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Identification of protein kinase C inhibitory activity associated with a polypeptide isolated from a phage display system with homology to PCM-1, the pericentriolar material-1 protein

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ABSTRACT

We had previously identified a protein kinase C (PKC) inhibitor in murine neuroblastoma cells (Chakravarthy et al. [1]). Similar PKC inhibitory activity was also found in adult rat brain. Using polyclonal antibodies raised against the partially purified PKC inhibitor from rat brain as bait, we isolated a putative brain PKC inhibitor using a T-7 phage display system expressing human brain cDNA library. After enriching the phage population expressing the putative PKC inhibitor with four rounds of biopanning using ELISA and *in vitro* PKC binding assays, we identified a phage clone that expressed a product with significant PKC inhibitory activity. We have cloned and expressed this cDNA in a bacterial system and purified the recombinant protein. This polypeptide (174 amino acids) is highly homologous to a region of the 228-kDa PCM-1, the human pericentriolar material 1 protein. We have mapped this polypeptide's PKC-inhibitory domain and shown its PKC inhibitory activity *in vitro*. However, it will need to be determined whether the full-length PCM-1 protein possesses PKC inhibitory activity *in vivo*, and if so, how this might contribute to PCM-1's recently demonstrated roles in ciliogenesis and neurogenesis.

1. Introduction

N1E-115 murine neuroblastoma cells have a PKC (protein kinase C) -inhibiting protein in their membranes which we initially enriched by 30–60% ammonium sulfate-precipitation [1]. We then found a similar PKC inhibitory activity in adult rat brain and primary cortical neurons. We partially purified the "putative" PKC inhibitor from rat brain extract with 30–60% ammonium sulfate precipitation and subsequent DE-52 anion exchange chromatography. The PKC inhibitory activity was further enriched in a fraction eluting at 150 mM NaCl, but further purification by conventional chromatographic methods failed because of low yields and loss of anti-PKC activity.

An alternative approach was taken to overcome this problem. We first raised polyclonal antibodies to this partially purified PKC inhibitor in rabbits. This antibody was able to immunoprecipitate PKC-inhibiting activity from the 30–60% ammonium sulfate fraction of N1E-115 neuroblastoma cell extracts, indicating that the polyclonal antibody did recognize the PKC-inhibitory protein. However, since this antibody was raised against only partially purified PKC inhibitor, it would also have recognized a number of other cellular proteins and could not be used to further purify the inhibitor. So we resorted to Phage Display technology using these polyclonal antibodies as bait and a commercial T7 Phage Display Library expressing human brain cDNA.

Here we show that one of the polypeptides isolated using this technology does inhibit PKC activity and that it is highly homologous to a region of PCM-1, the human pericentriolar material-1 protein [2]. This raises the very important possibility that the multi-functional full-length PCM-1 harbors intrinsic PKC inhibitory activity. PCM-1 recruits various protein components, including pericentrin, into pericentriolar satellites that are carried to the centrosome [3–6]. Thus, PCM-1 could potentially regulate PKCs, such as PKCβII which is anchored to the centrosome through pericentrin [7] and modulate various centrosomal activities including microtubule nucleation, mitotic spindle formation, ciliogenesis and cilium-driven processes such as neurogenesis [3–5,8–10].

2. Materials and methods

2.1. Materials

ATP, dsDNA-cellulose, 12-*O*-tetradecanoyl phorbol 13-acetate (TPA), cAMP, cAMP-dependent protein kinase (PKA), Kemptide (protein kinase A substrate), Syntide-2 (CaM kinase II substrate), trypsin and soybean trypsin inhibitor were purchased from Sigma Chemical Co. (St. Louis, MO). Protein reagent was from Bio-Rad

