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Bioorganic & Medicinal Chemistry 12 (2004) 4575–4583
Bioorganic & Medicinal Chemistry

Tumor promoter binding of the protein kinase C C1 homology domain peptides of RasGRPs, chimaerins, and Unc13s

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Received 21 June 2004; revised 5 July 2004; accepted 5 July 2004

Abstract—Recent investigations discovered nonkinase-type phorbol ester receptors, RasGRPs, chimaerins, and Unc13s. Phorbol ester binding occurs at the cysteine-rich sequences of about 50 residues in the C1 domains of these receptors. Fifty-one-residue RasGRP C1 peptides except for RasGRP2 showed significant phorbol 12,13-dibutyrate (PDBu) binding, but the K_d values of the RasGRP1 and RasGRP3 C1 peptides were about 10-fold larger than those for the corresponding whole enzymes. Addition of the C-terminal basic amino acid cluster decreased their K_d values about 10-fold, suggesting that the positive charges of these C1 peptides play an important role in the PDBu binding in the presence of negatively-charged phosphatidylserine. The 51-mer chimaerin C1 peptides showed potent PDBu binding, while the Unc13 and Munc13-1 C1 peptides without sufficient positive charges hardly bound PDBu. By the rapid screening system using this C1 peptide library, 5-prenyl-indolactam-V was identified as a promising lead for the novel protein kinase C isozyme specific ligands.

1. Introduction

Protein kinase C (PKC) isozymes are major receptors of tumor-promoting phorbol esters and also play a pivotal role in cellular signal transduction via the second messenger, 1,2-diacyl-*sn*-glycerol (DG).[1,2] PKC isozymes that bind phorbol esters[3] are classified into two classes: conventional PKCs (α, βI, βII, γ) which are calcium-dependent, and novel PKCs (δ, ε, η, θ) without a calcium binding domain. They consist of a regulatory region with phorbol ester binding domains designated as C1A and C1B,[4] and a catalytic region for protein phosphorylation (Fig. 1). Phorbol esters bind to the cysteine-rich sequences of about 50 amino acid residues [$HX_{12}CX_2CX_{13\text{-}14}CX_2CX_4HX_2CX_7C$ (H: histidine, C: cysteine, X: any amino acid residue)] in the presence of phospholipids, especially acidic phospholipids like phosphatidylserine.[5] The recently identified PKCμ, which is known as protein kinase D (PKD), also has two C1 domains with potent phorbol ester binding ability.[6]

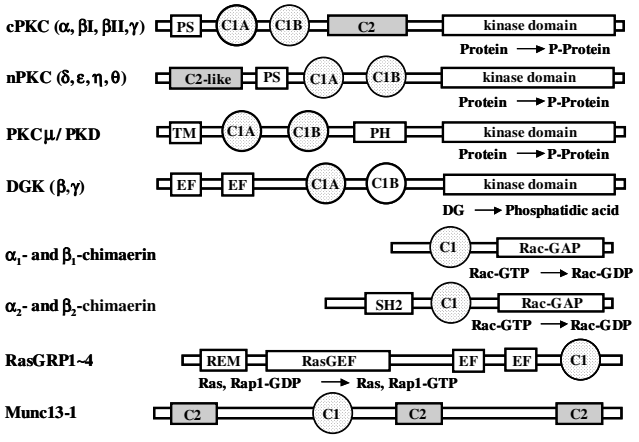

Figure 1. Structure of phorbol ester receptors. cPKC, conventional protein kinase C; nPKC, novel protein kinase C; PKD, protein kinase D; DGK, diacylglycerol kinase; PS, pseudosubstrate; EF and C2, Ca^{2+}-binding domain; C1A, C1B, and C1, cysteine-rich domain (zinc-finger motif); PH, pleckstrin homology domain; Rac-GAP, Rac-GTPase activating protein; SH-2, Src-homology domain 2; REM, Ras exchange motif; RasGEF, Ras guanine nucleotide exchange factor. The C1B domains of DGKβ and -γ do not bind phorbol esters.[9]

Keywords: Phorbol ester; Protein kinase C; RasGRP; Chimaerin; Unc13.
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doi:10.1016/j.bmc.2004.07.008

It has long been believed that PKC isozymes are the sole receptors of phorbol esters. Although many proteins have PKC C1 homology domains, most C1 domains lack the phorbol ester binding ability, as observed in PKCζ, Raf-1, and Vav.[4] However, the discovery that *n*-chimaerin (α_1-chimaerin), with a PKC C1 homology domain, showed potent phorbol ester binding affinity became a turning point.[7] Chimaerins consisting of four isoforms, α_1, α_2, β_1, and β_2, have a single C1 domain and a GTPase activating protein (GAP) domain that promotes GTP hydrolysis from the small GTPase Rac, resulting in Rac inactivation (Fig. 1).

