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BINDING OF PROTEIN KINASE C TO *N*-(6-AMINOHEXYL)-5-CHLORO-1-NAPHTHALENESULFONAMIDE THROUGH ITS ATP BINDING SITE*

CATHERINE A. O'BRIAN† and NANCY E. WARD

Department of Cell Biology, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030, U.S.A.

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Abstract—Protein kinase C (PKC) is a Ca^{2+}- and phospholipid-dependent protein kinase which has been implicated as a key enzyme in the regulation of cellular growth. The naphthalenesulfonamide W7 [*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide] is representative of a number of cationic amphiphilic inhibitors of PKC which appear to inhibit PKC by interacting with the acidic phospholipid cofactor of the enzyme, according to kinetic studies. In a previous report, we demonstrated that PKC binds directly to W7 when the naphthalenesulfonamide is immobilized on agarose. In the present report, we have defined the mechanism of the binding of PKC to W7-agarose, and its relevance to the inhibitory mechanism of the naphthalenesulfonamide. We demonstrate that PKC bound W7-agarose through the catalytic domain of the enzyme. An active catalytic fragment of PKC was generated by limited proteolysis, and we found that this fragment bound W7-agarose and coeluted with intact PKC upon the addition of Triton X-100. W7 inhibited PKC activity by two different mechanisms. As previously reported, W7 inhibited PKC by interacting with the phospholipid cofactor of the enzyme (IC_{50} = 260 μM). However, at higher concentrations of W7, we found that this naphthalenesulfonamide inhibited PKC by serving as a competitive inhibitor with respect to the substrate ATP, according to a kinetic analysis of the inhibition of the active catalytic fragment of PKC by W7. W7 inhibited the active catalytic fragment of PKC as well as PKC-catalyzed phosphorylation of protamine sulfate, a reaction which is independent of Ca^{2+} and phospholipid, with similar potencies. Consistent with the kinetic evidence that W7 serves as a competitive inhibitor of PKC with respect to ATP, we found that, in the presence of 10 mM $MgCl_2$, 1 mM ATP was sufficient to elute PKC from W7-agarose. Thus, naphthalenesulfonamide PKC inhibitors may include both agents which primarily function by interacting with the phospholipid cofactor of the enzyme and agents which primarily serve as active site inhibitors of PKC.

Protein kinase C (PKC) is a Ca^{2+}- and phospholipid-dependent protein kinase which binds phorbol ester tumor promoters and related compounds with high affinity [1, 2]. Since PKC can be directly activated by tumor promoters, such as TPA‡ [3], as well as by diglyceride, which is a second messenger of certain growth factors and hormones, PKC has been implicated as a critical enzyme in tumor promotion and cellular proliferation [2]. PKC is further implicated in cellular growth regulation since it phosphorylates growth factor receptors, such as the epidermal growth factor receptor [4], and proto-oncogene products, such as $pp60^{src}$ and the p21 ras product [5, 6]. The elucidation of the mechanisms of action of PKC inhibitors may facilitate the development of specific PKC inhibitors, which could be of value as inhibitors of cellular proliferation [7].

A number of cationic amphiphilic compounds, including the naphthalenesulfonamide W7 [8], the phenothiazine chlorpromazine [9], the antiestrogen tamoxifen [10, 11] and rhodamine 6G [12], are inhibitors of PKC. According to kinetic analyses of their inhibitory mechanisms, these agents inhibit PKC by their interactions with the phospholipid cofactor of the enzyme, since the inhibitory potencies of the agents are reduced at elevated phospholipid concentrations [8–10, 12]. Consistent with this mechanism, W7 has been shown to bind directly to PS [8]. However, PKC binds directly to immobilized phenothiazine, naphthalenesulfonamide [13] and triphenylethylene PKC inhibitors [14] in the absence of PKC cofactors, providing evidence that their inhibitory mechanisms may include direct interactions between PKC and the cationic amphiphilic inhibitors. In this report, we demonstrate that PKC binds W7-agarose through the ATP binding region of its active site.

MATERIALS AND METHODS

Materials. [γ-^{32}P]ATP was purchased from the Amersham Corp. (Arlington Heights, IL) and Whatman phosphocellulose paper, grade p81, was purchased from Fisher Scientific (Houston, TX).

