Pergamon

Bioorganic & Medicinal Chemistry Letters 9 (1999) 2487–2490

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

SYNTHESIS AND PHORBOL ESTER-BINDING STUDIES OF THE INDIVIDUAL CYSTEINE-RICH MOTIFS OF PROTEIN KINASE D

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Received 31 May 1999; accepted 15 July 1999

Abstract: To investigate the phorbol ester-binding properties of the individual cysteine-rich motifs of protein kinase D (PKD), the 52-mer peptides containing each cysteine-rich motif of PKD (PKD-C1A, PKD-C1B) have been synthesized. The [^{3}H]phorbol-12,13-dibutyrate (PDBu) binding to PKD-C1A was affected drastically by incubation temperature while that to PKD-C1B was not. Scatchard analysis of [^{3}H]PDBu binding to both PKD C1 peptides gave dissociation constants of 2.5 ± 0.4 and 2.7 ± 0.8 nM for PKD-C1A and PKD-C1B, respectively, indicating that the two cysteine-rich motifs of PKD are functionally equivalent like those of PKCγ.

Protein kinase C (PKC) isozymes mediate cellular signal transduction *via* the second messenger diacylglycerol and are also major receptors for phorbol ester-type tumor promoters.[1-3] Because of their individual involvement in diverse biological activities, these isozymes serve as novel targets for the design of a variety of new chemotherapeutic agents.[4-6] Tumor-promoters like the phorbol esters activate PKC by binding to the cysteine-rich C1 domains[7,8] designated as C1A and C1B (Fig. 1).[1] We have recently synthesized the C1A and C1B domains of all known PKC isozymes by a solid-phase Fmoc strategy and have determined the relative affinities of these domains for phorbol-12,13-dibutyrate (PDBu).[9,10] Importantly, both C1 domains of PKCγ exhibited high affinity for PDBu binding, providing a structural blueprint for the development of PKCγ-selective modulators, compounds of potential significance for the treatment of neuropathic pain.[5]

The recently identified protein kinase D (PKD), which is also known as PKCμ,[11,12] and PKC, differ in their respective catalytic domains. However, both contain a tandem repeat of cysteine-rich motifs that binds to phorbol esters with high affinity.[11] In order to identify which of the subdomains is the phorbol ester binding site of PKD, we have synthesized the 52-mer peptides corresponding to C1A and C1B of PKD, respectively, and examined in detail their PDBu binding affinity. The results clearly demonstrated that C1A and C1B of PKD are functionally equivalent, a situation similar to that found for PKCγ.

PKD surrogate peptides (PKD-C1A and PKD-C1B) were synthesized with a PerSeptive Biosystems Model 9030 (Pioneer Peptide Synthesizer™) using HATU[13] as an activator for Fmoc chemistry by the previously reported method.[10] Briefly, Fmoc amino acids (0.8 mmol) activated by HATU (0.8 mmol) in the presence of *N*,*N*-diisopropylethylamine (0.95 mmol) in DMF were coupled in a stepwise fashion with preloaded Fmoc-Gly-PEG-PS resin (0.2 mmol). Piperidine (20%) in DMF was used for removal of the Fmoc groups.

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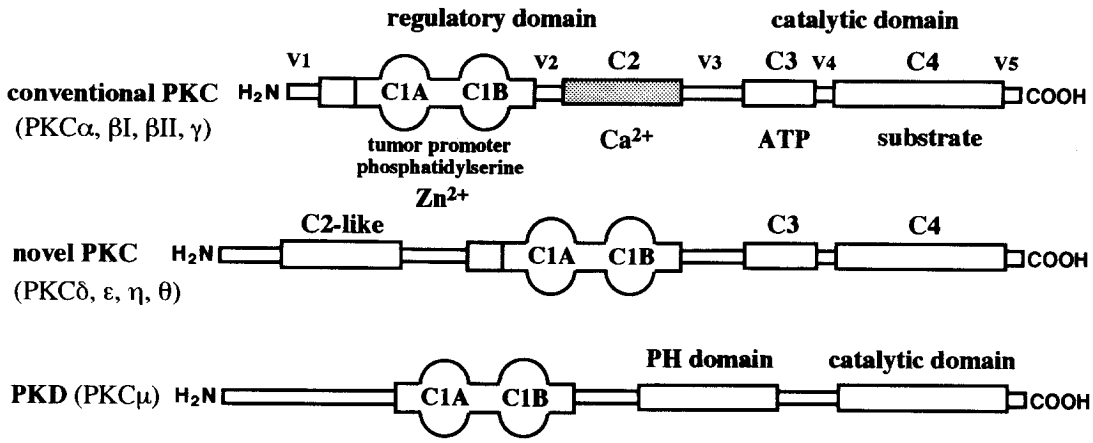

