)	ε-C1B	0.81 (0.03)	20.0 (3.2)
β-C1B	1.3 (0.4)	40.7 (9.9)	PKCε[b]	0.63	
PKCβ[b]	0.14				
			η-C1A	4.3 (0.2)	10.4 (5.1)
γ-C1A	1.5 (0.6)	14.5 (0.5)	η-C1B	0.45 (0.12)	32.7 (8.6)
γ-C1B	1.2 (0.2)	19.3 (0.7)	PKCη[b]	0.58	
γ-C1A-long[a]	0.97 (0.01)	17.7 (1.2)			
γ-C1B-long[a]	1.2 (0.0)	26.2 (4.0)	θ-C1A	>200	
PKCγ[b]	0.37		θ-C1B	0.72 (0.14)	31.7 (1.6)

[a]The 72-mer peptides in which 10 residues of both N- and C-termini of the original 51- or 52-mer peptides were elongated to improve solubility.
[b]The K_d values at 4 °C in the absence of calcium reported by Kazanietz et al.[19]
[c]Standard deviation of at least two separate experiments.

Determination of the K_d values of [^{3}H]PDBu for the C1 peptides of all PKC isozymes at 4 °C incubation. Scatchard analyses of the other C1 peptides were performed and the resultant K_d values of [^{3}H]PDBu are summarized in Table 2. As control references, the K_d values for native PKC isozymes reported by Kazanietz et al.[19] are also listed. All C1B peptides of novel PKC bound to PDBu strongly with nanomolar K_d values: 0.53, 0.81, 0.45, and 0.72 nM for the C1B peptides of PKCδ, ε, η, and θ, respectively. These values correspond almost exactly to the reported values for native novel PKC isozymes except for PKCθ whose K_d value is not available. In contrast, all C1A peptides of novel PKCs showed weaker PDBu binding. The PDBu binding affinity of δ-C1A and θ-C1A was especially low. Although specific PDBu binding of θ-C1A was detected, its K_d value could not be determined by Scatchard analysis. Low PDBu binding affinity of the C1A domain of PKCδ is also suggested by Szallasi et al.,[9] and Hunn and Quest.[26] These results indicate that the major PDBu binding site of novel PKC is C1B in agreement with previous reports.[14]

Unlike novel PKC, all C1A peptides of conventional PKC (α, β, γ) exhibited nanomolar K_d values: 1.1, 1.3, and 0.97 nM for the C1A-long peptides of PKCα, β, and γ, respectively. Moreover, both C1 peptides of PKCβ and γ showed strong PDBu binding affinities in the nanomolar range. Only α-C1B exhibited somewhat weak PDBu binding among the C1B peptides of conventional and novel PKC isozymes. The K_d values for conventional PKC C1 peptides determined by the new binding assay procedure at 4 °C incubation are in fairly good agreement with those reported for native PKC isozymes.[19] However, these values (0.97–1.3 nM) are, strictly speaking, 3–10-fold larger than the reported

Table 3. K_d Values of the [^{3}H]PDBu binding for several C1 mutants at 4 °C incubation

C1 mutants	K_d (nM)	B_{max} (%)	C1 mutants	K_d (nM)	B_{max} (%)
δ-C1A	51.9	38.5	α-C1B	7.4	7.6
16H-δ-C1A	25.3 (4.5)[a]	51.0 (0.0)	9S-α-C1B	14.8 (3.1)	6.0 (2.0)
9K-δ-C1A	2.2 (0.6)	32.4 (2.5)	42R-α-C1B	0.57 (0.19)	39.9 (4.7)
θ-C1A	>200		ϵ-C1A	5.6	10.0
16H-θ-C1A	>200		24-del-ϵ-C1A	6.1 (1.0)	14.4 (1.7)
9K-θ-C1A	2.9 (1.1)	40.9 (8.8)	η-C1A	4.3	10.4
			24-del-η-C1A	8.9 (2.1)	10.1 (0.1)

[a]Standard deviation of at least two separate experiments.

values (0.14–0.37 nM). In conventional PKCs, the calcium and phosphatidylserine binding domain (C2 domain) is proximate to the phorbol ester-binding domain (C1 domain). This structural feature might result in the minor variation in the K_d values. A range of the K_d values of PDBu binding for native PKCα, β, and γ is reported by several groups as shown in Table 1. The values determined for the C1 peptides of conventional PKC are clearly within this range.

