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CHARACTERIZATION OF SPECIFIC [^{3}H]DIMETHYLSTAUROSPORINE BINDING TO PROTEIN KINASE C

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Abstract—The microbial alkaloid staurosporine is a member of a recently described family of protein kinase inhibitors. [*N,N-dimethyl*-^{3}H]*N*-dimethylstaurosporine ([^{3}H]DMS) was prepared from staurosporine by methylation with [^{3}H]methyl iodide. Since staurosporine inhibits protein kinase C (PKC) most potently, the binding of [^{3}H]DMS to this enzyme was examined. Unlike [20-^{3}H(N)]phorbol-12,13-dibutyrate ([^{3}H]PDBu) binding to PKC, [^{3}H]DMS binding was not calcium or phosphatidylserine (PS) dependent. Binding was reversible, with a $T_{1/2}$ of 69 min and a K_{off} of 0.01/min. Non-specific binding was defined by a 500-fold molar excess of staurosporine and was less than 10% of total [^{3}H]DMS binding. Specific binding of [^{3}H]DMS was consistent with a single class of binding sites with a K_d of 3.8 ± 0.6 nM and a B_{max} of 675 ± 30 pmol/g tissue. In competition experiments, staurosporine inhibited [^{3}H]DMS binding with a K_i of 4.7 ± 0.6 nM, indicating that the two alkaloids had a similar potency for PKC. Also, unlabeled DMS and staurosporine inhibited [^{3}H]DMS binding and PKC catalysis with equivalent potencies. Highly purified rat brain PKC bound equimolar amounts of [^{3}H]PDBu and [^{3}H]DMS. In contrast, crude rat brain PKC, which had been proteolysed to generate a PS and Ca^{2+} independent enzyme (PK-M) retained the ability to bind [^{3}H]DMS, but not [^{3}H]PDBu. In addition, the kinase inhibitors K-252a and H-7 [1-(5-isoquinolinesulfonyl)-2-methylpiperazine] inhibited [^{3}H]DMS binding, whereas PDBu did not. These results indicate that [^{3}H]DMS is a useful ligand to identify catalytic inhibitors of kinase activity and to explore their mechanisms of action.

Protein kinases, a rapidly expanding family of diverse enzymes, regulate many responses of eukaryotic cells to extracellular signals through protein phosphorylation [1, 2]. PKC‡ is a family of Ca^{2+}- and phospholipid-regulated kinases specifically involved in the transduction of extracellular signals that produce increased phosphatidylinositol turnover [3, 4]. Like many other kinases [1], PKC consists of a regulatory and a catalytic domain [1, 5]. The catalytic domain is highly conserved among kinases and confers serine and threonine substrate specificity to PKC [1]. The regulatory domain is more variable among kinases. In the case of PKC, the regulatory domain shows specificity for phospholipids, such as phosphatidylserine, and *sn*-1,2-diacylglycerol (DAG), an endogenous intracellular second messenger generated from phosphatidylinositol degradation [1–4, 6]. Several classes of structurally distinct tumor promoters such as phorbol esters and aplysiatoxins also bind at the *sn*-1,2-diacylglycerol site of the regulatory domain of PKC [7, 8]. These findings have implicated PKC in cell growth, differentiation, cellular activation, and tumor promotion [3, 4].

Many structurally diverse compounds have been reported to inhibit PKC enzymatic activity. These include H-7 [9, 10], quercetin [10–12], aminoacridines [13], sangivamycin [14], sphingosine [15], tamoxifen [16], gossypol [10], trifluoperazine and other calmodulin antagonists [10, 17], and chloropromazine [18]. The direct interaction of some of these compounds with individual domains of PKC has been demonstrated by competition for [^{3}H]PDBu binding or by inhibition of isolated catalytic fragment activity [10]. Other compounds appear to inhibit by more complex or indirect mechanisms [9–18]. All of these compounds, however, are characterized by either low potency or a relative lack of selectivity for PKC [9–18].

A recently described class of indole carbazoles from natural products are potent inhibitors of several kinases including PKC, cAMP-dependent and cGMP-dependent protein kinases, calcium/calmodulin-dependent protein kinase, and tyrosine-specific protein kinase [19–23]. Each of the alkaloids of this class of compounds, such as staurosporine

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‡ Abbreviations: PKC, protein kinase C; [^{3}H]DMS, [*N,N-dimethyl*-^{3}H]*N,N*-dimethylstaurosporine; [^{3}H]PDBu, [20-^{3}H(N)]phorbol-12,13-dibutyrate; PS, phosphatidylserine; PK-M, calcium and phospholipid independent PKC; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine; DAG, *sn*-1,2-diacylglycerol; quercetin, 2-(3,4-dihydroxyphenyl)3,5,7-trihydroxy-4*H*-1-benzopyran-4-one; PC, phosphatidylcholine; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EGTA, ethyleneglycol-bis (β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N′*-(2-ethanesulfonic acid); TRIS, tris-(hydroxymethyl)aminomethane; and K-252, (8*R**,9*S**,11*S**) - (−) - 9 - hydroxy - 9 - methoxycarbonyl - 8 - methyl-2,3,9,10 - tetrahydro - 8,11 - epoxy-1*H*,8*H*,11*H* - 2,76,11a-triazadibenzo[a,g]cycloocta[cde]trinden-1-one.

[19, 20], K-252a [21, 22], and UCN-01 [23], exhibits a unique spectrum of specificity for kinases. Staurosporine is the most potent inhibitor of PKC activity reported, with a K_i of 2.7 nM, whereas it is a somewhat less potent inhibitor of cAMP-dependent, cGMP-dependent, and tyrosine-specific protein kinases (K_i, values of 7.0, 8.5 and 6.4 nM respectively) [19, 20]. Staurosporine appears to interact at the catalytic domain of PKC since it potently reduces the activity of the catalytic fragment of PKC but has no effect on phorbol ester binding to the intact enzyme [10, 19].