The next identified phorbol ester receptors were Unc13 of *C. elegans* and its mammalian homologs, Munc13-1, 13-2, and 13-3.[7] Munc13s act as scaffolding proteins that interact with elements of the exocytotic machinery. More recently, Ras guanyl nucleotide-releasing proteins (RasGRPs) were identified as new phorbol ester receptors.[7] RasGRPs are nucleotide exchange factors for Ras, leading to the activation of the downstream Raf-extracellular signal-regulated kinase cascade. Among the four RasGRPs, the PDBu binding affinities of RasGRP1 and RasGRP3 have been determined, while those of RasGRP2 and RasGRP4 remain unknown.[7] Furthermore, our own approach using synthetic C1 domain peptides to identifying new phorbol ester receptors led to the identification of diacylglycerol kinase (DGK) γ and β.[8,9] DGK phosphorylates DG to produce phosphatidic acid, whereas PKC is allosterically activated by DG. DGK may thus inhibit the activation of PKC by attenuating DG levels.

In order to understand the mechanism of tumor promotion by phorbol esters, these nonPKC-type phorbol ester receptors as well as PKCs should be taken into account. The design of agents that can discriminate PKC isozymes from the nonPKC-type phorbol ester receptors is urgently needed to reach this goal. In experiments using whole enzymes, however, there are several factors that abolish the PDBu binding ability (instability of the enzyme, folding problems, and post-transcriptional modifications). In fact, we experienced that rat-DGKγ expressed in the *E. coli* system did not show any detectable PDBu binding.[8,9] To circumvent this problem, we synthesized the cysteine-rich sequences of all PKC isozyme C1 domains (PKC C1 peptides) for high-throughput screening of new ligands with binding selectivity at each C1 domain.[10–12] This peptide library has enabled us to show that benzolactam and indolactam compounds could be new medicinal leads, with binding selectivity for C1B domains of novel PKC isozymes.[13,14] To identify new compounds with high selectivity for each C1 domain of PKCs and the nonPKC-type receptors, the C1 peptides of RasGRPs, chimaerins, and Unc13s have been synthesized. This paper describes the establishment of new C1 peptide library of nonkinase-type phorbol ester receptors and discusses the relationship between their K_d values in PDBu binding and their sequences. The identification of a ligand that discriminates novel PKC C1B domains from nonPKC-type phorbol ester receptors by using this new C1 peptide library is also mentioned.

2. Results

2.1. Synthesis and PDBu binding of RasGRP C1 peptides

Each PKC C1 domain has a typical core structure of 50 or 51 amino acid residues, $HX_{12}CX_2CX_{13-14}CX_2CX_4$-$HX_2CX_7C$ (X: any amino acid residue), that coordinates two zinc atoms in a tetrahedral geometry (Fig. 2).[15,16] Since this sequence is the minimum structure for potent PDBu binding,[17,18] the 51-mer C1 peptides of human RasGRP1∼4 were synthesized (Fig. 3 and Table 1) and their K_d values in PDBu binding were measured. Potent PDBu binding was detected in the 51-mer peptides of RasGRP1, RasGRP3, and RasGRP4, and subsequent Scatchard analysis determined their K_d values as 5.11, 11.5, and 1.20 nM, respectively (Table 2). The K_d values (5.11 and 11.5 nM) of RasGRP1 and RasGRP3 were about 10-fold larger than those of the whole enzymes (0.58 and 1.53 nM),[19,20] respectively. Since a recent investigation suggested that the basic amino acid residues in the C1 domain of murine RasGRP (KKRIK) play a critical role in the transforming ability of NIH 3T3 cells,[21] a similar basic amino acid sequence (KKR or KKRAK) in human RasGRP1 was added to its C-terminus. The Scatchard analysis showed that addition of the basic cluster significantly decreased the K_d values of these peptides with a drastic B_{max} increase (Fig. 4b and c). The K_d value of the 56-mer RasGRP1 C1 peptide (0.72 nM) was almost equal to that of whole RasGRP1 (0.58 nM).[19] However, further elongation of both N and C-terminal residues hardly changed the K_d value (Fig. 4d).

Similar results were obtained for the 56-mer C1 peptide of RasGRP3, whose K_d value (1.52 nM) in PDBu binding was equal to that of whole RasGRP3 (1.53 nM).[20] However, the C-terminal basic cluster of RasGRP4 C1

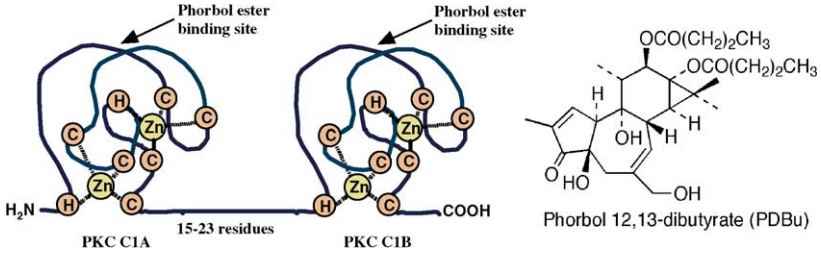

Figure 2. Structure of PKC C1 domains and phorbol 12,13-dibutyrate (PDBu).