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† Correspondence: Dr Catherine A. O'Brian, Department of Cell Biology, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030.

‡ Abbreviations: DEAE, diethylaminoethyl; DMSO, dimethyl sulfoxide; PKC, protein kinase C; PMSF, phenylmethylsulfonylfluoride; PS, phosphatidylserine; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; W7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; and BAEE, benzoyl-L-arginine ethyl ester.

Tris–HCl, bovine serum albumin, histone III-S, ATP, PS, PMSF, leupeptin, Triton X-100, soybean trypsin inhibitor type I, W7-agarose (*N*-6-aminohexyl-5-chloro-1-naphthalenesulfonamide coupled to CNBr-activated beaded agarose type X), and TPCK-treated trypsin from bovine pancreas (sp. act. 10,000–13,000 BAEE units/mg protein) were purchased from the Sigma Chemical Co. (St Louis, MO). Frozen rat brains were purchased from the Charles River Breeding Co. (Wilmington, MA).

Isolation and assay of PKC. PKC was partially purified from frozen rat brains to a specific activity of 230 nmol ^{32}P/min/mg protein as previously described [13]. In indicated experiments, PKC was purified to near homogeneity from frozen rat brains by the procedure of Huang *et al.* [15] through the polylysine chromatography step to a specific activity of 1300 nmol ^{32}P/min/mg protein. The phosphotransferase activities of both enzyme preparations were activated 10- to 30-fold by 1 mM Ca^{2+} and 30 μg/ml PS. Both PKC preparations were stored at −20° in 50% glycerol.

PKC assay reaction mixtures (120 μl) contained 20 mM Tris–HCl, pH 7.5, 5 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 1 mM $CaCl_2$ [or 1 mM ethyleneglycolbis(amino - ethylether)tetra - acetate (EGTA)], 30 μg/ml PS (or none), 70 μM [γ-^{32}P]ATP (150–400 cpm/pmol), 0.67 mg/ml histone III-S, and 1–4 μg isolated rat brain PKC. Reactions were initiated by the addition of PKC and proceeded from 5 to 10 min at 30°, which is within the linear phase of the time course. Reactions were terminated by pipetting a 40-μl aliquot of the reaction mixture on phosphocellulose paper, and the radioactivity incorporated into histone was measured as previously described [16].

Proteolysis of PKC. A solution of 1300 units/ml trypsin was prepared in 20 mM Tris–HCl, pH 7.5, immediately before use. Five hundred microliters of the trypsin-containing solution was incubated with 500 μl of partially purified PKC (14 nmol ^{32}P/min) for 30 min at 4°, and the reaction was terminated by the addition of 500 μl of 3 mM PMSF in 20 mM Tris–HCl, pH 7.5, 10% DMSO. For the proteolysis of nearly homogeneous PKC, 500 μl of a 650 units/ml solution of trypsin was incubated with an equal volume of PKC (7 nmol ^{32}P/min) at 4° for 30 min, and the reaction was terminated with PMSF by the procedure detailed above. After proteolysis, PKC activity could be enhanced by Ca^{2+} and PS by no more than 2-fold, indicating the generation of the active catalytic fragment of PKC [1, 17, 18].

Chromatography of proteolyzed PKC on DEAE Sephacel. All chromatographic procedures were done at 4°. A 3-ml DEAE Sephacel column was equilibrated in 20 mM Tris–HCl, 5 mM EGTA, 5 mM EDTA, 10 μg/ml leupeptin, 0.25 mM PMSF, 15 mM 2-mercaptoethanol at pH 7.5. Proteolyzed PKC (1.5 ml) was diluted to 5 ml with the DEAE equilibration buffer and then loaded onto the column. The column was washed with 10 ml of equilibration buffer, and the catalytic fragment of PKC and intact PKC were eluted into 2-ml fractions with a 30-ml linear gradient of 0–0.4 M NaCl. Intact PKC eluted at 0.08 M NaCl and the catalytic fragment eluted at 0.19 M NaCl. Fractions 10–13, which contained the catalytic fragment, were pooled for further chromatography on W7-agarose.

Chromatography of PKC on W7-agarose. 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. The indicated sample of PKC was loaded onto the column, which was then washed with 20 ml of the equilibration buffer. PKC was eluted with 60 ml of 0.1% Triton X-100 in the equilibration buffer, and 6-ml fractions were collected. In experiments where PKC was eluted with MgATP in the equilibration buffer, 1.2-ml fractions were collected.