Staurosporine and related compounds have shown a variety of effects, both *in vitro* and *in vivo* [24–28]. Staurosporine antagonizes phorbol ester-induced NADP oxidase activation in neutrophils [24], phorbol ester-mediated HL-60 macrophage differentiation [25], and dioctanoylglycerol-induced phosphorylation of the 47 kD protein in platelets [26]. Staurosporine has been reported to block tumor promotion caused by phorbol esters in mouse skin as well [27]. K-252a, a metabolite isolated from the culture broth of *Nocardiopsis* sp., has anti-inflammatory and anti-allergic activities *in vivo* [29]. Staurosporine and rebeccamycin are cytotoxic to human tumor cells *in vitro* [19, 30, 31]. UCN-01 and rebeccamycin have reported antitumor activities *in vivo* [23, 31].

The importance of staurosporine as an antagonist to PKC-mediated effects in cells has led to the development of a radiolabeled derivative of staurosporine, [*N,N - dimethyl* - ^{3}H]*N,N* - dimethylstaurosporine ([^{3}H]DMS). Conditions for the assay of [^{3}H]DMS binding to soluble PKC are presented in this report. The results indicate that [^{3}H]DMS binds specifically and with high affinity to the catalytic domain of PKC. This binding can be displaced by PKC inhibitors reported to interact with the catalytic domain of this enzyme, including staurosporine, K-252a, and H-7. [^{3}H]DMS is thus a useful ligand for the identification of kinase inhibitors and the characterization of their mechanisms of action.

MATERIALS AND METHODS

Materials. [20-^{3}H(N)]Phorbol-12,13-dibutyrate (20 Ci/mmol), ^{3}H]methyl iodide (80 Ci/mmol), [γ-^{32}P]ATP (3000 Ci/mmol), and Formula-989 scintillation fluid were from Dupont, NEN Products (Boston, MA). [*N,N-dimethyl*-^{3}H]*N,N*-dimethylstaurosporine was prepared at Dupont, NEN Products as described below and is commercially available. Phorbol 12,13-dibutyrate was from Chemsyn Science Laboratories (Lenexa, KA). H-7 was purchased from Seikagaku America (St. Petersburg, FL). Staurosporine and K-252a were obtained from Kyowa Hakko USA (New York City, NY). Synthetic phosphatidylserine (18:1 dioleoyl) and egg phosphatidylcholine (PC) were from Avanti Polar Lipids (Birmingham, AL). E-64 was from Boehringer Mannheim Biochemicals (Indianapolis, IN). Microtiter 96-well u-bottom plates were purchased from Costar (Cambridge, MA). The cell-harvesting apparatus and glass fiber filter mats were products of Skatron, Inc. (Sterling, VA). Phosphocellulose P-81 paper was from Whatman International Ltd. (Maidstone, U.K.). Highly purified (2.5 μmol/min/mg) and proteolysed (PK-M), partially purified PKC from rat brains were provided by Dr. Robert Bell (Duke University Medical Center, Durham, NC). CDF-1 mice were purchased from Charles River Laboratories, Inc. (Wilmington, MA). All other reagents were from the Sigma Chemical Co. (St. Louis, MO) or the Aldrich Chemical Co., Inc. (Milwaukee, WI).

Protein kinase C. Cytosolic PKC from brains of CDF-1 mice was partially purified by DEAE- and threonine-Sepharose chromatography as previously described [32].

Preparation of [^{3}H]*DMS.* Staurosporine (1 mg, 0.002 mmol) was dissolved in 50 μL of dry tetrahydrofuran and treated with 2 Ci of [^{3}H]methyl iodide for 18 hr at room temperature [33]. The material contained 165 mCi after removal of labiles. Thin-layer chromatography (TLC) on Analtech SiO_2 GHLF analytical plates eluted with *n*-butanol–acetic acid–water (4:1:1, by vol.) showed that 48% of the radioactivity appeared as a single spot at R_f 0.19, and the remainder of the radioactivity was at the solvent front. In this TLC, unlabeled staurosporine migrated as a highly fluorescent spot (UV) at R_f 0.54. The crude material was concentrated and streaked in a minimum volume of methanol onto an Analtech SiO_2 GHLF plate (20 cm × 20 cm; 250 μm). The plate was eluted with chloroform–methanol–water (100:10:1, by vol.), and bands were visualized with a UV lamp. Two strongly fluorescent bands appeared at origin and at the solvent front. The band at the origin was scraped and eluted with ethanol–water–acetic acid (50:50:1, by vol.) to give 69.7 mCi of material. Analysis of this material by the butanol system showed that only minor, more-polar impurities remained. A portion of this material was purified twice more by preparative TLC using the butanol system described. Bands at about R_f 0.15–0.25 were visualized with UV light, scraped, and eluted with ethanol–water–acetic acid (50:50:1, by vol.). Recovery from each TLC was greater than 80%. TLC showed that the radiochemical purity after the first and second purification was 95.2 and 97.5% respectively. Tritium NMR (CD_3OD) of this material gave a single peak at 3.19 ppm. The specific radioactivity of the [^{3}H]DMS was determined based on the specific activity of the [^{3}H]methyl iodide and was calculated to be 160 Ci/mmol. The unlabeled DMS was prepared in an equivalent manner and purified by preparative TLC.

[^{3}H]*DMS and* [^{3}H]*PDBu stocks.* [^{3}H]DMS or [^{3}H]PDBu was evaporated to dryness under a stream of nitrogen in the absence (total) or presence (nonspecific) of a 500-fold molar excess of either staurosporine or PDBu respectively. [^{3}H]DMS or [^{3}H]PDBu stocks were made 10 μM by the addition of dimethyl sulfoxide (DMSO). Subsequent dilutions of stocks were made in DMSO and stored at 4°.