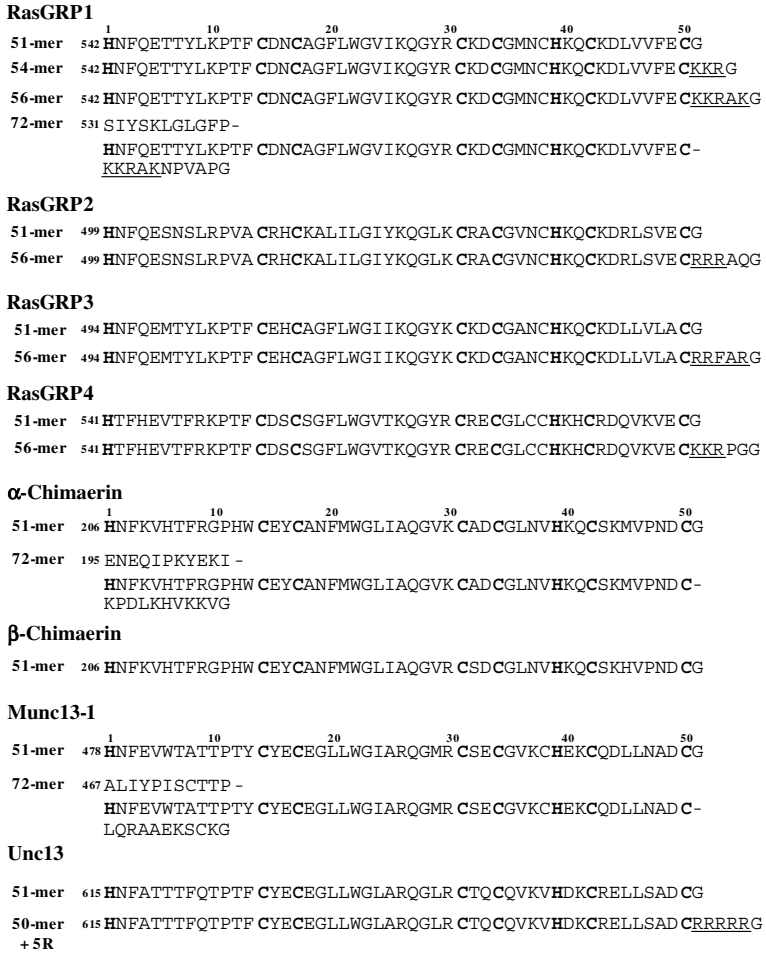

Figure 3. Sequences of the C1 peptides synthesized in this study. All of the C1 sequences were derived from humans. All sequences except for Unc13 50-mer + 5R are native. The basic amino acid cluster is underlined. To prevent racemization and oxidation during synthesis, the carboxyl terminus was extended in each peptide from the final residue to a glycine.

peptide hardly decreased the K_d value of the 51-mer C1 peptide of RasGRP4 (Table 2). RasGRP2 was completely inactive in the PDBu binding assay, even at the peptide and PDBu concentrations of 100 and 20 nM, respectively.

2.2. PDBu binding affinity of several C1 mutants of RasGRP isozymes

To reveal the mechanism of decrease in the K_d values of RasGRP1 and RasGRP3 C1 peptides by the C-terminal basic amino acid cluster, their 50-mer core structures were carefully compared with that of the RasGRP4 C1 peptide, in which addition of the basic cluster to the C-terminus of the 50-mer core structure did not change the K_d value significantly (Table 2). The comparison indicated that the number of basic amino acid residues within the 50-mer core-structure of the RasGRP4 C1 peptide is significantly larger than that of the C1 peptides of RasGRP1 and RasGRP3. To compare the basic amino acids roughly, the 'net charge' of the C1 peptides was defined as (number of arginines, lysines, and histidines)−(number of glutamic acids and aspartic acids), where the two histidines involved in the zinc folding were excluded in this calculation. The net positive charge of the 50-mer core-structure of the RasGRP4 C1 peptide (+5) is larger than that of the C1 peptides of RasGRP1 and RasGRP3 (+1 and +3), because His-4, His-41, and Lys-47 in RasGRP4 are replaced by Gln-4, Gln-41, and Val-47 in RasGRP1 and RasGRP3, respectively (Fig. 3). We focused on His-41 since our recent investigation on the PKC C1 peptides suggested the importance of the basic amino acid residue at position 41 in PDBu binding.[12] The 51-mer Q41H-RasGRP1 and Q41H-RasGRP3 C1 peptides were synthesized to confirm this speculation. As shown in Table 2, the K_d values of both C1 peptides decreased (1.79 and 2.69 nM), indicating that the replacement of His with Gln is one of the major reasons for the decrease in the PDBu binding affinity of the 51-mer RasGRP1 and RasGRP3 C1 peptides. In contrast, the 51-mer H41Q-RasGRP4 showed similar binding potency compared with the original 51-mer peptide.

The RasGRP2 C1 peptides (51- and 56-mer) did not show any PDBu binding affinity though they have sufficient positive charges (+7 and +10). Since recent investigations[9,12,22,23] suggested that aromatic amino acid residues at positions 8 and 13, and Thr-12 are necessary for PDBu binding, S8Y-, and V12T, A13F-RasGRP2

Table 1. Yields and MALDI-TOF-MS data of the C1 peptides synthesized in this study