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Laboratories (Richmond, CA). Pure PKC (total) and PKC α, ß, γ isoforms were obtained from Upstate Biotechnology Inc. (Millipore, Bellerica, MA). Isozyme-specific PKC antibodies were obtained from GIBCO-BRL (Burlington, Ontario, Canada). [γ-32P] ATP (6000 Ci/mmol) was purchased from New England Nuclear (Du Pont Canada Inc., Mississauga, Ontario, Canada). TALON-IMAC resin was from Clontech Laboratories Inc. (Mountain View, CA) and Phage-coat (T-7.Tag) antibody linked to horseradish peroxidase (HRP) was purchased from Novagen (EMD Millipore). PVDF Western blotting membranes were from Bio-Rad (Mississauga, ON, Canada). The PKC-selective peptide substrate Ac-FKKSFKL-NH_2 and the DNA-PK-selective peptide Ac-PESQEAFADLWKK-NH_2 were synthesized in this laboratory as described previously [11,12].

3. Methods

3.1. Biopanning

Biopanning was carried out using T7select Biopanning Kit (Novagen) essentially according to the manufacturer's instructions. Tissue culture plates (24-well) were coated overnight at 4 °C with polyclonal antibody raised against partially purified PKC inhibitor (PK-Ab) from rat brain [0.3 μg/ml of antibody in 0.5% skim milk in Tris-buffered saline containing 0.05% Tween-20 (TBS-T)]. T-7 phage library (10^9 pfu) was added and incubated for 30 min at 22 °C and bound phage was eluted after washing the plate five times with TBS-T. Bound phage was eluted with Tris-buffered saline (TBS) containing 1% sodium dodecyl sulfate (SDS), amplified in BLT5615 bacterial cultures and subjected to three more rounds of biopanning as described above. Amplified phage samples were tested after each round of selection for PKC binding and inhibitory activity. Phage isolates from the fourth biopanning were serially diluted and subjected to Plaque-Assay to select and amplify individual clones on agar plates according to the supplier's instructions (Novagen). cDNAs from select plaques were extracted and amplified by PCR with T7Select UP and DOWN primers according to T7Select System manual (Novagen) and cDNA inserts were analysed on an agarose gel. A few selected clones were subjected to ELISA assay with polyclonal PKC inhibitor antibody (PK-Ab), and partially purified and commercially available purified PKC. The cDNAs from these Phage isolates were cloned and the peptide was expressed with an N-terminal His tag using the T7 TOPO TA (pCRT7/NT-TOPO) cloning kit (Invitrogen). The expressed protein was purified on TALON-IMAC resin column. A recombinant polypeptide of ~19 kDa (~25 kDa with the His-tag) was isolated corresponding to the ~500 bp human cDNA insert in the Phage.

3.2. Elisa

Maxisorp 96-well plates were coated overnight at 4 °C with PK-Ab, pure or partially purified PKC in TBS-T, blocked with 0.5% skim milk in TBS-T for 30 min and incubated with isolated phage for 30 min. Following three washes with TBS-T, Phage binding was determined with phage-coat antibody linked to HRP.