[^{3}H]*PDBu and* [^{3}H]*DMS binding assay.* Conditions for measuring [^{3}H]PDBu binding using a soluble binding assay have been described previously [32]. Briefly, a 200-μL reaction mixture containing 20 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 500 μg/mL bovine γ-globulin, 10 μg/mL sonicated PS, 40 μg/mL sonicated PC, PKC, and

[^{3}H]PDBu (total or non-specific) was incubated in 96-well microtiter plates for 1 hr at room temperature. Reaction mixtures were aspirated with a cell-harvesting apparatus onto glass fiber filter mats which had been soaked previously for 1 hr in 0.3% (w/v) aqueous polyethyleneimine. Filter mats were treated automatically with two successive 10-sec water washes followed by a 10-sec air treatment. Filter disks containing PKC–ligand complexes were removed from mats with forceps and added to scintillation vials containing 4 mL of fluid. Radioactivity was measured with a Packard Tricarb 2000CA liquid scintillation counter.

[^{3}H]DMS binding was performed similarly. The composition of the binding assay was 50 mM Tris–HCl (pH 8.0), 500 μg/mL bovine γ-globulin, 1 mM dithiothreitol (DTT), PKC, and [^{3}H]DMS (total or non-specific) unless otherwise indicated. Binding was performed on ice for 15 min followed by filtration as described above. The PKC used in both binding assays was 2.5 to 5 μg of crude rat or mouse brain preparations or 25 ng of purified PKC. These amounts of PKC were within the linear region for both [^{3}H]PDBu and [^{3}H]DMS binding. For measurements of K_d, the concentrations of ligands were from 0.1 to 30 nM; otherwise, the ligand concentrations were as stated. For [^{3}H]DMS competition experiments, compounds were added in a total volume of 5 μL in DMSO prior to the addition of [^{3}H]DMS. Specific binding was the difference between total and non-specific binding. All measurements were the average of triplicate determinations. Binding parameters were determined by non-linear regression curve fitting using RS/1 (BBN Research Systems, Cambridge, MA).

PKC catalytic assay. Basal kinase activity was measured in reaction mixtures of 20 mM Tris–HCl (pH 7.5), 5 mM EGTA, 400 μg/mL histone H1, 25 ng purified rat brain PKC, various concentrations of compound in DMSO and 20 μM [^{32}P]ATP. To measure agonist-stimulated kinase activity, EGTA was replaced with 0.5 mM $CaCl_2$, 20 μg/mL PS, 5 mM $MgCl_2$, and 40 nM phorbol myristate acetate. Reactions were initiated by the addition of enzyme, incubated for 15 min at room temperature, and terminated by spotting 50-μL aliquots in triplicate onto phosphocellulose squares. The squares were washed four times (10 min) with water and once with acetone. Filters were air dried and radioactivity was measured as described above [34]. The PKC catalytic activity was the difference between agonist-stimulated and basal activities, measured in the presence of compound.

Protein determination. Protein was measured according to the method of Lowry *et al.* [35] using bovine serum albumin as a standard.

RESULTS

[^{3}H]DMS binding to mouse brain PKC was first optimized by examining several conditions of the binding reaction including pH, ionic strength and composition, and temperature. Total [^{3}H]DMS binding was not affected significantly when assays were performed in three buffers ranging in pH from 5.8 to 9.7 (data not shown). Since total [^{3}H]DMS binding was also increased slightly (10%) at pH 8.1 in 50 mM Tris–HCl and by an additional 14% by the inclusion of 1 mM DTT, further binding studies were performed in 50 mM Tris–HCl (pH 8.1) with 1 mM DTT (data not shown). Although total [^{3}H]DMS binding was stable for up to 3 hr when assays were incubated on ice, [^{3}H]DMS binding was carried out routinely for 15 min on ice, since binding reached equilibrium within this time (data not shown).

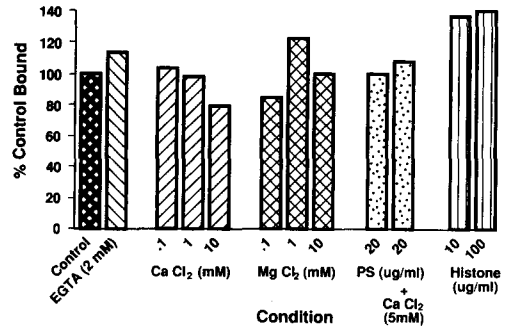

Fig. 1. Effect of assay components on [^{3}H]DMS binding to PKC. Mouse brain PKC was incubated for 15 min on ice in 50 mM Tris–HCl (pH 8.1) with 1 mM DTT, 500 μg/mL γ-globulin and 2 nM [^{3}H]DMS with or without (control) additions described. Total binding was measured in triplicate. Non-specific binding was less than 10% of total binding. Specific binding of control samples was 130 ± 8 fmol [^{3}H]DMS (mean ± SD, N = 3) and represents 100%. The data are representative of two separate experiments.

Conditions required for either [^{3}H]PDBu binding to PKC or for PKC catalysis were examined for their effects on [^{3}H]DMS binding. As shown in Fig. 1, calcium and PS, which are necessary for [^{3}H]PDBu binding to PKC, had no effect on [^{3}H]DMS binding, either alone or in combination. Interestingly, agents involved in PKC catalysis, such as 1 mM $MgCl_2$ and substrate histone H1, increased [^{3}H]DMS binding by 22% and 40% respectively (Fig. 1).

[^{3}H]DMS binding to PKC was reversible. [^{3}H]DMS binding to PKC was allowed to reach equilibrium and then was incubated for various amounts of time at 4° with a 250-fold molar excess of unlabeled staurosporine. As shown in Fig. 2, the kinetics of dissociation of [^{3}H]DMS from PKC were linear. The time required for 50% dissociation of bound [^{3}H]DMS from PKC was 69 min ($T_{1/2}$), with an apparent K_{off} of 0.01 min^{-1}.