C1 peptides[a]	Yield (%)	Obsd mass	Cald mass (MH^+)
RasGRP1 (51-mer)	4.8	5882.33	5881.93
RasGRP1 (54-mer)	18.4	6294.06	6294.46
RasGRP1 (56-mer)	13.9	6494.21	6493.71
RasGRP1 (72-mer)	8.1	8136.31	8135.67
Q41H-RasGRP1 (51-mer)	6.6	5890.65	5890.94
RasGRP2 (51-mer)	11.6	5718.22	5717.85
RasGRP2 (56-mer)	17.1	6385.01	6385.61
S8Y-RasGRP2 (56-mer)	10.8	6461.32	6461.71
V12T, A13F-RasGRP2 (51-mer)	5.7	5794.77	5795.90
R15D, K18A-RasGRP2 (56-mer)	8.6	6287.43	6287.41
RasGRP3 (51-mer)	15.3	5797.65	5796.95
RasGRP3 (56-mer)	13.4	6483.63	6483.75
Q41H-RasGRP3 (51-mer)	6.5	5805.01	5805.95
RasGRP4 (51-mer)	15.3	5925.84	5925.95
RasGRP4 (56-mer)	13.4	6492.32	6492.65
H41Q-RasGRP4 (51-mer)	5.1	5916.50	5916.94
α-Chimaerin (51-mer)	4.8	5784.83	5783.84
α-Chimaerin (72-mer)	3.2	8330.12	8329.89
β-Chimaerin (51-mer)	14.4	5834.13	5833.80
Unc13 (51-mer)	4.4	5780.80	5781.70
Uuc13 (50-mer + 5R)	3.1	6562.73	6562.63
Munc13-1 (51-mer)	6.7	5772.99	5772.65
Munc13-1 (72-mer)	1.8	8049.43	8048.38

[a] Sequences of these C1 peptides are shown in Figure 3. To prevent racemization and oxidation during synthesis, glycine was added to the carboxyl terminus in each peptide. Since the MALDI-TOF-MS was measured under the acidic condition, the molecular masses without zinc ions were observed. In the binding assay of tumor promoters, zinc coordination was carried out in a helium-purged distilled water solution of each C1 peptide as described in the Experimental section.

Table 2. K_d values for PDBu binding of various C1 peptides synthesized in this study

C1 peptides	K_d (nM)	B_{max} (%)	Net charge[a]
RasGRP1 (51-mer)	5.11 (0.085)[b]	18.9 (2.4)	+1
RasGRP1 (54-mer)	1.43 (0.12)	28.5 (1.1)	+4
RasGRP1 (56-mer)	0.72 (0.21)	30.6 (1.7)	+5
RasGRP1 (72-mer)	0.68 (0.01)	25.2 (1.4)	+6
Q41H-RasGRP1 (51-mer)	1.79 (0.63)	29.1 (8.4)	+2
Whole RasGRP1	0.58[19]		
RasGRP2 (51-mer)	No binding		+7
RasGRP2 (56-mer)	No binding		+10
S8Y-RasGRP2 (56-mer)	No binding		+10
V12T, A13F-RasGRP2 (51-mer)	No binding		+7
R15D, K18A-RasGRP2 (56-mer)	No binding		+7
Whole RasGRP2	Not determined		
RasGRP3 (51-mer)	11.5 (0.43)	18.6 (2.4)	+3
RasGRP3 (56-mer)	1.52 (0.37)	16.2 (4.8)	+6
Q41H-RasGRP3 (51-mer)	2.69 (0.60)	12.1 (4.1)	+4
Whole RasGRP3	1.53[20]		
RasGRP4 (51-mer)	1.20 (0.16)	11.9 (1.3)	+5
RasGRP4 (56-mer)	1.06 (0.08)	16.0 (1.2)	+8
H41Q-RasGRP4 (51-mer)	1.35 (0.37)	13.3 (1.5)	+4
Whole RasGRP4	Not determined		
α-Chimaerin (51-mer)	4.83 (0.19)	30.3 (4.7)	+4
α-Chimaerin (72-mer)	1.79 (0.07)	13.9 (1.0)	+7
Whole α_1-chimaerin	0.17[25] or 29[26]		
β-Chimaerin (51-mer)	4.49 (0.09)	19.2 (0.6)	+5
Whole β_2-chimaerin	1.9[24]		
Unc13 (51-mer)	Little binding		±0
Unc13 (50-mer + 5R)	3.05 (0.17)	17.2 (4.3)	+5
Whole Unc13	1.1[27]		
Munc13-1 (51-mer)	Little binding		−3
Munc13-1 (72-mer)	Little binding		−1

[a] Net charge = (number of Arg, Lys, and His) − (number of Asp and Glu). Two histidines involved in the zinc-folding are excluded.
[b] Standard deviation.