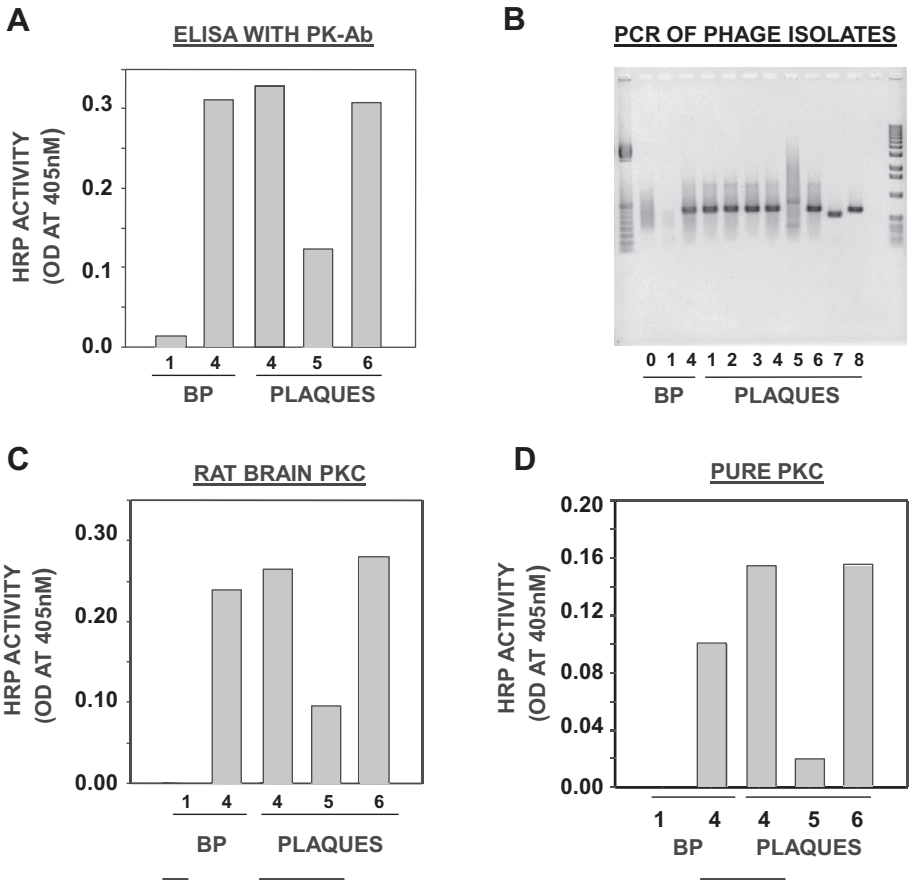

Fig. 1. Isolation of a PKC inhibitor with a phage display system: Using a polyclonal antibody against a partially purified rat brain PKC inhibitor (PK-Ab) as a bait phage clones binding to the antibody were enriched after 4 rounds of biopanning of T-7 phage library expressing human brain cDNA (A) as described in Section 2. PCR amplification followed by agarose gel electrophoresis revealed ~500 bp cDNA inserts in the isolated phage clones (B). ELISA assays by anchoring partially purified rat brain PKC (C) or a pure PKC (D) revelaed that the isolated T-7 phage clones bound PKC and enrichment of binders with four rounds of biopanning. Abbreviations: BP, biopanning; HRP, horseradish peroxidase.