RESULTS

Binding of the catalytic fragment of PKC to W7-agarose. Previously, we have shown that partially purified PKC (sp. act. = 230 nmol ^{32}P/min/mg protein) binds directly to W7-agarose in the absence of PKC cofactors, and that Triton X-100 elutes the bound PKC. The eluted PKC has a specific activity of 1120 nmol ^{32}P/min/mg protein and is nearly homogeneous according to silver-stained gel analysis [13]. Figure 1A shows that a nearly homogeneous preparation of PKC (sp. act. = 1300 nmol ^{32}P/min/mg protein) also bound to W7-agarose and was eluted with the detergent Triton X-100. It is evident from Fig. 1A that PKC bound W7-agarose quantitatively. To determine whether the catalytic domain of PKC contained binding sites for the immobilized W7, we trypsinized nearly homgeneous PKC, thus producing an active catalytic fragment of the enzyme. While the enzyme was activated 14-fold by 1 mM Ca^{2+} and 30 μg/ml PS prior to proteolysis, it was activated by these cofactors only 1.2-fold after proteolysis, indicating the generation of an active catalytic fragment. The overall yield of catalytic activity was 50%. Figure 1B illustrates that, when proteolyzed PKC was chromatographed on W7-agarose according to the procedure employed with the intact enzyme, the Ca^{2+}- and PS-independent activity of the catalytic fragment coeluted with the PKC that remained intact after limited proteolysis. The overall yield of catalytic activity from this chromatography was greater than 90%.

To further examine the capacity of the catalytic fragment of PKC to bind W7-agarose, we employed ion exchange chromatography to isolate the catalytic fragment of PKC from the PKC which remained intact after limited tryptic digestion. Nearly homogeneous, proteolyzed PKC was chromatographed on DEAE Sephacel with a linear NaCl gradient of 0 to 0.4 M NaCl. This chromatographic procedure separated intact PKC, which eluted in the first peak of phosphotransferase activity, from the active catalytic fragment of the enzyme (Fig. 2). The isolated catalytic fragment bound to W7-agarose and was eluted in a single major peak with Triton X-100 (Fig. 3).

Inhibition of PKC activity by W7. W7 is an established PKC inhibitor which is believed to inhibit the enzyme by its interactions with the phospholipid cofactor [8]. We determined that the Ca^{2+}- and PS-dependent activity of PKC was inhibited by W7 with an IC_{50} of 260 ± 30 μM (data not shown), a value

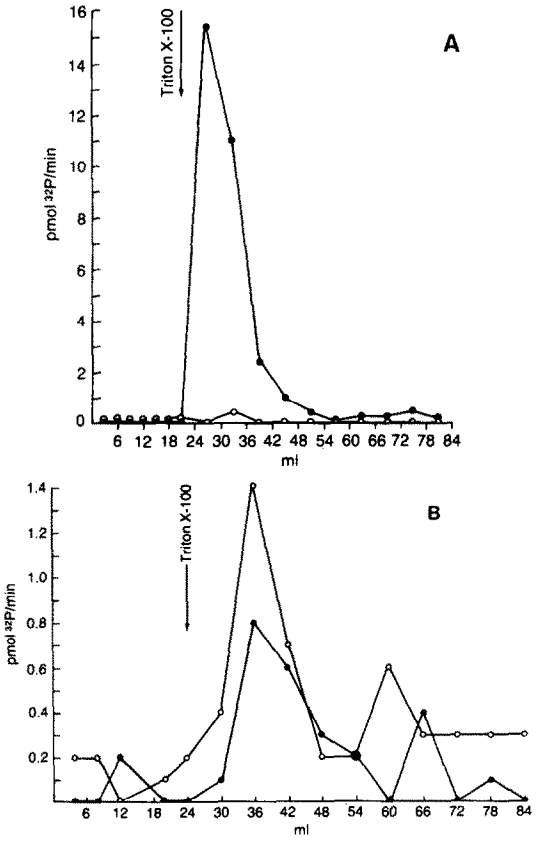

Fig. 1. Chromatography of PKC and its catalytic fragment on W7-agarose. (A) PKC (sp. act = 1300 nmol ^{32}P/min/mg protein) was loaded onto a 1.5-ml W7-agarose column. The column was washed with 20 ml of equilibration buffer, and the enzyme was eluted with 0.1% Triton X-100 in equilibration buffer. Key: (●) Ca^{2+}- and PS-dependent protein kinase activity; (○) Ca^{2+}- and PS-independent protein kinase activity. For further experimental details, see Materials and Methods. (B) PKC was trypsinized to a limited extent in order to generate an active catalytic fragment of the enzyme. The resultant solution, which contained proteolytic fragments of PKC and the remaining intact enzyme, was chromatographed on W7-agarose as described in panel A.