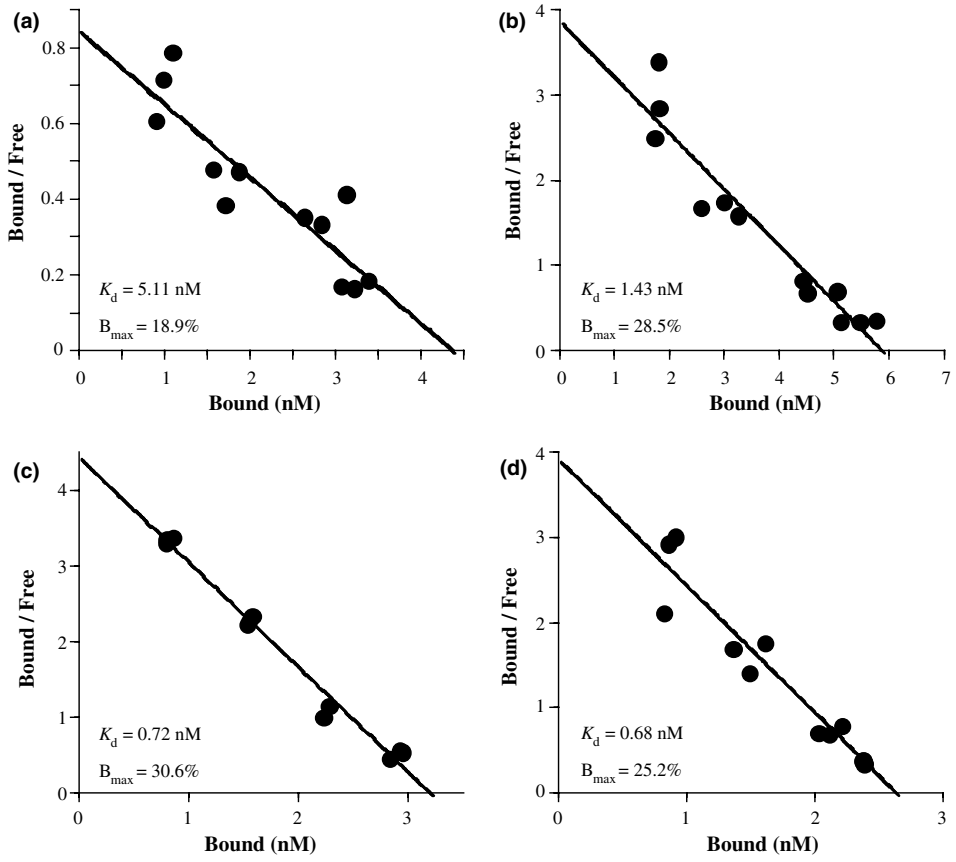

Figure 4. Scatchard analyses of [^{3}H]PDBu binding to the RasGRP1 C1 peptides. (a) RasGRP1 (51-mer), (b) RasGRP1 (54-mer), (c) RasGRP1 (56-mer), (d) RasGRP1 (72-mer). In the Scatchard analyses, each representative experiment is shown. Similar results were obtained in other experiments.

(56- and 51-mer, respectively) were synthesized. R15D, K18A-RasGRP2 (56-mer) was also synthesized, as these basic amino acid residues are not contained in the C1 peptides of other RasGRP isozymes with potent PDBu binding affinity. However, these three mutants did not bind PDBu at all.

2.3. Synthesis and PDBu binding of chimaerin C1 peptides

51-mer C1 peptides of human α-chimaerin and β-chimaerin were synthesized to determine their K_d values in PDBu binding (Fig. 3). Because the cysteine-rich sequence in the C1 domains of α_1- and α_2-chimaerin (or β_1- and β_2-chimaerin) is the same, the C1 peptides of the chimaerins were named α-chimaerin and β-chimaerin. The K_d values for PDBu of 51-mer α-chimaerin and β-chimaerin were 4.83 and 4.49 nM, respectively (Table 2). The K_d value of β-chimaerin C1 peptide (4.49 nM) was similar to that of whole β-chimaerin (1.9 nM).[24] However, there was a marked difference between the K_d values of α-chimaerin C1 peptide (4.83 nM) and whole α_1-chimaerin (0.17 nM) reported by the Blumberg's group.[25] Since elongation of both the N- and C-termini of the core cysteine-rich sequence of 50 amino acid residues sometimes decreases the K_d value, as observed for RasGRP1 and RasGRP3, the 72-mer C1 peptide of α-chimaerin (Fig. 3) was synthesized. The Scatchard analysis gave a K_d of 1.79 nM, suggesting that this elongation is not effective for increasing the PDBu binding affinity. On the other hand, Ahmed et al.[26] reported another K_d value for α_1-chimaerin (29 nM). The large variation in the K_d values is one of the major defects of using whole enzymes for rapid evaluation of the PDBu binding potency of ligands. The K_d value of our α-chimaerin C1 peptide was between the values reported by the two groups.

2.4. Synthesis and PDBu binding of Unc13 and Munc13-1 C1 peptides

Finally, the 51-mer peptides containing the cysteine-rich sequence of *C. elegans* Unc13 and human Munc13 were synthesized (Fig. 3). Unlike the C1 peptides described above, amount of the specific PDBu binding of Unc13 C1 peptide was extremely low. Since Munc13-1 C1 peptide was hardly soluble in water, a corresponding 72-mer C1 peptide was synthesized to improve its solubility. This peptide was soluble in water as expected, but amount of specific PDBu binding was too low to determine the K_d value for PDBu.