The binding stoichiometries calculated from the B_{max} values of each C1 peptide are also listed in Table 2. All C1B peptides including the 72-mer peptides showed relatively high B_{max} values (20–40%), while the values of most C1A peptides except for γ-C1A and δ-C1A were less than 10%. Since the B_{max} values correspond to the actual concentration of the C1 peptide in the assay mixture, they reflect in part the efficiency of the zinc folding. This folding efficiency was not related to the temperature dependent inactivation mentioned above since α-C1A-long and β-C1A-long did not experience this inactivation.

Identification of the C1 peptide residues that play an important role in the PDBu binding. Most C1 peptides of conventional and novel PKC isozymes showed strong PDBu binding affinities with K_d's in the nanomolar range (0.45–7.4 nM) as shown in Table 2. However, two C1 peptides (δ-C1A and θ-C1A) bound PDBu over 100-fold less tightly. To identify the residues which contribute to this affinity difference, several mutants of these C1 peptides were synthesized on the basis of the sequence analysis. We focused at first on the 16V residue of these peptides since other C1 peptides with potent PDBu binding affinity have histidine at position 16. The binding affinity of both the V16H mutants of δ-C1A and θ-C1A (16H-δ-C1A and 16H-θ-C1A), however, did not increase significantly compared with the original peptides (Table 3). We examined next the 9G and 9P residues of these two peptides which are close to the putative PDBu-binding domain,[27] since glycine and proline residues influence the conformation of the main chain differently from other amino acid residues. These residues were mutated to lysine because many C1 peptides have lysine at position 9. As shown in Table 3, both the G9K mutant of δ-C1A and the P9K mutant of θ-C1A showed K_d's of 2–3 nM, indicating that the residues at position 9 play an important role in the PDBu binding of δ-C1A and θ-C1A. PKCδ has a tumor-suppressor role in PKCδ-depleting cells[11] and in the skin of transgenic mice overexpressing PKCδ.[12] Bögi et al.[28] investigated the translocation of PKCδ based on selectively mutated C1A or C1B domains. All tumor promoters studied depended selectively on the C1B domain of PKCδ to cause enzyme translocation to the membrane fractions, whereas non-tumor-promoting bryostatin 1[29] depended on both C1A and C1B domains. Wang et al.[30] have recently reported the differential localization of PKCδ by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and bryostatin 1 using a fusion protein with the green fluorescent protein. TPA induced plasma membrane translocation of PKCδ followed by slower nuclear membrane translocation. In contrast to TPA, bryostatin 1 induced almost exclusively nuclear membrane translocation, suggesting the possibility that the binding selectivity to each C1A and C1B domain of PKCδ controls the translocation of PKCδ. The present results provide a useful procedure to determine the role of each C1 domain of PKCδ in translocation by point mutation.

Only α-C1B showed weak PDBu binding among the C1B peptides of all PKC isozymes. The sequences of α-C1B and β-C1B resemble each other and differ only in the four amino acid residues at positions 9, 36, 42, and 45. We reasoned that 9G and/or 42Q residues might be responsible for weak PDBu binding affinity of α-C1B on the basis of binding data of 9K-δ-C1A and conservation of the basic amino acid residue at position 42 of other C1 peptides. 9S-α-C1B and 42R-α-C1B were thus synthesized and their PDBu binding was measured. Although the K_d value for 9S-α-C1B was almost equal to that for α-C1B, 42R-α-C1B showed a 10-fold stronger PDBu binding affinity along with high B_{max} value (39.9%), indicating the importance of the basic amino acid residue at position 42.