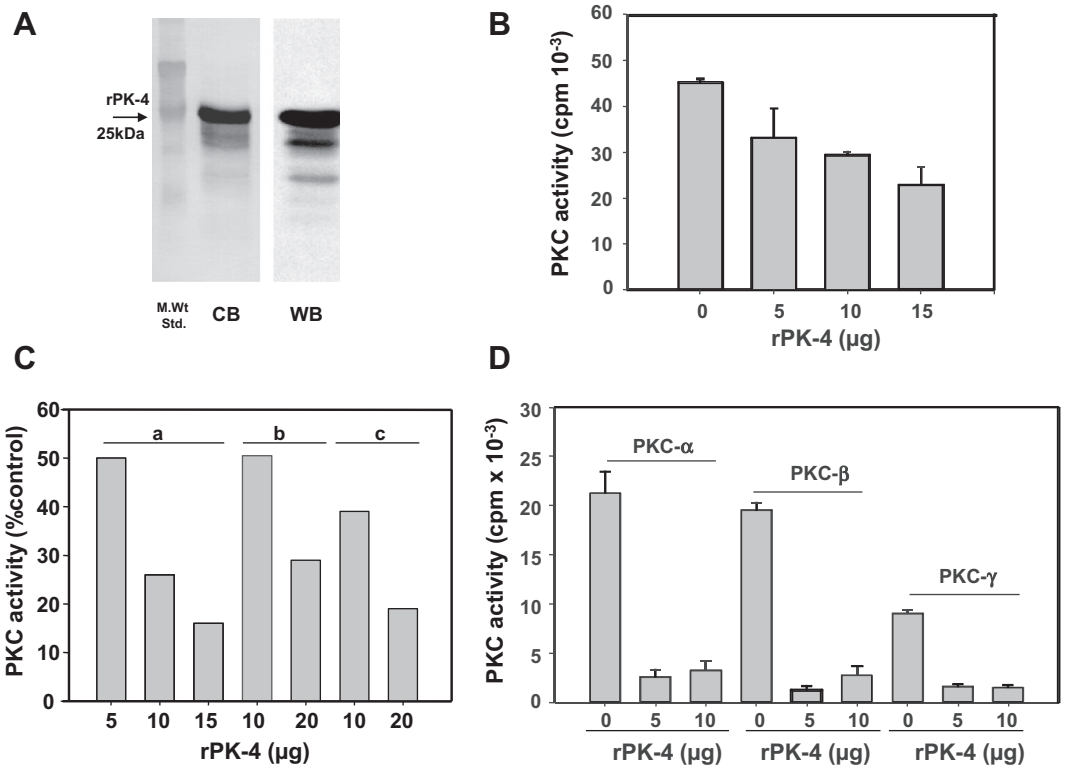

Fig. 2. Expression of phage cDNA in bacterial system and assessment of the biological activity of the expressed protein. The phage cDNA was amplified by PCR and expressed in a bacterial system using the TOPO cloning kit as described in Section 2. A recombinant protein with His-tag (rPK-4) of ~25 kDa was isolated (A). PKC inhibitory activity of rPK-4 was measured as described in Section 2. rPK-4 inhibited PKC activity in crude rat brain extract (B), rat brain membrane-assocaited PKC (C-a), partially purified PKC (C-b), rat brain cytosol PKC (C-c) and PKC isforms, α, ß, and γ (D). Bar graphs represent the Means ± SD of three separate assays (B and D) and the mean of two separate assays (C) done in triplicates. CB, Coomassie blue staining; WB, Western blot with His-tag antibody.

3.3. PKC assay

PKC activity was measured as described [1,11] using either partially purified rat brain PKC or pure PKC (Upstate Biotechnology Inc.) after reconstituting the enzyme in PS/TPA. MARCKS peptide, Ac-FKKSFKL-NH2, was used as PKC-selective substrate.

3.4. Measurement of PKC in intact rat brain membranes

Whole rat brain was homogenized in ice-cold Tris–HCl buffer (20 mM, pH 7.4, 5 μg/ml AEBSF, 0.8 μg/ml aprotonin, pepstatin, and leupeptin) with 10 up-and-down strokes in a Potter–Elvehjem (Glas-Col) tissue homogenizer at maximum speed (4000 rpm). The tissue homogenates were centrifuged at 600*g* for 5 min at 4 °C and the supernatants were centrifuged again at 100,000*g* for 30 min at 4 °C in a Beckman Optima MAX-XP ultracentrifuge to separate particulate (total membranes) fraction. Isolated total membranes were suspended in PKC assay buffer and membrane-associated PKC was measured in intact membranes using PKC-selective peptide Ac-FKKSFKL-NH2 as described previously [11].

3.5. Other protein kinase assays

cAMP-dependent protein kinase (PKA), Ca^{2+}-CaM-dependent protein kinase and DNA-dependent protein kinase activities in the rat brain were determined essentially as described previously [1,11–13] using Kemptide, syntide-2 and Ac-PESQEAFADLWKK-NH_2 as specific peptide substrates, respectively.

4. Results

We had previously reported the presence of a protein kinase C (PKC) inhibitor protein in N1E-115 murine neuroblastoma cell membranes [1]. We subsequently found similar PKC inhibitory activity in adult rat brain and primary cortical neurons. In order to identify the putative inhibitory protein, we raised a rabbit polyclonal antibody against partially purified PKC inhibitor (PK-Ab) and used this antibody as a bait to isolate and purify the putative PKC inhibitor from a phage display library expressing human brain cDNA.