The extremely low amount of specific PDBu binding of the 51-mer Unc13 and the 72-mer Munc13 C1 peptides was deduced to be ascribable to a relatively large number of acidic amino acid residues in its cysteine-rich

region. The net charges of the Unc13 and Munc13 C1 peptides are ±0 and −1, respectively, while those of the C1 peptides of all PKC isozymes (51–53-mer) are over +4. Since addition of the basic amino acid cluster to the C-terminus of RasGRP1 and RasGRP3 peptides increased their PDBu binding amount, five arginine residues were added to the C-terminus of the Unc13 C1 peptide (51-mer). The specific PDBu binding of the 56-mer peptide [Unc13 (50-mer + 5R)] increased drastically as observed for the large B_{max} value of 17.2%. The K_d of this peptide (3.05 nM) was almost equal to that of whole Unc13 (1.1 nM).[27]

2.5. Identification of a new agent with significant binding selectivity for novel PKC isozymes using the C1 peptide library

Recent investigations revealed that PKCδ has a tumor-suppressor role using transgenic mice.[28] The involvement of PKCε and -η in tumor promotion has also been suggested using transgenic and knockout mice, respectively.[29,30] To understand the mechanism of tumor promotion at a molecular level, the development of new agents with novel PKC isozyme selectivity is urgently needed. Our recent effort to find new ligands with novel PKC isozyme selectivity led to the identification of 5-prenyl-indolactam-V (Fig. 5), a *trans*-amide restricted analogue of indolactam-V, as reported in the preliminary communication.[31] Indolactam-V existing as two stable conformers at room temperature (Fig. 5), the *cis*-amide (twist) and the *trans*-amide (sofa) conformers,[32,33] could not discriminate novel PKCs from conventional PKCs (Table 3).

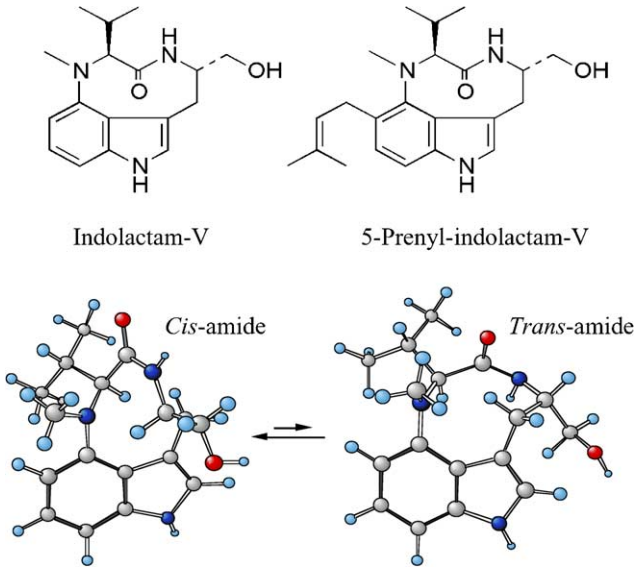

Figure 5. Structure of indolactam-V and 5-prenyl-indolactam-V along with the two stable conformations of indolactam-V. The *cis*-amide conformation is slightly more stable than the *trans*-amide conformation (ΔG^0 at 25 °C = ca. 1.0 kcal/mol).[32]

The establishment of the C1 peptide library containing all of the PKC C1 homology domain peptides enabled us to examine whether 5-prenyl-indolactam-V can discriminate novel PKC isozymes from new phorbol ester receptors other than PKC. The dissociation constants of 5-prenyl-indolactam-V for all C1 peptides (K_i), determined by inhibition of specific [^{3}H]PDBu binding, are summarized in Table 3. Indolactam-V as well as PDBu

Table 3. Selectivity in the C1 peptide binding of indolactam-V and 5-prenyl-indolactam-V

C1 peptides	Indolactam-V [K_i (nM)]	5-Prenyl-indolactam-V [K_i (nM)]	PDBu [K_d (nM)]
PKCα-C1A (72-mer)[a]	20.8 (1.2)[b]	5970 (730)	1.1
PKCα-C1B (72-mer)	4000 (870)	11,300 (900)	5.3
PKCβ-C1A (72-mer)	18.9 (4.5)	8160 (830)	1.3
PKCβ-C1B (51-mer)	136 (4.4)	321 (25)	1.3
PKCγ-C1A (52-mer)	138 (13.5)	11,800 (2400)	1.5
PKCγ-C1B (51-mer)	213 (5.0)	792 (142)	1.2
PKCδ-C1A (52-mer)	1900 (190)	22,800 (4300)	51.9
PKCδ-C1B (51-mer)	8.3 (1.1)	21.0 (4.4)	0.53
PKCε-C1A (53-mer)	4110 (50)	14,800 (3,100)	5.6
PKCε-C1B (51-mer)	7.7 (1.2)	12.0 (2.4)	0.81
PKCη-C1A (52-mer)	3770 (480)	8600 (1400)	4.3
PKCη-C1B (51-mer)	5.5 (0.6)	6.2 (0.4)	0.45
PKCθ-C1B (51-mer)	8.7 (1.2)	26.3 (2.2)	0.72
PKD-C1A (52-mer)	52.5 (5.0)	552 (130)	2.5
PKD-C1B (51-mer)	213 (32)	1900 (41)	2.7
DGKβ-C1A (51-mer)	1930 (150)	8890 (540)	14.6
DGKγ-C1A (51-mer)	576 (46)	1270 (40)	2.8
α-Chimaerin (72-mer)	43.6 (3.8)	6420 (760)	1.8
β-Chimaerin (51-mer)	119 (12)	9540 (570)	4.5
RasGRP1 (56-mer)	12.9 (2.9)	3350 (300)	0.72
RasGRP3 (56-mer)	53.6 (4.9)	4340 (780)	1.5
RasGRP4 (56-mer)	8.4 (1.5)	2060 (430)	1.1
Unc13 (50-mer + 5R)[c]	59.9 (17)	450 (120)	3.1

[a] PKC C1 peptides were synthesized as reported previously.[10,12] Sequences of nonPKC-type C1 peptides are shown in Figure 3. DGK and PKD C1 peptides were synthesized as reported previously.[8,9]

[b] Standard deviation.