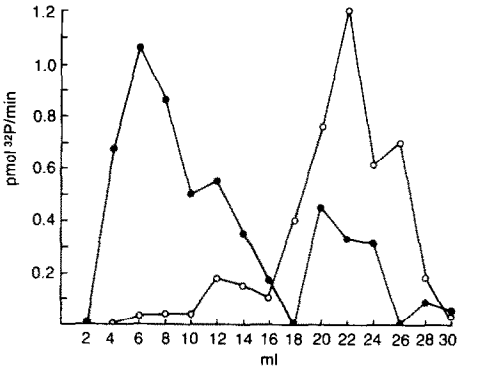

Fig. 2. Resolution of PKC and its catalytic fragment by DEAE chromatography. PKC was trypsinized to a limited extent to produce an active catalytic fragment of the enzyme. After proteolysis was terminated with PMSF, the resultant solution, which contained intact PKC and its active catalytic fragment, was loaded onto a 3-ml DEAE Sephacel column. The column was washed with 10 ml of equilibration buffer and then eluted with a linear NaCl gradient (0 to 0.4 M NaCl). See Materials and Methods for further experimental details. Key: (●) Ca^{2+}- and PS-dependent protein kinase activity; (○) Ca^{2+}- and PS-independent protein kinase activity.

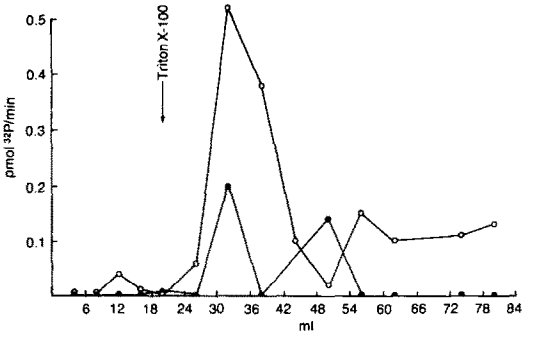

Fig. 3. Chromatography of purified catalytic fragment of PKC on W7-agarose. PKC was trypsinized as described in Fig. 1B, and the catalytic fragment of PKC was separated from the remaining intact PKC by ion exchange chromatography (see Fig. 2). The isolated catalytic fragment was chromatographed on W7-agarose as described for Fig. 1A.

which is consistent with its reported inhibitory potency [8]. However, we found that at higher concentrations of the inhibitor, W7 also inhibited the Ca^{2+}- and PS-independent activity of the enzyme. Nearly homogeneous PKC was proteolyzed, and the capacity of W7 to inhibit the resultant Ca^{2+}- and PS-independent activity of the enzyme was determined. Figure 4A shows that W7 inhibited this activity with an IC_{50} value of approximately 1.5 mM. According to Michaelis–Menten kinetics, the inhibition of the Ca^{2+}- and PS-independent activity of the enzyme was apparently competitive with MgATP (Fig. 4B). The phosphorylation of protamine sulfate is catalyzed by PKC in a reaction which is independent of Ca^{2+} and PS [19]. We found that W7 inhibited PKC-catalyzed protamine sulfate phosphorylation and the activity of the catalytic fragment of PKC with similar potencies (data not shown).

Elution of PKC from W7-agarose with MgATP. To determine whether W7 could elute intact PKC from W7-agarose, we attempted to elute PKC (7 nmol ^{32}P/min) from the resin with 0.5 mM W7; the use of higher concentrations of W7 was precluded by the solubility of W7 at 4°. Sixty milliliters of 0.5 mM W7 in equilibration buffer eluted no detectable PKC activity from a 1.5-ml W7-agarose column. The inability to detect PKC activity could not be ascribed to the presence of the PKC inhibitor W7 in the collected fractions, since the phosphotransferase reaction mixtures contained only 40 μM W7, and this concentration of W7 has little effect on PKC activity.

