Biochemical Pharmacology, Vol. 36, No. 23, pp. 4179–4181, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00
Pergamon Journals Ltd.

Binding of protein kinase C to napthalenesulfonamide- and phenothiazine-agarose columns: evidence for direct interactions between protein kinase C and cationic amphiphilic inhibitors of the enzyme

(*Received* 12 *March* 1987; *accepted* 20 *May* 1987)

Protein kinase C (PKC*) is a Ca^{2+}- and phospholipid-dependent protein kinase which has been implicated in tumor promotion and cellular proliferation (for review, see Ref. 1). PKC is inhibited by several cationic amphiphiles, including the phenothiazine chlorpromazine [2], the napthalenesulfonamide W7 [3, 4], tamoxifen [5–7], rhodamine 6G [8], and sphingosine [9]. These PKC inhibitors are also pharmacologically related in that many of them, including chlorpromazine and W7, are calmodulin antagonists, which function by interacting directly with calmodulin [3, 10–13]. Kinetic analyses of the inhibition of PKC by tamoxifen [5], chlorpromazine [2], W7 [3], and rhodamine 6G [8] provide evidence that their mechanisms of inhibition of PKC involve drug–lipid interactions, although direct interactions between PKC and the drugs have not been excluded. We report here that PKC interacts directly with immobilized W7 and with an immobilized chlorpromazine analog. This is the first report to demonstrate direct interactions between PKC and cationic amphiphilic PKC inhibitors. An understanding of the direct interactions between PKC and these drugs may facilitate the design of more specific PKC inhibitors.

Materials and methods

[γ-^{32}P]ATP was purchased from the Amersham Corp, Hydrofluor was from National Diagnostics, YM-30 ultrafiltration membranes were from Amicon, and phosphocellulose paper (grade P81) was from Whatman Inc. SDS–PAGE reagents and CAPP affigel 10 [2-chloro-10-(3-aminopropyl)phenothiazine coupled to affigel 10] were from BioRad Laboratories. Tris–HCl, bovine serum albumin, histone III-S, ATP, PS, PMSF, Triton X-100, beaded agarose type X, and W7 agarose (*N*-6-aminohexyl-5-chloro-1-napthalenesulfonamide coupled to beaded agarose type X) were from the Sigma Chemical Co. Leupeptin was a gift of the U.S.–Japan Cooperative Cancer Research Program.

Enzyme assay. Rat brain PKC was partially purified as previously described [14] and then chromatographed on a second DEAE Sephacel column with a linear gradient of 0 to 0.3 M NaCl. The resultant PKC preparation had a specific activity of 230 nmol ^{32}P/min/mg and was activated over 10-fold by PS plus either 1 mM Ca^{2+} or 100 nM TPA. PKC was assayed by measuring the Ca^{2+}- and PS-dependent phosphotransferase reaction between [γ-^{32}P]ATP and histone IIIS, as previously described [14].

Chromatography of PKC on W7 agarose, CAPP agarose, and unmodified agarose. All chromatographic procedures were done at 4°. A 1.5-ml column of W7 agarose was equilibrated with 20 mM Tris–HCl/200 mM KCl/4 mM EDTA/4 mM EGTA/15 mM 2-mercaptoethanol/100 μg/ml leupeptin/0.4 mM PMSF at pH 8.3 (Equilibration Buffer). From 100 to 150 μg of the rat brain PKC preparation was loaded onto the column, which was then washed with 21 ml of Equilibration Buffer (14 column volumes). Three-milliliter wash fractions were collected into tubes containing 450 μl of 50% glycerol. The majority of the PKC activity was then eluted with 60 ml of 0.1% Triton X-100 (v/v) in Equilibration Buffer. Fractions (6 ml) were collected in tubes containing 900 μl of 50% glycerol and were assayed for PKC activity. While 0.1% Triton X-100 inhibited PKC activity >90%, the concentration of Triton X-100 in the PKC assay reaction mixtures (0.08% Triton X-100) had no detectable effect on PKC activity. The PKC-containing fractions that were eluted by Triton X-100 were pooled, and the yield of PKC activity was determined. The pooled fractions were concentrated approximately 10-fold by ultrafiltration with a YM30 membrane, and the protein concentration was determined with the BioRad protein assay solution, using bovine serum albumin as a standard. This procedure for chromatographing PKC on W7 agarose was also used for chromatographing the enzyme on CAPP agarose and on unmodified agarose. SDS-PAGE on 7.5% gels, and subsequent silver staining of the gels, were done by standard techniques [15, 16].