[c] Since the C-terminal five arginine residues do not exist in the native Unc13, the results may not reflect the binding affinity to whole Unc13.

did not discriminate PKC isozymes from nonPKC-type phorbol ester receptors. However, the binding affinity of 5-prenyl-indolactam-V for C1 peptides of chimaerins and RasGRPs was far less than that of indolactam-V (K_i >1 μM). 5-Prenyl-indolactam-V bound potently only to novel PKC C1B peptides with a K_i of 6.2–26.3 nM.

3. Discussion

The discovery of new phorbol ester receptors other than PKCs (Fig. 1) suggests complexity in the mechanism of tumor promotion elicited by phorbol esters. Since all new phorbol ester receptors have PKC C1 homology domains, various C1 peptides containing the cysteine-rich sequences of 50 amino acid residues were synthesized to establish a rapid screening system for developing new medicinal leads with high selectivity for each receptor.

Our previous research indicated that many PKC C1 peptides with 51 or 52 amino acid residues bound PDBu with K_d values quite similar to those of whole PKC isozymes.[12] The 51-mer α-chimaerin and β-chimaerin C1 peptides showed substantial PDBu binding ability (K_d = 4.84 and 4.49 nM, respectively) comparable to corresponding whole chimaerins (K_d = 0.19 or 29 nM for whole α_1-chimaerin[25,26] and 1.9 nM for β_1-chimaerin[24]), suggesting that these peptides become surrogates of the whole chimaerins. In contrast, the K_d values of the 51-mer C1 peptides of RasGRP1 and RasGRP3 (5.11 and 11.5 nM, respectively) were significantly larger than those for whole RasGRP1 and RasGRP3 (0.58 and 1.53 nM, respectively).[19,20] In this case, the 56-mer peptides with the basic amino acid cluster at their C-termini (K_d = 0.72 and 1.52 nM, respectively) are suitable surrogates of whole RasGRP1 and RasGRP3. Although the K_d of whole RasGRP4 has not been reported, addition of several basic amino acid residues to the C-terminus did not change the K_d of RasGRP4 C1 peptide (51-mer), suggesting that both the 51-mer and 56-mer RasGRP4 C1 peptides are surrogates of whole RasGRP4. Since Unc13 and Munc13-1 C1 peptides did not show sufficient PDBu binding, it is difficult to use these peptides for screening.

The lack of PDBu binding ability of RasGRP2 C1 peptides (51- and 56-mer) was an unexpected result, since many amino acid residues critical for PDBu binding were retained.[7] Mutation experiments on RasGRP2 C1 peptides could not identify the residues responsible for abolishing the PDBu binding ability. However, the present result seems to be reasonable since DG, the endogenous ligand of PKC, did not cause any detectable change in the subcellular localization of Ras-GRP2.[34]

Our previous research on PKC C1 peptides indicated that elongation of the cysteine-rich sequence at both the N- and C-termini did not change the K_d values significantly.[12] In contrast, the C-terminal elongation of RasGRP1 and RasGRP3 C1 peptides (51-mer) decreased their K_d values while that of RasGRP4 (51-mer) did not. To interpret this phenomenon, we focused on the net positive charges of the C1 peptides (Table 2), since negatively-charged phosphatidylserine is involved in an ionic interaction with a positively-charged C1 peptide, to which PDBu binds. The weak PDBu binding ability of the 51-mer RasGRP1 and RasGRP3 C1 peptides can be explained by the shortage of the positive charges. It is interesting that the basic cluster of RasGRP4 does not contribute to the enhancement of PDBu binding, possibly because the 50-mer cysteine-rich sequence has a sufficient positive charge. All PKC C1 peptides (51–53-mer)[10,12] have also enough net positive charges over +4. The importance of positive charges in the C1 peptides was also supported by the fact that addition of five arginine residues to the C-terminus of the 50-mer cysteine-rich sequence of Unc-13 drastically enhanced the amount of PDBu binding. Although total positive charges of the C1 peptides are critical for potent PDBu binding in the presence of negatively charged phosphatidylserine, the position of the basic amino acid residues also seems to be important in some cases, since Q41H-RasGRP1 and Q41H-RasGRP3 C1 peptides showed K_d values similar to those of the corresponding 54-mer peptides. These results indicate that the 50-mer cysteine-rich sequence ($HX_{12}CX_2CX_{13}CX_2CX_4HX_2CX_7C$) is the minimum structure required for binding phorbol esters, but that additional basic amino acid residues are necessary when the net positive charge of this core structure is insufficient, as observed for the 51-mer C1 peptides of RasGRP1, RasGRP3, and Unc13.