Staurosporine competed as effectively as [^{3}H]DMS for binding to PKC. Using various concentrations of [^{3}H]DMS in the presence of a 500-fold molar excess of staurosporine, non-specific binding was less than 10% of total binding (Fig. 3A). Analysis of specific [^{3}H]DMS binding data by the method of Scatchard [36] was consistent with a single class of binding sites with a K_d of 3.8 ± 0.6 nM and a B_{max} of 675 ± 30 pmol/g brain tissue (Fig. 3B). The calculated B_{max} for [^{3}H]PDBu binding to the same PKC preparation was 650 ± 20 pmol/g brain tissue, suggesting that most of the [^{3}H]DMS binding was to PKC. These preparations of mouse brain PKC did not contain any cAMP- or cGMP-dependent kinase activity (data not shown).

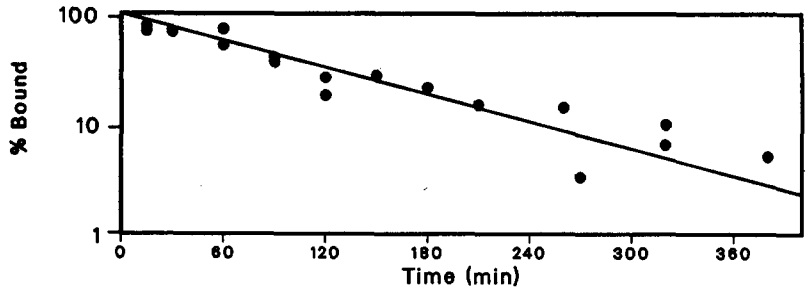

Fig. 2. Determination of K_{off} of [^{3}H]DMS bound to PKC. Mouse brain PKC was incubated in 50 mM Tris–HCl (pH 8.1), 1 mM DTT, 500 μg/mL γ-globulin, and 3 nM [^{3}H]DMS for 15 min on ice to reach equilibrium. At equilibrium, specific binding of control samples was 119 ± 11 fmol [^{3}H]DMS (mean ± SD, N = 3) and represents 100% bound. Unlabeled staurosporine (1 μM) or DMSO (control) was added. At various times thereafter, triplicate aliquots were removed and specific binding was measured by filtration. The data are representative of two separate experiments.

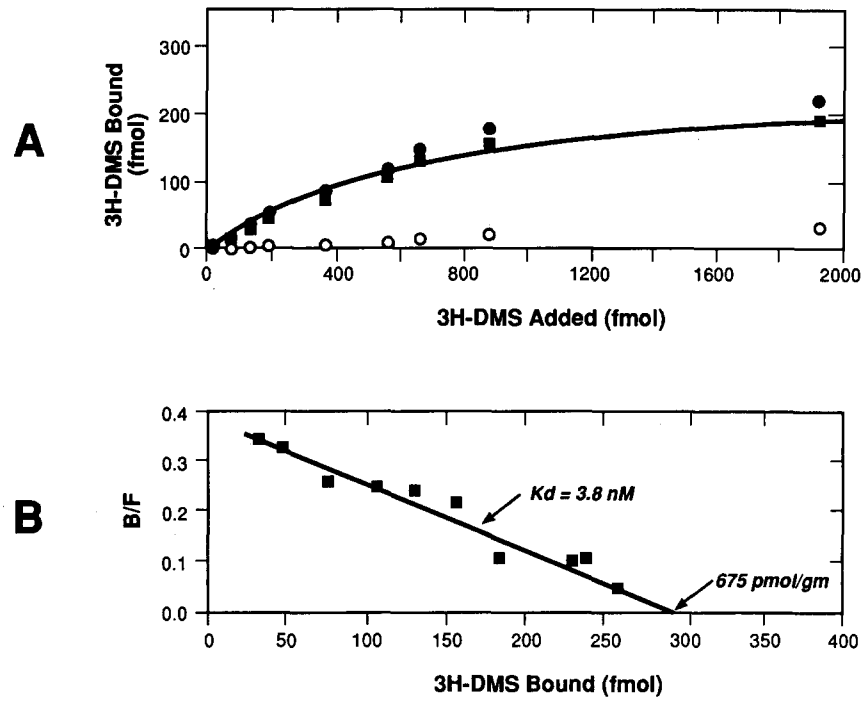

Fig. 3. Specific [^{3}H]DMS binding to PKC. (A) Mouse brain PKC was incubated for 15 min on ice in 50 mM Tris–HCl (pH 8.1) with 1 mM DTT, 500 μg/mL γ-globulin, and various concentrations (0.25 to 30 nM) of [^{3}H]DMS. Specific binding (■) was the difference between total (●) and non-specific binding (○), measured in the presence of a 500-fold excess of unlabeled staurosporine. Data are the average of triplicate measurements and are representative of a typical experiment. (B) [^{3}H]DMS binding data were calculated according to the method of Scatchard [36].

In competition experiments using three concentrations of [^{3}H]DMS, staurosporine effectively inhibited specific binding of [^{3}H]DMS with a K_i of 4.7 ± 0.6 nM (Fig. 4A). The K_d of [^{3}H]DMS for PKC estimated in the same experiment was 5.0 nM (Fig. 4B), suggesting that staurosporine and [^{3}H]DMS bound with equivalent potencies to PKC.

Further evidence that staurosporine and dimethylstaurosporine were equivalent in their effects on PKC is presented in Table 1. In competition experiments to mouse brain PKC, unlabeled dimethylstaurosporine and staurosporine were similar in potency at inhibiting [^{3}H]DMS binding, with K_i values of 4.7 and 4.1 nM respectively. The PS-and-calcium stimulatable catalytic activity of highly purified rat brain PKC was also inhibited concentration-dependently by dimethylstaurosporine and staurosporine. The IC_{50} values for catalytic inhibition by dimethylstaurosporine and staurosporine were similar in potency and were 4.2 and 4.3 nM respectively (Table 1).