As shown in Fig. 1A, after four rounds of biopanning, samples were enriched with phage clones binding to the PKC inhibitor antibody (compare BP 1 and 4). When the phages from biopanning 4 were plated for plaque assay, we found that the phages in about 60–70% of the plaques bound PKC inhibitor antibody (plaques 4, 5 and 6) which confirmed the enrichment of this phage clone during four rounds of biopanning. This was further confirmed by analyzing the human cDNA insert by agarose gel electrophoresis after PCR amplification of phage DNA using the T7Select UP and DOWN primers. As shown in Fig. 1B, there was a prominent ~500-bp DNA band that was enriched by four rounds of biopanning (BP 1 and 4). The phages in 6 out of 8 isolated plaques tested had the same size DNA inserts with identical sequences.

To determine whether the isolated, PKC inhibitor antibody-binding, phage clones in Fig. 1A also expressed PKC inhibitor, they were then tested for PKC-binding assay by ELISA. As shown in Fig. 1C, when phage isolates were allowed to bind to partially purified PKC from rat brain coated onto ELISA plates, more PKC binding was also observed after four rounds of biopanning (BP 1 and 4) and the phage in plaques 4, 5 and 6 bound coated PKC similarly to their

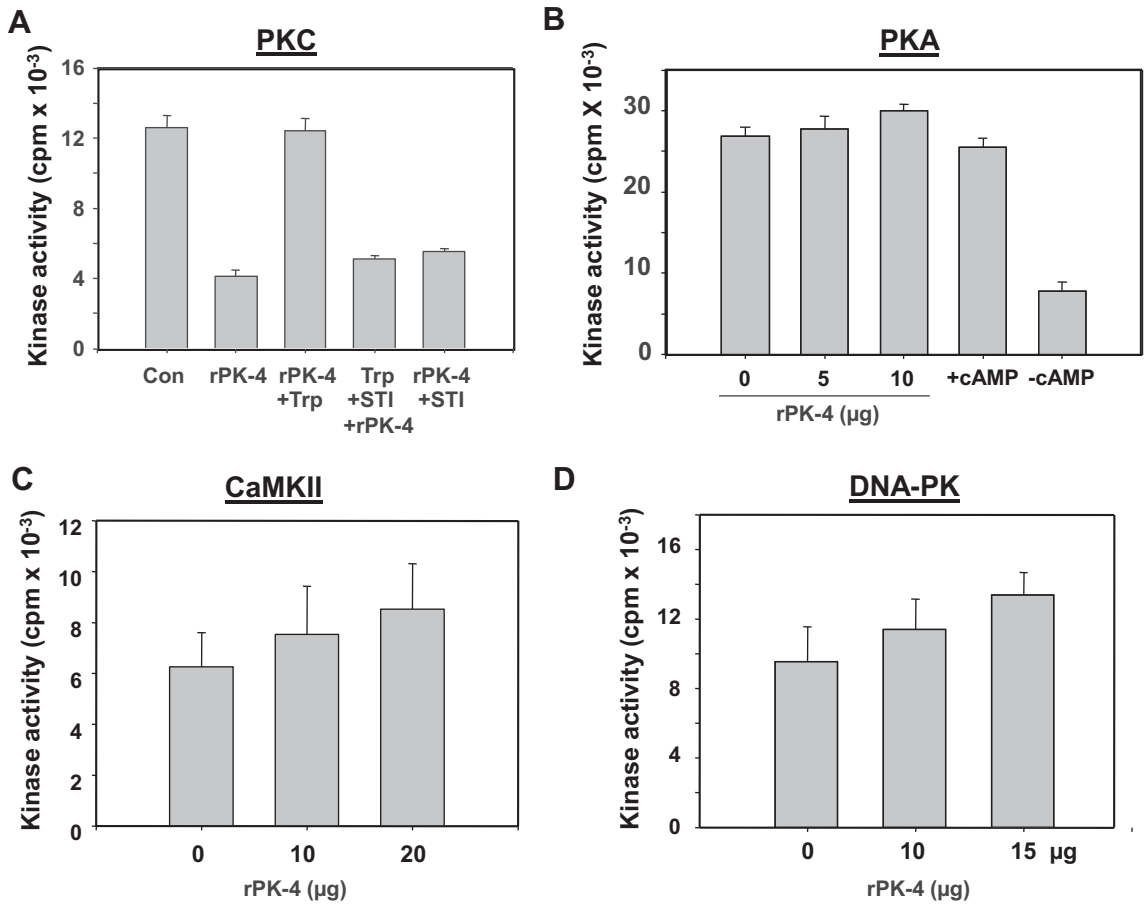

Fig. 3. rPK-4 is trypsin-sensitive and selectively inhibits PKC. Prior treatment of recombinant rPK-4 with trypsin (as described in Ref. [1]) completely eliminated rPK-4's ability to inhibit the PKC activity (A). Inactivation of rPK-4 was blocked when trypsin was premixed with 3-fold excess of soybean trypsin inhibitor before incubating with PK-4 (A, Trp + STI + rPK-4). Soybean trypsin inhibitor alone did not affect rPK-4 activity (A, rPK-4 + STI). rPK-4 did not affect the activities of cAMP-dependent protein kinase (PKA, B), calmodulin-dependent protein kinase (CaMK II, C) or DNA-dependent protein kinase (DNA-PK, D). Bar graphs represent the Means ± SD of three separate assays done in triplicates. Trp, trypsin; STI, soybean trypsin inhibitor; Con, control.