Since the kinetic analysis of the inhibition of the activity of the catalytic fragment of PKC by W7 (Fig.

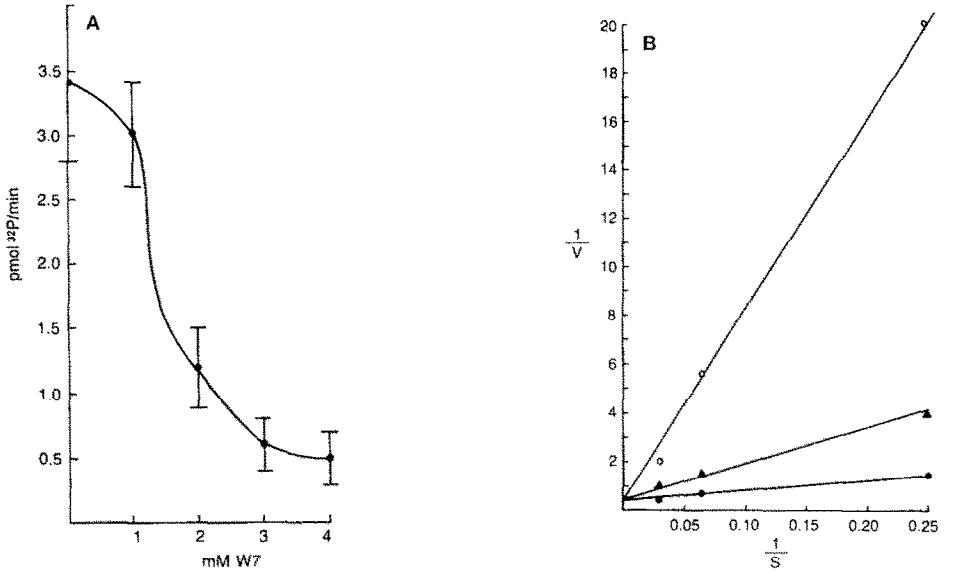

Fig. 4. Inhibition of the phosphotransferase activity of the catalytic fragment of PKC by W7. (A) Phosphotransferase activity was measured in the absence of Ca^{2+} and PS by the procedure detailed in Materials and Methods. (B) Phosphotransferase activity was measured as in panel A, and the [γ^{32}P]ATP concentration was varied (the specific activity of the nucleotide remained constant). Key: (●) 0 mM W7; (▲) 1 mM W7; and (○) 3 mM W7.

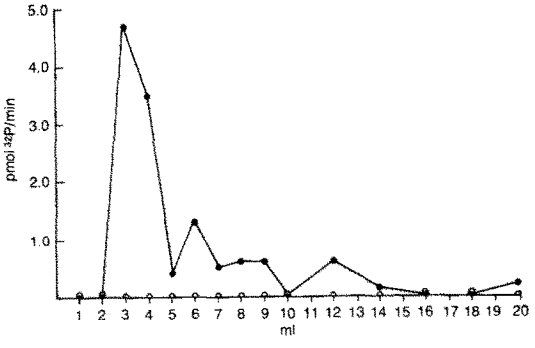

Fig. 5. Elution of PKC from W7-agarose by MgATP. PKC was loaded onto W7-agarose, and the column was washed as described in the legend to Fig. 1A. PKC activity was eluted from the column in a single peak by 10 mM $MgCl_2$ and 1 mM ATP in equilibration buffer. A 10 mM concentration of $MgCl_2$ in the equilibration buffer (in the absence of ATP) could not elute the activity. Key: (●) Ca^{2+}- and PS-dependent protein kinase activity; (○) Ca^{2+}- and PS-independent protein kinase activity.

4B) suggested that W7 was a competitive inhibitor with respect to ATP, we tested whether MgATP could elute intact PKC from W7-agarose. We loaded PKC (6.7 nmol ^{32}P/min) onto a 1.5-ml W7-agarose column and washed the column with 20 ml of equilibration buffer. We found that 10 mM $MgCl_2$ and 1 mM ATP in equilibration buffer eluted PKC (6.7 nmol ^{32}P/min) with a yield of 15% (Fig. 5). In the absence of ATP, 10 mM $MgCl_2$ did not elute the enzyme (data not shown). Taken together, these data provide strong evidence that PKC binds to W7-agarose through the ATP binding region of its active site.