The PKC C1 homology domain peptides established in this study allowed us to identify a compound that shows novel PKC isozyme selectivity. 5-Prenyl-indolactam-V, a *trans*-amide restricted analogue of indolactam-V, is one of the promising ligands with novel PKC isozyme C1B domain selectivity. This compound bound to novel PKC C1B peptides with K_i values of 6.2–26.3 nM, while the binding potency for C1 peptides of conventional PKCs and other phorbol ester receptors was very weak. This is the first example of discrimination of novel PKCs from nonPKC-type phorbol ester receptors. Although fixation to the *trans*-amide conformation seems to be an effective approach for developing new medicinal leads with novel PKC isozyme selectivity, position of the prenyl group might also be involved in this selectivity as exemplified in the 1-hexyl-indolinelactam-V.[35] Further investigation on the effect of the substituents at various positions of the indole ring is indispensable.

In summary, we have established a C1 peptide library of nonPKC-type phorbol ester receptors as an effective screen for the identification of new ligands with selectivity for each receptor containing PKC C1 homology domains. Extensive analysis on the sequence and PDBu binding ability of RasGRP C1 peptides indicated that positive charges of the C1 peptides are critical for potent PDBu binding in the presence of negatively charged phosphatidylserine. To establish a C1 surrogate peptide of the whole enzyme, positive charge of the C1 peptide should also be considered. This newly established C1 peptide library, along with our previous PKC C1 peptides, allowed the identification of

5-prenyl-indolactam-V as a promising lead for novel PKC isozyme specific ligands, which are quite useful for understanding the mechanism of tumor promotion. The application of 5-prenyl-indolactam-V to the analysis of biological events through novel PKC isozymes is in progress, in collaboration with several laboratories.

4. Experimental

4.1. General procedures

The following spectroscopic and analytical instruments were used: matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), Applied Biosystems Voyager-DE™ STR (20 kV); peptide synthesizer, Pioneer™ peptide synthesizer Model 9030 (Applied Biosystems); HPLC, Waters Model 600E with Model 2487UV detector. MALDI-TOF-MS measurements were carried out as follows: each 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 at a 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 a YMC-packed SH-342-5 (ODS, 20 mm id×150 mm) column (Yamamura Chemical Laboratory) for preparative purposes. [^{3}H]PDBu (17.0 Ci/mmol) was purchased from Perkin-Elmer Life Science and American Radiolabeled Chemicals, Inc., USA. Indolactam-V and 5-prenyl-indolactam-V were prepared as reported previously.[36,37] *N*-[(Dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU),[38] piperidine, Fmoc amino acids, Fmoc-Gly-PEG-PS resin, and *N,N*-diisopropylethylamine (DIPEA) were purchased from Applied Biosystems. Unless otherwise noted, reagents were purchased from Sigma, Wako Pure Chemical Industries, or Nacalai Tesque.

4.2. Synthesis of the C1 peptides of RasGRPs, chimaerins, and Unc13s

The 51–72-mer peptides containing the cysteine-rich sequences of the C1 domains of RasGRPs, chimaerins, and Unc13s were synthesized in a stepwise fashion on 0.1 mmol of preloaded Fmoc-Gly-PEG-PS resin (Applied Biosystems) by a Pioneer™ using the Fmoc method as reported previously.[10–12] The purity of the C1 peptides was confirmed by HPLC (>98%). Each purified peptide exhibited satisfactory mass spectrometric data. The yields and mass data of the C1 peptides synthesized in this study are summarized in Table 1.

4.3. [^{3}H]PDBu binding assay of the C1 peptides of RasGRPs, chimaerins, and Munc13s

The PDBu binding assay was carried out using the procedure of Sharkey and Blumberg[39] as reported previously.[10–12] The standard assay mixture (250 μL), in 1.5 mL Eppendorf tubes, contained 50 mM Tris–maleate (pH 7.4), 50 μg/mL 1,2-di-(*cis*-9-octadecenoyl)-*sn*-glycero-3-phospho-L-serine, 3 mg/mL bovine γ-globulin, [^{3}H]PDBu (17.0 Ci/mmol), and each C1 peptide. For the determination of the PDBu saturation curves for Scatchard analysis, concentrations of free [^{3}H]PDBu between 1.25 and 40 nM were used.

Zinc coordination of the C1 peptides was carried out in a helium-purged distilled water solution (pH 5.5–6.0). Five mol equivalents 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 10 μL of the peptide solution were diluted with 990 μL of helium-purged distilled water, the resultant solution (2.9 μL) was added to the standard assay mixture described above (247.1 μL), and the solution was incubated at 4 °C for 10 min. To the tubes were added 187 μL of 35% (w/w) poly(ethyleneglycol) (average molecular weight, 8000), and the mixture was vigorously stirred. The tubes stood at 4 °C for 10 min, and then were 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%).

In competition experiments using the C1 peptides, various concentrations of an inhibitor in ethanol solution were added to the reaction mixture mentioned above. The effective concentrations of [^{3}H]PDBu and each C1 peptide were 20 and 5 nM, respectively. The final ethanol 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.[40] The dissociation constant of each ligand (K_i) was calculated from the IC_{50} values and K_d for PDBu of each C1 peptide by the method of Sharkey and Blumberg.[39]

Acknowledgements

This research was partly supported by a Grant-in-Aid for Scientific Research (A) (2) (No. 15208012) (H.O. and K.I.), for the Promotion of Science for Young Scientists (Y.N.) from the Ministry of Education, Science, Culture, Sports, Science, and Technology, of the Japanese Government.

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