The stoichiometry of [^{3}H]DMS binding to highly

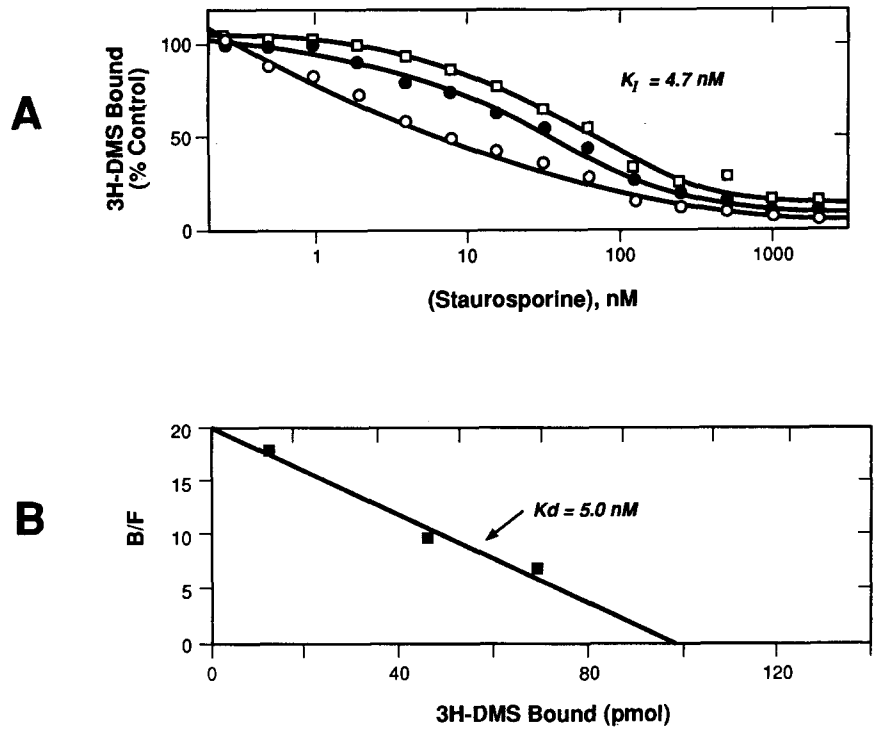

Fig. 4. Competition of [3H]DMS binding to PKC by staurosporine. (A) Mouse brain PKC was incubated on ice for 15 min in 50 mM Tris–HCl (pH 8.1), 1 mM DTT, 500 μg/mL γ-globulin, and various concentrations of unlabeled staurosporine in the presence of 1 nM (○), 6 nM (●) or 12.5 nM [3H]DMS (□). Specific [3H]DMS binding values of control samples at 1, 6 and 12.5 nM [3H]DMS were 61.9 ± 3.9, 230 ± 18, and 350 ± 33 fmol respectively (mean ± SD, N = 3). The data are representative of two separate experiments. (B) [3H]DMS binding data were calculated according to the method of Scatchard [36].

Table 1. Comparison of staurosporine and dimethylstaurosporine on [3H]DMS binding and catalytic activity of PKC

	[3H]DMS binding K_i (nM)	PKC catalysis IC_{50} (nM)
Dimethylstaurosporine	4.7 ± 1.0	4.2 ± 0.7
Staurosporine	4.1 ± 0.8	4.3 ± 0.9

Specific [3H]DMS binding to mouse brain PKC was measured in the presence of various concentrations of unlabeled dimethylstaurosporine or staurosporine. The K_i value was calculated from the IC_{50}, as described in Materials and Methods. The concentration of [3H]DMS was 9.9 nM. The inhibition of calcium and phospholipid dependent kinase activity of purified rat brain PKC was measured as described in Materials and Methods.

purified rat PKC was measured. As shown in Table 2, rat brain PKC bound approximately equivalent amounts of [3H]DMS and [3H]PDBu (6.1 and 6.3 pmol/μg PKC) as determined by estimation of B_{max} from analysis of specific binding data. Assuming that this PKC preparation was greater than 95% pure as judged by polyacrylamide gel electrophoresis*, the stoichiometry of both [3H]DMS and [3H]PDBu binding was approximately 0.5 pmol ligand bound/pmol PKC. The K_d values for [3H]DMS and [3H]PDBu to intact protein kinase C were 2.5 and 0.81 nM respectively (Table 2). The K_d values of these ligands were not significantly different (2.4 and 0.87 nM respectively) to a crude preparation of rat brain protein kinase M, which was auto-proteolysed so that it was no longer phospholipid and calcium dependent for enzymatic activity. The small amount of high-affinity [3H]PDBu binding to the protein kinase M preparation (0.03 pmol/μg, Table 2) was most likely to be incompletely digested PKC, since intact and proteolysed PKC were not chromatographically separated.

The interaction of staurosporine with the catalytic domain of PKC has been demonstrated [10]. To substantiate a similar conclusion for [3H]DMS, binding of [3H]DMS to intact PKC and protein kinase M was compared. As shown in Table 2, protein kinase M bound 23% of the [3H]PDBu, compared to the [3H]DMS (0.03 pmol/μg [3H]PDBu compared to 0.13 pmol/μg [3H]DMS respectively). Since protein

* Bell R, personal communication, cited with permission.

Table 2. [^{3}H]PDBu and [^{3}H]DMS binding to rat brain protein kinase C and M

	[^{3}H]DMS		[^{3}H]PDBu	
	K_d (nM)	B_{max} (pmol/μg)	K_d (nM)	B_{max} (pmol/μg)
Protein kinase C	2.5	6.1	0.81	6.3
Protein kinase M	2.4	0.13	0.87	0.03

Specific [^{3}H]DMS and [^{3}H]PDBu binding was measured on the rat brain protein kinase preparations by filtration. The PKC preparations were of different degrees of purity. The K_d and B_{max} values were estimated from data analysis according to the method of Scatchard [36].