We formerly thought that one extra valine at position 24 unlike the other C1 peptides might interfere with the proper folding and PDBu binding of these peptides.[14] However, ϵ-C1A and η-C1A bound significantly to PDBu at 4 °C incubation (Table 2), indicating that the additional valine residue makes little influence on the PDBu binding of these peptides. This fact is reasonable since the 24th residue is suggested to be in the loop which is not involved in forming the tertiary structure of the C1 peptides.[24,27] However, the PDBu binding affinity of ϵ-C1A and η-C1A was one order of magnitude weaker than that of the corresponding C1B peptides. To investigate the effect of the 24th valine residue on the PDBu binding, two mutants without this valine residue (24-del-ϵ-C1A and 24-del-η-C1A) were synthesized. The

K_d values of these mutants were similar to those of the original peptides, suggesting that existence of the additional valine residue is not the main reason for weak PDBu binding affinity of ε-C1A and η-C1A.

Conclusion

In summary, we synthesized the C1A and C1B peptides (about 50–70 amino acids) of all conventional and novel PKC isozymes by the solid-phase Fmoc strategy and examined their PDBu binding properties. The current study found that incubation temperature at 30 °C in the binding assay significantly decreases the specific PDBu binding of some C1 peptides. This temperature dependent inactivation was shown to be sequence specific and occurs even with the GST fusion proteins of the PKC C1 domains prepared by DNA recombination techniques. We determined the dissociation constants (K_d) of [^{3}H]PDBu for all PKC C1 peptides except for θ-C1A at 4 °C incubation, enabling the measurement of the binding constants (K_i) of PKC activators other than PDBu for each PKC C1 domain. The PKC C1 peptides are now established as an effective screen for identifying new compounds that show PKC isozyme selective binding and are especially useful for identifying C1 domain selective binding agents for individual isozymes. In addition to their ready availability, these PKC surrogate binding domains offer greater purity relative to recombinant proteins and as a result allow for greater reproducibility from batch to batch preparation. The ready synthesis of these and related peptides allowed also for identification of the C1 peptide residues that play a significant role in PDBu binding. These data are valuable not only for estimating the binding affinity of a variety of C1 domains like diacylglycerol kinases other than PKC but also for determining the role of each PKC C1 domain by point mutation.

Experimental

General methods and materials. The following spectroscopic and analytical instruments were used: matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), PerSeptive Biosystems Voyager-DE™ STR (20 kV) Peptide synthesizer, PerSeptive Biosystems Model 9030 (Pioneer™ Peptide Synthesizer); HPLC, Waters Model 600E with Model 484 UV detector. MALDI-TOF-MS was measured as follows: each C1 peptide dissolved in 0.1% trifluoroacetic acid (TFA) aqueous solution (50 pmol/μL) was mixed with saturated α-cyano-4-hydroxy-cinnamic 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 PROTEIN-RP (C4, 20 mm i.d. ×150 mm) columns for preparative purpose (Yamamura Chemical Laboratory). [^{3}H]PDBu (20.0 Ci/mmol) was purchased from NEN Research Products. GST-γ-C1A (26–90) and GST-γ-C1B (91–172) were prepared by the method of Quest and Bell.[21] Human recombinant PKCγ was purchased from Biomol Research Laboratories Inc. Unless otherwise noted, reagents were obtained from Sigma, Allexis, or Wako Pure Chemical Industries.

Synthesis of the C1 peptides of all conventional and novel PKC isozymes. The 51- or 52-mer C1 peptides were prepared and identified as reported previously.[14] The 72-mer C1 peptides (α-C1A-long, α-C1B-long, β-C1A-long, γ-C1A-long, and γ-C1B-long) were synthesized in a stepwise fashion on 0.2 mmol of preloaded Fmoc-Gly-PEG-PS resin (PerSeptive Biosystems) by Pioneer™ Peptide Synthesizer using the Fmoc method as previously reported[14] with a slight modification. The 51- or 52-mer C1 mutants were similarly synthesized on 0.1 mmol of the Fmoc-Gly-PEG-PS resin. 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 *N,N*-dimethylformamide (DMF) for 5 min (flow rate: 5.0 mL/min). For 0.1 mmol scale synthesis, the coupling reaction was carried out using each Fmoc amino acid (0.4 mmol), HATU[31] (0.4 mmol), and *N,N*-diisopropylethylamine (DIPEA, 1.0 mmol) in DMF for 30 min (flow rate: 30 mL/min). Each Fmoc amino acid (0.4 mmol) weighed in a test tube was dissolved in 1.9 mL of the reagent solution (0.21 M HATU and 0.52 M DIPEA in DMF). The solution 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 0.21 M.