Results

Figure 1 illustrates the chromatography of PKC on W7 agarose. A 110 μg sample of PKC (230 nmol ^{32}P/min/mg) was loaded onto a 1.5-ml W7-agarose column. Greater than 85% of the applied PKC was retained by the column during a 14-column volume wash with Equilibration Buffer, a Tris–HCl buffer (pH 8.3) containing 4 mM EDTA and 4 mM EGTA. PKC activity was then eluted with 0.1% Triton X-100 (v/v) in Equilibration Buffer. The yield of PKC activity in the Triton X-100 eluant was 340 ± 50%, indicating that the chromatography had unmasked additional PKC activity, perhaps by separating an endogenous PKC inhibitor from the enzyme. The specific activity of the Triton X-100 eluted enzyme was 1120 ± 170 nmol ^{32}P transferred/min/mg, representing a 4.9-fold purification of the enzyme.

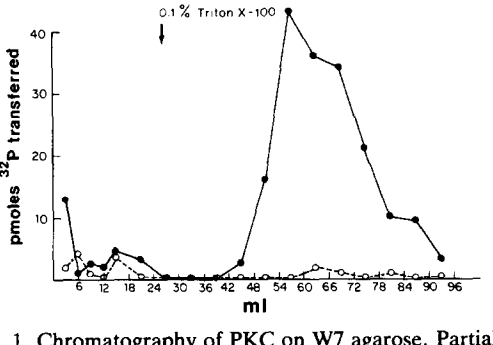

Fig. 1. Chromatography of PKC on W7 agarose. Partially purified rat brain PKC (110 μg of a 230 nmol ^{32}P/min/mg preparation) was chromatographed on a 1.5-ml W7-agarose column as described in the text. PKC activity was eluted with 0.1% Triton X-100. All fractions were assayed for PKC activity as described in Materials and Methods. Key: (●—●) PKC activity; and (○---○), Ca^{2+}- and phospholipid-independent protein kinase activity.

* Abbreviations: PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; W7, *N*-6-aminohexyl-5-chloro-1-napthalenesulfonamide; CAPP, 2-chloro-10-(3-aminopropyl)phenothiazine; PS, phosphatidylserine; EGTA, ethyleneglycolbis (β-amino-ethylether)-*N*,*N*,*N*^{1},*N*^{1}-tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; and PAGE, polyacrylamide gel electrophoresis.

To ensure that the binding of PKC to W7 agarose was not due to interactions between PKC and the agarose resin itself, PKC was chromatographed on a 1.5-ml agarose column using the procedure employed with the W7-agarose column. One hundred ±ten percent of the applied PKC activity eluted in the first fraction of the wash, and negligible levels of activity were subsequently eluted with Triton X-100 (Fig. 2). Thus, the binding of PKC to W7 agarose involves direct interactions between PKC and W7, since it occurred in the absence of PKC cofactors and was not seen with agarose itself.

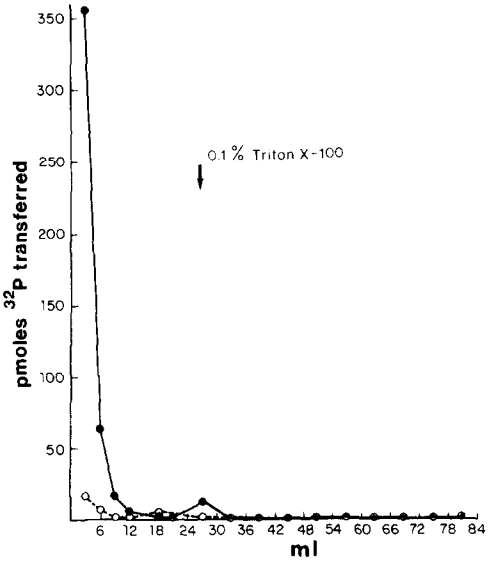

Fig. 2. Chromatography of PKC on unmodified agarose. Partially purified PKC was chromatographed on unmodified agarose by the procedures described in the legend to Fig. 1. Key: (●—●) PKC activity; and (○---○), Ca^{2+}- and phospholipid-independent protein kinase activity.

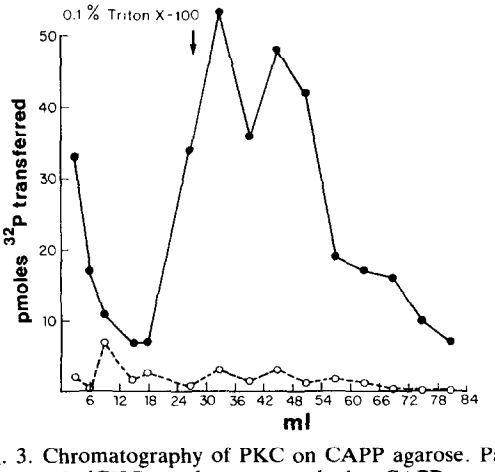

Fig. 3. Chromatography of PKC on CAPP agarose. Partially purified PKC was chromatographed on CAPP agarose by the procedures described in the legend to Fig. 1. Key: (●—●) PKC activity; (○---○) Ca^{2+}- and phospholipid-independent protein kinase activity.