binding PKC inhibitor antibody. This was further confirmed using pure PKCs (Fig. 1D). These results thus confirmed that the phage clones isolated from the phage library using PKC inhibitor antibody did interact with PKC and potentially inhibit PKC activity.

We then cloned and expressed the phage cDNA in a bacterial expression system using the T7 TOPO TA cloning kit. As shown in Fig. 2A, both coomassie blue staining and Western blot analysis with His-tag antibody revealed a recombinant polypeptide of approximately ~25 kDa which we called rPK-4. The His-Tagged rPK-4 was tested for its PKC inhibitor activity. As shown in Fig. 2B, rPK-4 dose-dependently inhibited the PKC activity in a crude rat brain extract. An unrelated polypeptide carrying His-tag did not affect PKC activity suggesting that the inhibitory activity was not associated with His-tag (data not shown). Further studies revealed that rPK-4 inhibited PKC activity in native intact-membranes as well as partially purified PKC and three PKC isoforms tested, PKCα, PKCß and PKC γ (Fig. 2C and D). These results further confirmed that the polypeptide isolated from the phage library using anti-PKC-inhibitor antibody was indeed a PKC inhibitor. As expected rPK-4's activity was trypsin-sensitive. PKC inhibitory activity was completely lost after trypsin treatment (Fig. 3A). rPK-4 appeared to be selective towards PKC as it had very little effect on cAMP-dependent protein kinase (PKA), calmodulin kinase (CaM Kinase II) and DNA-dependent protein kinase (DNA-PK) activities (Fig. 3B–D, respectively).

The isolated PK-4 was found to be a 174-amino acid polypeptide (Fig. 4) with high homology to human pericentriolar material-1 protein (PCM-1). We then determined PK-4's PKC-interacting domain. To do this we made six overlapping 40-amino acid peptides spanning the whole length of PK-4 (Fig. 4) and tested each peptide for its abilityto inhibit PKC. As shown in Fig. 4, peptide 5, KDKTPKSKSKKRNSTQLKSRVKNITHARRILQQSNRNACN, in the C-terminus region of PK-4 had the PKC inhibitory activity which inhibited partially purified rat brain PKC activity in a does-dependent manner. Nestling between last 10 amino acids of peptide 4 and the first 15 amino acids of peptide 5 is the KRNSTQLKSRVKNI region which might be PK-4's PKC-inhibiting core domain.