DISCUSSION

The naphthalenesulfonamide W7 is representative of a class of PKC inhibitors which are cationic amphiphiles that interact with phospholipids and inhibit calmodulin-dependent activities [8, 13]. While the mechanism of inhibition of PKC activity by W7 had been hypothesized to involve merely the interactions of W7 with the lipid cofactor of the enzyme, it is now evident that W7, as well as a phenothiazine PKC inhibitor [13] and a triphenylethylene PKC inhibitor [14], have direct interactions with PKC in the absence of lipid and other cofactors of the enzyme.

In this report we have further investigated the mechanism of inhibition of PKC by W7. We demonstrated that a catalytic fragment of PKC, generated by limited tryptic digestion of the enzyme, bound W7-agarose and coeluted with intact PKC when 0.1% Triton X-100 was applied to the column. These results provide evidence that the binding sites for W7 on PKC are in the catalytic rather than the regulatory domain of the enzyme.

We determined that W7 inhibited PKC activity by two distinct mechanisms. Consistent with a previous report [8], W7 inhibited the Ca^{2+}- and PS-dependent activity of the enzyme with an IC_{50} of 260 ± 30 μM. However, W7 did not inhibit the activity of the catalytic fragment of PKC at this inhibitor concentration. Higher concentrations of W7 inhibited the activity of the catalytic fragment of PKC, and we observed 50% inhibition of the activity of the catalytic fragment at a W7 concentration of approximately 1.5 mM. According to kinetic analysis of the inhibition of the catalytic fragment by W7, W7 was

a competitive inhibitor of the catalytic fragment with respect to ATP. W7 also inhibited the PKC-catalyzed phosphorylation of protamine sulfate. Since this phosphotransferase reaction is independent of Ca^{2+} and PS, the inhibition of protamine sulfate phosphorylation by W7 provides another line of evidence that W7 can serve as an active site inhibitor of PKC. MgATP eluted PKC from W7-agarose, substantiating the kinetic evidence that W7 binds at the ATP binding region of the active site of PKC. Consistent with these kinetic results, which suggest that W7 binds weakly to the active site of PKC, 0.5 mM W7 did not elute PKC activity from W7-agarose, indicating that the binding interaction between W7 and PKC is of low affinity.

N - (2 - Aminoethyl) - 5 - isoquinolinesulfonamide (H9) is a competitive inhibitor of PKC with respect to ATP [20]. PKC binds to H9-coupled Sepharose, and the bound enzyme can be eluted from the immobilized inhibitor with 30 mM ATP [21]. The mechanistic studies of the isoquinolinesulfonamide PKC inhibitor H9 and our present study of the naphthalenesulfonamide PKC inhibitor W7 suggest that isoquinolinesulfonamide and naphthalenesulfonamide PKC inhibitors may inhibit PKC through related mechanisms. The relative potencies of a given naphthalenesulfonamide in the inhibition of PKC by interactions with the lipid cofactor of the enzyme and in the inhibition of PKC by binding the ATP binding region of the active site of the enzyme may determine the relatedness of the overall mechanism of inhibition of PKC by the naphthalenesulfonamide to the mechanism of inhibition of PKC by isoquinolenesulfonamides.

Because of the critical role of PKC in tumor promotion and in numerous signal transduction mechanisms, we and other investigators are interested in identifying specific inhibitors of PKC, and, ultimately, specific inhibitors of particular PKC isozymes. Currently, no specific PKC inhibitors have been reported. While our original observation that PKC binds specifically and directly to W7-agarose [13] suggested that the enzyme may contain inhibitor binding sites which could be exploited in the design of specific PKC inhibitors, we now demonstrate that W7 bound to PKC through the ATP binding region of the active site of the enzyme, and that the disruption of the binding interaction between the ATP binding region of PKC and W7 was sufficient to release the enzyme from the immobilized inhibitor. Considering the dual inhibitory activities of W7 against the activation of PKC by phospholipid and against the binding of ATP to the active site of the enzyme, we conclude that the napthalenesulfonamide W7 may be useful in establishing a rationale for the design of more selective PKC inhibitors.

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