To determine whether PKC can also interact directly with phenothiazines, PKC was chromatographed on a 1.5-ml CAPP-agarose column (Fig. 3), using the procedure employed with W7 agarose. The affinity ligand present in CAPP agarose is a structural analog of chlorpromazine [17]. A 130 μg sample of PKC was loaded onto a CAPP-agarose column, and the column was washed with 21 ml of Equilibration Buffer. The majority of the PKC was bound to the column and was eluted with 0.1% Triton X-100 (Fig. 3). The yield of PKC activity in the Triton X-100 eluant was 180 ± 19%, indicating that, like W7-agarose chromatography of PKC, CAPP-agarose chromatography unmasks additional PKC activity. The specific activity of the Triton X-100-eluted enzyme was 1720 ± 170 nmol ^{32}P transferred/min/mg, representing a 7.4-fold purification of the enzyme. PKC activity eluted from CAPP agarose in two distinct peaks (Fig 3); this bimodal distribution of PKC activity was observed in five different chromatographic runs.

Figure 4 shows a silver-stained gel of a PKC preparation before chromatography on W7 agarose or CAPP agarose (lane A), the PKC preparation after elution from CAPP

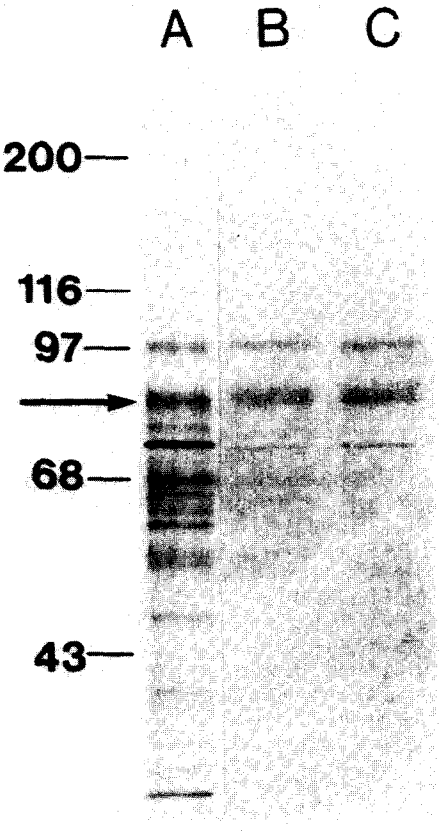

Fig. 4. SDS–PAGE analysis of PKC containing samples. SDS–PAGE analysis was employed in order to assess the enrichment of a PKC preparation by chromatography on CAPP agarose and on W7 agarose. SDS–PAGE and silver staining of the gels (7.5%) were done as described in Materials and Methods. Samples containing 1–2 μg protein were applied to each lane. Lane A: the PKC preparation prior to CAPP- or W7-agarose chromatography. Lane B: the pooled PKC-containing fractions which eluted from CAPP agarose. Lane C: the pooled PKC-containing fractions which eluted from W7 agarose.

agarose (lane B), and the PKC preparation after elution from W7 agarose (lane C). All three lanes contained an 82 kD band characteristic of our PKC preparations [18], and it is apparent that contaminating bands were less abundant in the preparations purified on the W7-agarose and CAPP-agarose columns.

Discussion

In view of the central role of PKC in numerous signal transduction mechanisms, it is of great interest to develop specific PKC inhibitors for mechanistic studies of the enzyme and for potential therapeutic purposes. Several PKC inhibitors that have been identified, including chlorpromazine [2] and W7 [3], appear to inhibit PKC by drug–lipid interactions, but the possibility that there are direct interactions between these drugs and PKC has not been investigated prior to this report. In this paper, we show that PKC interacted directly with W7 and also with a chlorpromazine analog. These interactions did not require the presence of phospholipid, Ca^{2+}, or other PKC cofactors. Our evidence for direct interactions betwen PKC and these inhibitors suggests that the mechanism of inhibition of PKC by cationic amphiphilic drugs does not merely involve drug–lipid interactions.

Certain PKC inhibitors also inhibit calmodulin-dependent enzymes by binding to calmodulin in a Ca^{2+}-dependent manner [3, 10]. In addition, calmodulin binds to W7 Sepharose and to CAPP Sepharose, in a Ca^{2+}-dependent manner [12, 13, 17, 19]. In contrast, we find the PKC binds to W7 agarose and CAPP agarose in a Ca^{2+}-independent manner. A chemically reactive chlorpromazine analog, [^{3}H] norchlorpromazine isothiocyanate, forms a covalent one-to-one complex with calmodulin [20]. It will be of interest to determine whether chemically reactive derivatives of PKC inhibitors bind covalently to the enzyme and to determine the specific sites on the enzyme to which these drugs might bind. Such studies could lead to a rationale for the design of specific PKC inhibitors.