The 52-mer PKD surrogate peptides

PKD-C1A: H_2N-^{145}HALFVHSYRAPAFCDHCGEMLWGLVRQGLKCEGCGLNYHKRCAFKIPNNCS^{196}G-CO_2H

PKD-C1B: H_2N-^{277}HTFVIHSYTRPTVCQFCKKLLKGLFRQGLQCKDCRFNCHKRCAPKVPNNCL^{328}G-CO_2H

Figure 1. Structures of PKD and the 52-mer C1 peptides (PKD-C1A and PKD-C1B) along with conventional and novel PKC isozymes. PKD surrogate peptides derive from mouse PKD.[11]

After completion of the chain assembly, the 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 preparative C4 and C18 reversed phase columns to give PKD-C1A and PKD-C1B in 22% and 18% yield, respectively, which exhibited satisfactory mass spectral data by MALDI-TOF-MS: for PKD-C1A (MH^+, average molecular mass: obsd 5841.05, cald for $C_{256}H_{388}N_{77}O_{67}S_7$, 5840.84) and for PKD-C1B (MH^+, average molecular mass: obsd 6040.61, cald for $C_{265}H_{425}N_{82}O_{66}S_7$, 6040.27). Furthermore, the amino acid composition of these peptides, which was determined by the method of Hayashi and Sasagawa,[14] coincided well with that of the theoretical values except for Cys and Trp residues (data not shown).

PKC C1 domains have six conserved cysteines and two histidines. Each C1 domain coordinates two atoms of zinc with each metal bound by three sulfur atoms of cysteines and one nitrogen atom of histidine.[15,16] Since all of these amino acids are strictly conserved in both PKD C1 peptides, zinc coordination was carried out by the method reported previously.[10] [^{3}H]PDBu binding to the PKD C1 peptides was measured by the method of Sharkey and Blumberg.[17] The results showed the importance of zinc in the folding and phorbol ester binding of PKD-C1A and PKD-C1B as observed for the PKC C1 peptides.[10]

Scatchard analysis of [^{3}H]PDBu binding to PKD-C1A did not give a straight line as observed for PKD-C1B and most PKC-C1B peptides.[10] The B/F values at low concentrations of [^{3}H]PDBu were especially small, suggesting that the PDBu binding to PKD-C1A decreased significantly under the binding assay conditions employed. Since the incubation temperature (30 °C) and time (20 min) were considered possible causes of this reduced affinity, the effect of the former on PDBu binding to both PKD C1 peptides was examined.

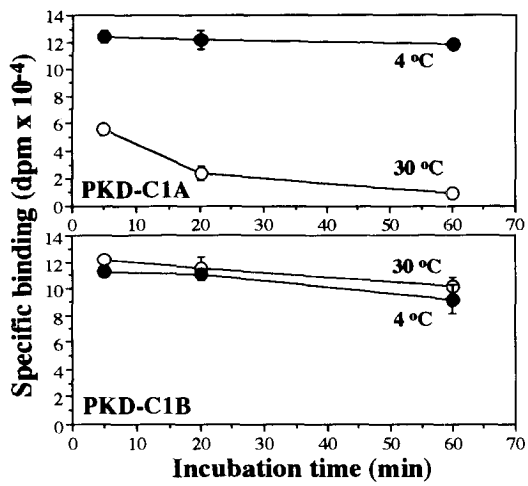

Figure 2. Temperature effects on the [^{3}H]PDBu binding to PKD-C1A and PKD-C1B. Zinc coordination was carried out in a distilled water solution of each PKD C1 peptide (100 μg/100 μL) using 5-molar equiv. of $ZnCl_2$ at 4 °C for 10 min. After dilution with distilled water, an aliquot of the peptide solution (2.9 μL) was added to the reaction mixture (247.1 μL) consisting of 50 mM Tris-acetate (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 *ca.*10 nM, which was determined by Scatchard analysis. The reaction mixture was incubated at 4 °C or 30 °C for 5 – 60 min. The PDBu-peptide complex was precipitated at 4 °C by adding poly(ethyleneglycol) as reported previously.[10,17] ●: PKD-C1A (top) and PKD-C1B (bottom) at 4 °C; ○: PKD-C1A (top) and PKD-C1B (bottom) at 30 °C. The bars represent standard deviations.

Importantly, as shown in Fig. 2, the PDBu binding to PKD-C1A decreased drastically at 30 °C while the binding was constant at 4 °C over a 60 min incubation. Interestingly, no significant temperature effect was observed for PKD-C1B, indicating that the temperature effect is sequence dependent. Decrease in the PDBu binding to PKD-C1A was not attributable to degradation of PKD-C1A since zinc-folded PKD-C1A in Tris buffer (pH 7.4) did not decompose at 30 °C for 60 min. The mechanism of this inactivation is under investigation.