5. Discussion

We have isolated the DNA from a human brain cDNA library that codes for a 174-amino-acid polypeptide containing PKC-inhibiting domain of approximately 20–25 amino acid. This could be an important finding because this peptide and its PKC-inhibiting region are homologous to the 1171–1323 amino acid region of the 228-kDa human PCM-1 protein (GenBank: AAA60120.1). PCM-1 recruits various components for the pericentriolar material that ensheaths the centrioles [3,4,6]. For example PCM-1 forms complexes with proteins such as Htt (huntingtin), HAP-1 and Hook proteins that drive ciliogenesis and neurogenesis [4,9,10].

If the PKC-inhibiting domain of PCM-1 is both accessible and functional in the centrosomal complexes *in vivo* where might be its PKC targets? One target might be the PKCβII that is anchored

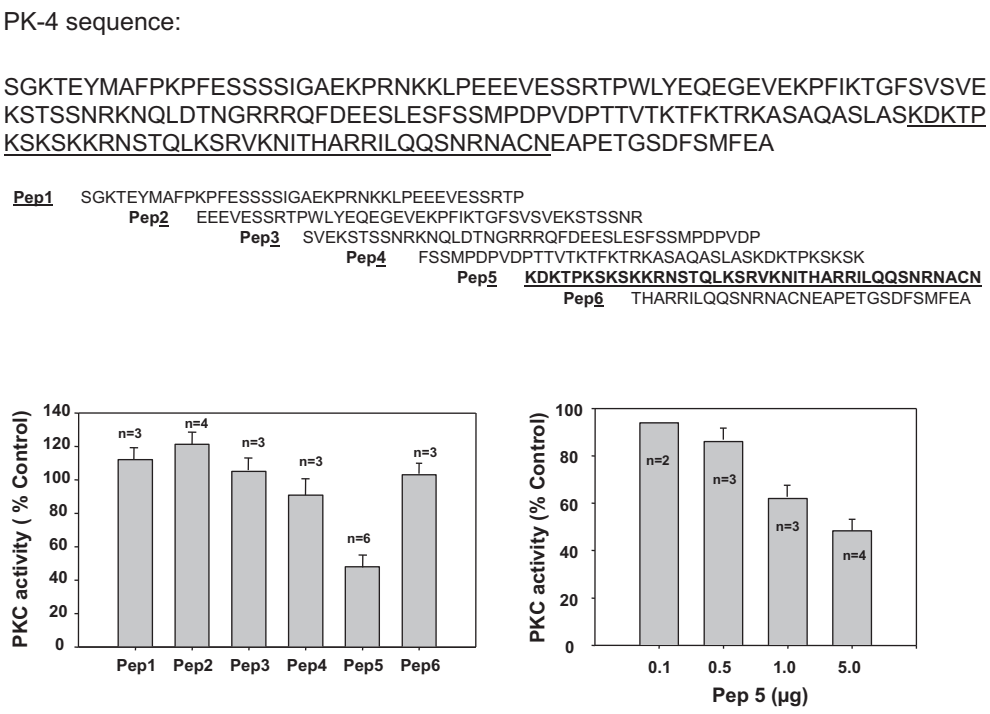

Fig. 4. The PKC-inhibitor domain on PK-4. A series of overlapping peptides spanning the entire length of PK-4 were synthesized and tested for PKC-inhibiting activity using partially purified rat brain PKC as described in Section 2. Peptide 5 in the C-terminus region of PK-4 had the PKC-inhibitory activity. Bar graphs represent the Means ± SD of two-six separate assays done in triplicates. Pep, Peptide.

by its CA1 region to the 494 and 593 region of the pericentrin molecule [7]. Pericentrin is a large coiled-coil protein component of the pericentriolar material that is involved in a very wide range of cellular functions and PCM-1 might target these functions via its PKC-inhibiting domain [5]. However any association of PCM-1 with pericentrin remains to be investigated. Interestingly, PCM-1 complexes with the related pericentrin-B, but is not known whether pericentrin-B has an attached PKC [6,7]. Clearly, it will be important to assess the role(s) of PCM-1 and its PKC-inhibiting domain in the several wide-ranging centrosomal- centriolar functions.

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