After completion of the chain assembly, each peptide-resin (about 1.5 g) was treated with a cocktail containing TFA, *m*-cresol, ethanedithiol, and thioanisole (16, 0.4, 2.4, 1.2 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 (about 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 (about 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 PROTEIN-RP column with elution at 8 mL/min by a 120-min linear gradient of 25–50% CH_3CN in 0.1% TFA, followed by using the SH-342-5 column in the same condition. 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%). Each purified peptide

exhibited satisfactory mass spectrometric data. The yields and mass data of the C1 peptides synthesized this time are summarized in Table 4.

[^{3}H]PDBu binding assay of the PKC C1 peptides. The PDBu binding to the PKC C1 peptides was evaluated using the procedure of Sharkey and Blumberg.[18] The standard assay mixture (250 μL) in a 1.5 mL of Eppendorf tube contained 50 mM Tris-maleate (pH 7.4), 50 μg/mL 1,2-di-(*cis*-9-octadecenoyl)-*sn*-glycero-3-phospho-L-serine, 4 mg/mL bovine γ-globulin, [^{3}H]PDBu (20.0 Ci/mmol), and each PKC C1 peptide (10–20 nM). For determination of PDBu saturation curves for Scatchard analysis, concentrations of free [^{3}H]PDBu between 1 and 50 nM were used. Phosphatidylserine was suspended in 50 mM Tris-maleate (pH 7.4) by sonication (1 min) and added to the above reaction mixture.

Metal coordination was carried out in helium-purged distilled water solution (pH 5.5–6.0) of each C1 peptide (190 μM). Five mol equiv of 10 mM $ZnCl_2$ in helium-purged distilled water were added to the peptide solution, and the solution (174 μM) was allowed to stand at 4 °C for 10 min. After 5 or 10 μL of the peptide solution was diluted, respectively, with 995 or 990 μL helium-purged distilled water, an aliquot of the peptide solution (2.9 μL) was added to the standard assay mixture (247.1 μL), and the solution was incubated at 4 °C for 20 min. To the tubes, 187 μL of 35% (w/w) poly-(ethyleneglycol) (average molecular weight, 8000) was added, and the mixture was vigorously stirred. The tubes were incubated at 4 °C for 20 min and centrifuged for 10 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. The nonspecific binding for each tube was calculated from its measured free [^{3}H]PDBu concentration and its partition coefficient to the pellet (about 3%).

Table 4. Yields and MALDI-TOF-MS data of the C1 peptides synthesized in this study

C1 peptides	Yield (%)	Obsd mass	Cald mass (MH^+)
α-C1A-long[a]	3.1	8329.56	8328.54
α-C1B-long[b]	3.1	8033.40	8032.24
β-C1A-long[c]	2.3	8258.49	8258.08
γ-C1A-long[d]	4.3	8216.24	8215.40
γ-C1B-long[e]	5.0	8159.50	8159.32
16H-δ-C1A	3.6	5966.38	5966.02
9K-δ-C1A	4.1	5998.71	5999.13
16H-θ-C1A	2.5	5974.32	5974.94
9K-θ-C1A	6.0	5967.97	5967.05
9S-α-C1B	2.6	5713.15	5712.67
42R-α-C1B	2.8	5710.67	5710.74
24-del-ε-C1A	8.1	6064.16	6063.17
24-del-η-C1A	3.1	5978.48	5978.74

[a]Mouse PKCα 27–98.
[b]Mouse PKCα 92–162.
[c]Mouse PKCβ 27–98.
[d]Mouse PKCγ 26–97.
[e]Mouse PKCγ 91–161.

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