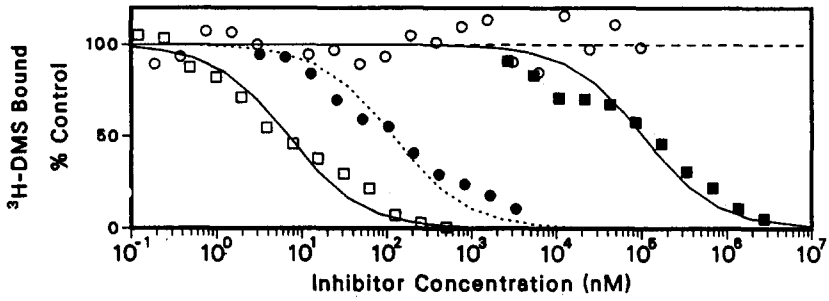

Fig. 5. Competition of [^{3}H]DMS binding to PKC by PKC active agents. Mouse brain PKC was incubated for 15 min on ice in 50 mM Tris–HCl (pH 8.1), with 1 mM DTT, 500 μg/mL γ-globulin, 3 nM [^{3}H]DMS, and various concentrations of PDBu (○), staurosporine (□), K-252a (●), or H-7 (■). Specific [^{3}H]DMS bound by control samples (100% bound) was 102 ± 8 fmol (mean ± SD, N = 3), as determined by the difference between total and non-specific binding. The calculated K_i values for the representative PKC antagonists were the average from competition curves performed at 1.1, 3.0, 5.4, and 11.2 nM [^{3}H]DMS. The calculated K_i values for the respective PKC antagonists were 4.7 nM, 66 nM, and 79 μM. The solid and dashed lines represent theoretical inhibition curves.

kinase M retained [^{3}H]DMS binding, but did not bind an equivalent amount of [^{3}H]PDBu (Table 2), [^{3}H]DMS most likely bound to the catalytic domain of PKC.

PDBu and other compounds reported to interact with PKC [10] were tested for their abilities to displace [^{3}H]DMS from the mouse brain enzyme. As shown in Fig. 5, PDBu could not displace [^{3}H]DMS binding to PKC when tested at concentrations up to 100 μM. These results were consistent with the conclusion that [^{3}H]DMS bound to the catalytic domain of PKC and could not be displaced by a ligand which bound specifically to the regulatory domain. In addition, the following data suggested that [^{3}H]DMS bound to the catalytic domain of PKC but not to the catalytic or ATP-binding site of PKC. Using binding conditions described in this report, the K_i for Mg^{2+}-ATP for [^{3}H]DMS displacement was 2.5 mM (unpublished data), which is at least two orders of magnitude higher than the reported K_m of PKC for ATP [9].

The displacement of [^{3}H]DMS from PKC by other compounds reported to interact with the catalytic domain of PKC is also presented in Fig. 5. As shown previously (Fig. 4A), staurosporine inhibited [^{3}H]DMS binding with a calculated K_i of 4.7 nM. K-252a inhibited [^{3}H]DMS binding less potently than staurosporine with a calculated K_i of 66 nM (Fig. 5). Of the compounds tested, the kinase inhibitor H-7 was the least potent inhibitor of [^{3}H]DMS binding to PKC; the estimated K_i was 79 μM. The relative potency of these compounds at inhibiting [^{3}H]DMS binding to PKC was in agreement with reports in the literature [9, 10, 19, 21]. However, with the exception of staurosporine, the IC_{50} values for [^{3}H]DMS displacement were higher than values reported to inhibit PKC activity *in vitro* [9, 10, 19, 21] (Fig. 5). The slopes of the displacement curves were also less than one, the slope of the theoretical displacement curve, suggesting some heterogeneity in the affinities of [^{3}H]DMS and/or the inhibitors for the PKC isozymes.

DISCUSSION

Characteristics of a soluble binding assay using the ligand [^{3}H]DMS have been presented. Total [^{3}H]DMS binding was performed in a simple buffer containing γ-globulin and DTT to prevent non-specific binding to surfaces and to stabilize PKC [37] respectively. Total [^{3}H]DMS binding was stable and unaffected by many of the conditions tested. In addition, this ligand binding assay required fewer components than the corresponding [^{3}H]PDBu binding assay, which needs phospholipid and calcium for maximal binding [32, 38, 39]. The reasons for the

increased [^{3}H]DMS binding observed in the presence of magnesium and histone are not yet understood. However, magnesium has been also reported to increase [^{3}H]PDBu and [^{3}H]TPA (12-*O*-tetradecanoylphorbol-13-acetate) binding to PKC [37, 40]. The interaction of PKC with its substrate, histone, may stabilize [^{3}H]DMS–PKC interactions as well.

Methylation of staurosporine apparently did not alter characteristics of the radiolabeled ligand, since [^{3}H]DMS bound as effectively as staurosporine to PKC. Also, staurosporine and dimethylstaurosporine were equipotent as antagonists to PKC catalytic activity. Because of these observations, commercially available staurosporine was used to define non-specific binding in this assay. Using these conditions, [^{3}H]DMS was shown to bind reversibly and potently to PKC from mouse and rat brain. The potencies of [^{3}H]DMS binding (2.4 to 5.0 nM) to rat and mouse brain PKC were similar to the potencies of staurosporine reported to inhibit PKC catalysis [10, 19, 24].

[^{3}H]PDBu has been reported to bind PKC with a stoichiometry of 0.9:1 [41]. Using the soluble binding assays described in this paper, both [^{3}H]PDBu and [^{3}H]DMS bound with a 0.48:1 (ligand:protein) stoichiometry to highly purified rat brain PKC, assuming a molecular weight of 77 kD for the PKC isozymes [41]. Although the reasons for this discrepancy have not yet been clarified, the techniques used to separate [^{3}H]PDBu–receptor complexes from free ligand differed (gel filtration [41] versus rapid filtration). It is also possible that the highly-purified PKC used in the experiments reported in this paper contained some denatured protein which was incapable of binding either ligand.