Scatchard analysis of [^{3}H]PDBu binding to both PKD C1 peptides at 4 °C gave dissociation constants (K_d) of 2.5 ± 0.4 and 2.7 ± 0.8 nM, for PKD-C1A and PKD-C1B, respectively (Fig. 3). This means that the two cysteine-rich motifs of PKD are functionally equivalent like those of PKCγ.[10] After completion of this work, Iglesias *et al.* reported dissimilar PDBu binding properties of the individual cysteine-rich motifs of PKD using the GST-fusion proteins containing C1A and C1B with mutations.[18] They proposed that the PKD C1B is the major PDBu binding site in native PKD. Although they did not report the K_d values of each cysteine-rich domain, their conclusion differs from our results. This difference might arise from the binding assay conditions since the PDBu binding to PKD-C1A is quite sensitive to the incubation temperature as shown above.

We have recently reported the K_d values of the C1 peptides of all PKC isozymes.[10] The K_d values of the C1 peptides of PKCγ (γ-C1A and γ-C1B) differed by 50-fold from those reported by Kazanietz *et al.*[19] but agreed with those reported by Dimitrijevic *et al.*[20] for native PKCγ. Prompted by the novel temperature dependency noted above, we determined the K_d values of γ-C1A and γ-C1B using the new binding assay conditions at 4 °C. The Scatchard analysis gave the K_d values of 1.6 ± 0.6 and 1.4 ± 0.2 nM for γ-C1A and γ-C1B, respectively, which were very close to the K_d value of native PKCγ (0.4 nM) reported by Kazanietz *et al.*[19] On the other hand, the K_d values of α-C1B, β-C1B, δ-C1B, ε-C1B, η-C1B, and θ-C1B did not change significantly between the two incubation conditions (30 °C and 4 °C). The temperature dependency of PDBu binding to the C1A peptides, almost all of which were considered to be weak PDBu binders,[10,21,22] was also investigated preliminarily. Importantly, a drastic temperature effect like that found for PKD-C1A was observed in ε-C1A and η-C1A. The K_d values of these peptides were determined to be 5.6 ± 0.62 and 4.3 ± 0.18 nM, respectively, which were 10-fold larger than the corresponding C1B peptides (ε-C1B: 0.81 ± 0.03; η-C1B: 0.45 ± 0.12 nM). The PDBu binding ability of α-C1A, β-C1A, δ-C1A, and θ-C1A did not change significantly between the two incubation conditions.

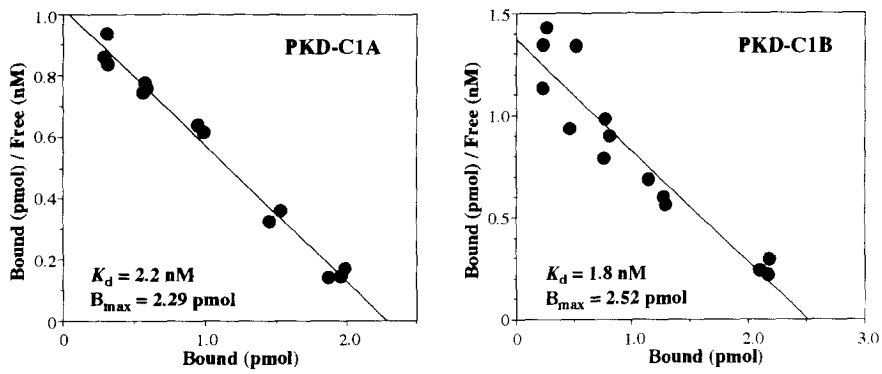

Figure 3. Scatchard analyses of [^{3}H]PDBu binding to PKD-C1A and PKD-C1B. The zinc-folded PKD C1 peptides (20 nM) were incubated with increasing concentrations of [^{3}H]PDBu in the presence of 50 μg/mL phosphatidylserine at 4 °C for 20 min as described in Fig. 2. Each representative experiment is shown. The binding stoichiometries calculated from the B_{max} values of each PKD-C1A and PKD-C1B were 46% and 50%, respectively. Similar results were obtained in additional experiments.

In summary, we have synthesized the two C1 domains of PKD and have shown that they bind to PDBu with similar affinities. This conclusion differs from that of Iglesias *et al.*[18] derived from the GST-fusion proteins of PKD C1 domains. The current study also demonstrates that the incubation temperature drastically affects the PDBu binding to some of the C1 peptides of PKD and PKC. It is of special importance that this temperature effect is dependent on the sequence of the C1 peptides. The mechanism of this temperature dependency and modified K_d values of all PKC C1 peptides will be reported in due course.

Acknowledgments: This research was partly supported by a grant-aid for Scientific Research (C) (No. 11660109) from the Ministry of Education, Science, Culture, and Sports of Japan (K.I.), and by a grant (CA31841) from the National Institutes of Health (P.A.W.).

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