PKC eluted from the W7-agarose column as an asymmetrical peak and from the CAPP-agarose column in two peaks (Figs. 1 and 3). These elution profiles may reflect structural heterogeneity of PKC molecules within the enzyme preparation. Such heterogeneity would be consistent with the evidence for multiple and distinct PKC-encoding cDNAs [18, 21, 22]. Thus, the affinity column procedures described in the present study may also be useful in resolving multiple forms of PKC.

Acknowledgements—We acknowledge the excellent secretarial assistance of Mrs. Nancy Mojica and Ms. Lintonia Sheppard. This work was supported by NCI Grant CA 02656 to I. Bernard Weinstein.

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REFERENCES

1. Y. Nishizuka, *Science* **233**, 305 (1986).
2. T. Mori, Y. Takai, R. Minakuchi, B. Yu and Y. Nishizuka, *J. biol. Chem.* **255**, 8378 (1980).
3. T. Tanaka, T. Ohmura, T. Yamakado and H. Hidaka, *Molec. Pharmac.* **22**, 408 (1982).
4. M. Ito, T. Tanaka, M. Inagaka, K. Nakanishi and H. Hidaka, *Biochemistry* **25**, 4179 (1986).
5. C. A. O'Brian, R. Liskamp, D. H. Solomon and I. B. Weinstein, *Cancer Res.* **45**, 2462 (1985).
6. H-D. Su, G. J. Mazzei, W. R. Vogler and J. F. Kuo, *Biochem. Pharmac.* **34**, 3649 (1985).
7. C. A. O'Brian, R. M. Liskamp, D. H. Solomon and I. B. Weinstein, *J. natn. Cancer Inst.* **76**, 1243 (1986).
8. C. A. O'Brian and I. B. Weinstein, *Biochem. Pharmac.* **36**, 1231 (1987).
9. Y. A. Hannun, C. R. Loomis, A. H. Merrill and R. M. Bell, *J. biol. Chem.* **261**, 12604 (1986).
10. D. R. Marshak, T. J. Lukas and D. M. Watterson, *Biochemistry* **24**, 144 (1985).
11. H-Y. P. Lam, *Biochem. biophys. Res. Commun.* **118**, 27 (1984).
12. D. R. Marshak, D. M. Waterson and L. J. Van Eldyk *Proc. natn. Acad. Sci. U.S.A.* **78**, 6793 (1981).
13. J. F. Harper, *J. Cyclic Nucleotide Protein Phosphorylat. Res.* **9**, 3 (1983).
14. C. A. O'Brian, D. S. Lawrence, E. T. Kaiser and I. B. Weinstein, *Biochem. biophys. Res. Commun.* **124**, 296 (1984).
15. U. K. Laemmli, *Nature, Lond.* **227** 680 (1970).
16. C. R. Merril, R. C. Switzer and M. L. Van Keuren, *Analyt. Biochem.* **105**, 361 (1980).
17. G. A. Jamieson and T. C. Vanaman, *Biochem. biophys. Res. Commun.* **90**, 1048 (1979).
18. G. M. Housey, C. A. O'Brian, M. D. Johnson, P. Kirschmeier and I. B. Weinstein, *Proc. natn. Acad. Sci. U.S.A.*, **84**, 1065 (1987).
19. C. R. Caldwell and A. Haug, *Analyt. Biochem.* **116**, 325 (1981).
20. D. L. Newton, T. R. Burke, K. C. Rice and C. B. Klee, *Biochemistry* **22**, 5472 (1983).
21. L. Coussens, P. J. Parker, L. Rhee, T. L. Yang-Feng, E. Chen, M. D. Waterfield, U. Francke and A. Ullrich, *Science* **233**, 859 (1986).
22. J. L. Knopf, M-H. Lee, L. A. Sultzman, R. W. Kriz, C. R. Loomis, R. M. Hewick and R. M. Bell, *Cell* **46**, 491 (1986).

Biochemical Pharmacology, Vol. 36, No. 23, pp. 4181–4185, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00
Pergamon Journals Ltd.

Cytotoxic activity of cyclic peptides of marine origin and their derivatives: importance of oxazoline functions

(*Received* 6 *May* 1987; *accepted* 10 *June* 1987)

A series of cytotoxic cyclic peptides have been isolated from marine organisms in the last few years [1–6]. All of these peptides contain unusual amino acid moieties involving the thiazole ring as their constituents. Furthermore, except dolastatin 3[5], all of the other peptides have the unique oxazoline ring. Their intriguing structures and cytotoxic activities led us to synthesize some of these peptides: dolastatin 3 (the proposed structure and its 15 isomers) [7, 8], ascidiacyclamide [9, 10], patellamides A[11], B[12, 13], and C[12, 13] (their proposed and revised struc-