The concentrations of K-252a and H-7 reported to inhibit PKC enzymatic activity [9, 10, 21] were lower than those which inhibited [^{3}H]DMS binding to PKC. Since it is known that the preparation of mouse brain PKC which was used contained at least three isoforms of this enzyme (unpublished observations), it is likely that these differences were from heterogeneity in [^{3}H]DMS binding or from differences in the effectiveness of compounds on individual PKC isoenzymes. This was supported by the deviation of the slopes of the inhibition curves from the theoretical curve. The affinities of [^{3}H]DMS for the PKC isozymes, and the inhibition profiles of [^{3}H]DMS binding by K-252a and H-7 for each of the isozymes remain to be determined.

Compounds which interact with PKC have been identified by competition with [^{3}H]PDBu or by inhibition of the kinase activity of intact enzyme or catalytic fragment [10]. A limitation of using [^{3}H]PDBu competition to detect PKC inhibitors has been demonstrated, since some inhibitors have been shown to indirectly inhibit PKC by disturbing phospholipid/enzyme interactions [10]. Also, inhibitors at the catalytic site may not be detected in [^{3}H]PDBu competition studies (for example, H-7), and, conversely, agents interacting at the regulatory site may not be detected in catalytic assays or with [^{3}H]DMS competition studies. Indeed, this was reported in this paper, where PDBu was ineffective in competing for [^{3}H]DMS binding to PKC. More direct interactions of compounds with PKC have been demonstrated by examining their effects on the isolated catalytic fragment of PKC [10]. Since [^{3}H]DMS bound to the catalytic domain of PKC, this binding assay can be used directly to identify compounds which interact at or near the [^{3}H]DMS binding site on the catalytic domain of PKC. The effects of a compound on [^{3}H]PDBu and [^{3}H]DMS binding to PKC, as well as its effects on enzymatic activity, should help define its mode of interaction with PKC.

Another application of [^{3}H]DMS is to follow PKC purification. The combined activities of [^{3}H]PDBu and [^{3}H]DMS binding should provide a complete profile of PKC through purification protocols. Since [^{3}H]DMS has been synthesized to a high specific activity, this ligand provides even more sensitivity than [^{3}H]PDBu under comparable conditions.

The results presented indicate that [^{3}H]DMS is a useful ligand to identify catalytic inhibitors of kinase activity and to explore their mechanisms of action.

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REFERENCES

1. Hanks SK, Quinn AM and Hunter T, The protein kinase C family: Conserved features and deduced phylogency of the catalytic domains. *Science* **241**: 42–52, 1988.
2. Blackshear PJ, Nairn AC and Kuo JF, Protein kinases 1988: A current perspective. *FASEB J* **2**: 2957–2969, 1988.
3. Nishizuka Y, Studies and perspectives of protein kinase C. *Science* **233**: 305–312, 1986.
4. Kikkawa U and Nishizuka Y, The role of protein kinase C in transmembrane signalling. *Annu Rev Cell Biol* **2**: 149–178, 1986.
5. Coussens L, Parker PJ, Rhee L, Yang-Feng TL, Chen E, Waterfield MD, Franke U and Ullrich A, The complete primary structure of protein kinase C—The major phorbol ester receptor. *Science* **233**: 853–859, 1986.
6. Takai Y, Kishimoto A, Kikkawa U, Mori T and Nishizuka Y, Unsaturated diacylglycerol as a possible messenger for the activation of calcium-activated, phospholipid-dependent protein kinase system. *Biochem Biophys Res Commun* **91**: 1218–1224, 1979.
7. Ashendel CL, Staller JM and Boutwell RK, Identification of a calcium- and phospholipid-dependent phorbol ester binding activity in the soluble fraction of mouse tissues. *Biochem Biophys Res Commun* **111**: 340–345, 1983.
8. Moore RE, Patterson GML, Entzeroth M, Morimoto H, Suganuma M, Hakii H, Fujiki H and Sugimura T, Binding studies of [^{3}H]lyngbyatoxin A and [^{3}H]debromoaplysiatoxin to the phorbol ester receptor in a mouse epidermal particulate fraction. *Carcinogenesis* **7**: 641–644, 1986.
9. Hidaka H, Inagaki M, Kawamoto S and Sasaki Y, Isoquinoline sulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. *Biochemistry* **23**: 5036–5041, 1984.
10. Nakadate T, Jeng AY and Blumberg PM, Comparison of protein kinase C functional assays to clarify mechanisms of inhibitor action. *Biochem Pharmacol* **37**: 1541–1545, 1988.
11. Gschwendt M, Horn F, Kittstein W and Marks F, Inhibition of the calcium- and phospholipid-dependent

protein kinase activity from mouse brain cytosol by quercetin. *Biochem Biophys Res Commun* **117**: 444–447, 1983.
12. Horn F, Gschwendt M and Marks F, Partial purification and characterization of the calcium-dependent and phospholipid-dependent protein kinase C from chick oviduct. *Eur J Biochem* **148**: 533–538, 1985.
13. Hannun YA and Bell RM, Aminoacridines, potent inhibitors of protein kinase C. *J Biol Chem* **263**: 5124–5131, 1988.
14. Loomis CR and Bell RM, Sangivamycin, a nucleoside analog, is a potent inhibitor of protein kinase C. *J Biol Chem* **263**: 1682–1692, 1988.
15. Hannun YA and Bell RM, Lysosphingolipids inhibit protein kinase C: Implications for the sphingolipidoses. *Science* **235**: 670–674, 1987.
16. O'Brian CA, Liskamp RM, Solomon DH and Weinstein IB, Inhibition of protein kinase C by tamoxifen. *Cancer Res* **45**: 2462–2465, 1985.
17. Schatzman RC, Raynor RL and Kuo JF, *N*-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), a calmodulin antagonist, also inhibits phospholipid-sensitive calcium-dependent protein kinase. *Biochim Biophys Acta* **755**: 144–147, 1987.
18. Mori T, Takai Y, Minakuchi R, Yu B and Nishizuka Y, Inhibitory action of chlorpromazine, dibucaine, and other phospholipid-interacting drugs on calcium-activated, phospholipid-dependent protein kinase. *J Biol Chem* **255**: 8378–8380, 1980.
19. Tamoki T, Nomoto H, Takahashi I, Kato Y, Morimoto M and Tomita F, Staurosporine, a potent inhibitor of phospholipid/Ca^{++} dependent protein kinase. *Biochem Biophys Res Commun* **135**: 397–402, 1986.
20. Nakano H, Kobayashi E, Takahashi I, Tamaoki T, Kuzuu Y and Iba J, Staurosporine inhibits tyrosine-specific protein kinase activity of Rous sarcoma virus transforming protein p60. *J Antibiot (Tokyo)* **40**: 706–708, 1987.
21. Kase H, Iwahashi K and Matsuda Y, K-252a, a potent inhibitor of protein kinase C from microbial origin. *J Antibiot (Tokyo)* **39**: 1059–1065, 1986.
22. Kase H, Iwahashi K, Nakanishi S, Matsuda Y, Yamada K, Takahashi M, Murakata C, Kato A and Kaneko M, K-252 compounds, novel and potent inhibitors of protein kinase C and cyclic nucleotide-dependent protein kinases. *Biochem Biophys Res Commun* **142**: 436–440, 1987.
23. Takahashi I, Kobayashi E, Asano K, Yoshida M and Nakano H, UCN-01, a selective inhibitor of protein kinase C from *Streptomyces*. *J Antibiot (Tokyo)* **40**: 1782–1784, 1987.
24. Sako T, Tauber AI, Jeng AY, Yuspa SH and Blumberg PM, Contrasting actions of staurosporine, a protein kinase C inhibitor, on human neutrophils and primary mouse epidermal cells. *Cancer Res* **48**: 4646–4650, 1988.
25. Okazaki T, Kato Y, Mochizuka T, Tashima M, Sawada H and Uchino H, Staurosporine, a novel protein kinase inhibitor, enhances HL-60-cell differentiation induced by various compounds. *Exp Hematol* **16**: 42–48, 1988.
26. Watson SP, McNally J, Shipman LJ and Godfrey PP, The action of the protein kinase C inhibitor, staurosporine, on human platelets. *Biochem J* **249**: 345–350, 1988.
27. Yamada S, Hirota K, Chida K and Kuroki T, Inhibition of phorbol ester-caused induction of ornithine decarboxylase and tumor promotion in mouse skin by staurosporine, a potent inhibitor of protein kinase C. *Biochem Biophys Res Commun* **157**: 9–15, 1988.
28. Koizumi S, Contreras ML, Matsuda Y, Hama T, Lazarovici P and Guroff G, K-252a: A specific inhibitor of the action of nerve growth factor on PC12 cells. *J Neurosci* **82**: 715–721, 1988.
29. Ohmori K, Ishii H, Manabe H, Satoh H, Tamura T and Kase H, Antiinflammatory and antiallergic effects of a novel metabolite of *Nocardiopsis* sp. as a potent protein kinase C inhibitor from microbial origin. *Arzneimittelforschung* **38**: 809–814, 1988.
30. Nettleton DE, Doyle TW, Krishnan B, Matsumoto GK and Clardy J, Isolation and structure of rebeccamycin—A new antitumor antibiotic from *Nocardia aerocolonigenes*. *Tetrahedron Lett* **25**: 4011–4014, 1985.
31. Bush JA, Long BH, Catino JJ and Bradner WT, Production and biological activity of rebeccamycin, a novel antitumor agent. *J Antibiot (Tokyo)* **40**: 668–678, 1987.
32. Gross JL and Stephens LE, An automated filtration assay for phorbol ester receptors. *Anal Biochem*, in press.
33. Switor JE and Pittman KE, Synthesis of [^{3}H]Porfiromycin. *J Label Comp Radiopharm* **21**: 789–791, 1984.
34. Perrella FW, Hellmig BD and Diamond L, Up regulation of the phorbol ester receptor–protein kinase C in HL-60 variant cells. *Cancer Res* **46**: 567–572, 1986.
35. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
36. Scatchard G, The attraction of proteins for small molecules and ions. *Ann NY Acad Sci* **51**: 660–672, 1949.
37. Ashendel CL, Staller JM and Boutwell RK, Protein kinase activity associated with a phorbol ester receptor from mouse brain. *Cancer Res* **43**: 4333–4337, 1983.
38. Leach KL, James ML and Blumberg PM, Characterization of a specific phorbol ester aporeceptor in mouse brain cytosol. *Proc Natl Acad Sci USA* **80**: 4208–4212, 1983.
39. Tanaka Y, Miyake R, Kittawa U and Nishizuka Y, Rapid assay of binding of tumor-promoting phorbol esters to protein kinase C. *J Biochem (Tokyo)* **99**: 257–261, 1986.
40. Sando JJ and Young MC, Identification of high-affinity phorbol ester receptor in cytosol of EL4 thymoma cells: Requirement for calcium, magnesium, and phospholipids. *Proc Natl Acad Sci USA* **80**: 2642–2646, 1983.
41. Kikkawa U, Takai Y, Tanaka Y, Miyake R and Nishizuka Y, Protein kinase C as a possible receptor protein of tumor-promoting phorbol esters. *J Biol Chem* **258**: 11442–11445, 1983.