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ATP-Sensitive Binding of Melittin to the Catalytic Domain of Protein Kinase C

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SUMMARY

Protein kinase C (PKC) is a Ca^{2+}- and phospholipid-dependent protein kinase that appears to play a critical role in the regulation of cell growth. Melittin was previously shown to inhibit Ca^{2+}- and phosphatidylserine (PS)-dependent PKC activity with an inhibitory potency that was reduced as the PS concentration was elevated. In this report, we found that melittin could inhibit activation of PKC by Ca^{2+} and PS, with an IC_{50} of 3 μM. When the enzyme activity was released from regulation by Ca^{2+} and PS by the generation of an active catalytic fragment of PKC through limited proteolysis, melittin inhibited the enzyme activity with an IC_{50} of 25 μM. Through inhibitor binding studies and enzyme kinetics, we established that melittin binds directly to the catalytic domain of PKC and that the substrate MgATP can release bound melittin from PKC. Melittin bound to PKC in the absence of PKC cofactors, and MgATP completely disrupted the binding of melittin to PKC, whereas phosphoacceptor substrates did not. The catalytic fragment of PKC, which contains two potential ATP-binding sites according to sequence analysis of PKC-encoding cDNAs, also bound melittin. The kinetics of inhibition of the catalytic fragment were consistent with a noncompetitive inhibition with respect to the substrate ATP, providing evidence that the antagonism of the binding of melittin to PKC by MgATP is not due to a direct competition between MgATP and melittin at the active site of PKC.

PKC, the Ca^{2+}- and phospholipid-dependent protein kinase, is composed of a famiy of closely related isozymes (1–3) that appear to play a critical role in the regulation of cell growth, according to three major lines of evidence. First, phorbol esters and related tumor promoters bind to PKC with high affinity and specifically activate the enzyme (4–6). Second, certain growth factors can stimulate a phospholipase C-catalyzed production of diacylglycerol, an endogenous activator of PKC (6, 7). Finally, the specific introduction of PKC-encoding cDNA into cultured fibroblasts can result in an overexpression of PKC activity concomitant with a disruption of cellular growth control mechanisms (8, 9).

In view of the evidence that PKC plays a fundamental role in the mediation of extracellular signals that regulate growth, an understanding of the mechanisms of action of PKC inhibitors may facilitate the rational design of antiproliferative agents targeted against PKC. A number of PKC inhibitors have been identified, including melittin (10), chlorpromazine (11), staurosporine (12), the antitumor agents tamoxifen (13, 14) and sangivamycin (15), and sphingosine, which may be an endogenous inhibitor of the enzyme (16).

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Melittin, a toxic peptide isolated from bee venom, was previously shown to inhibit calmodulin-sensitive protein kinase activity and the Ca^{2+}- and PS-dependent activity of PKC (10). This report demonstrates that melittin binds the catalytic domain of PKC in the absence of lipid and other cofactors. The primary structure of melittin is established, and its secondary structure is well defined (17, 18). Therefore, the binding of melittin to the catalytic domain of PKC that we report here suggests that melittin, along with structural analogs of this 26-amino acid polypeptide, may be valuable as probes of the structure and function of the catalytic domains of PKC isozymes.

Experimental Procedures

Materials. [γ-^{32}P]ATP was purchased from Amersham Corporation (Arlington Heights, IL). Whatman phosphocellulose paper, grade p81, was purchased from Fisher Scientific (Houston, TX). Tris-HCl, bovine serum albumin, histone III-S, ATP, PS, PMSF, leupeptin, Triton X-100, soybean trypsin inhibitor type I, CNBr-activated Sepharose 4B, *N*-tosyl-L-phenylalanyl chloromethyl ketone-treated trypsin from bovine pancreas (specific activity, 10,000 to 13,000 *N*-α-benzoyl-L-arginine ethyl ester units/mg of protein), kemptide, purified catalytic subunit of bovine heart PKA (36 nM units/mg of protein), and melittin (approximately 70% pure by high performance liquid chromatography

ABBREVIATIONS: PKC, protein kinase C; H7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; IC_{50}, concentration at which 50% inhibition is observed; PKA, cAMP-dependent protein kinase; PMSF, phenylmethylsulfonyl fluoride; PS, phosphatidylserine; W7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

and containing <20 units of phospholipase A_2/mg of solid) were purchased from Sigma Chemical Co. (St. Louis, MO). Pure melittin (>97% pure by high performance liquid chromatography) and [Ser^{25}]PKC(19–31) were purchased from Peninsula Laboratories (Belmont, CA). Frozen rat brains were purchased from Charles River Breeding Co. (Wilmington, MA). Protein concentrations were determined using the Bio-Rad protein assay solution, and polyacrylamide gels were stained using the Bio-Rad silver stain kit (Bio-Rad, Richmond, CA).

Preparation of melittin-agarose. Three grams of CNBr-activated Sepharose 4B were washed with 600 ml of 1 mM HCl and subsequently with 10 ml of 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 9.0 (coupling buffer). Fifty-milligrams of melittin (Sigma) were dissolved in 25 ml of coupling buffer and then added to the washed resin. The mixture was end-over-end rotated for 20 hr at room temperature, and active groups remaining on the resin were then blocked by mixing the resin with 10 ml of 0.2 M Tris·HCl, pH 8.3, for 2 hr. The resin was washed with 100 ml of coupling buffer and 100 ml of 0.1 M $NaHCO_3$, pH 9.0. The coupling efficiency was determined to be 1.5 μmol of melittin/ml of gel, based on the absorbance of the coupled gel, suspended in glycerol, at 280 nm (melittin contains one tryptophan residue). Tris-coupled resin served as a blank.

Isolation of PKC. Rat brain PKC was partially purified, to a specific activity of 230 nmol ^{32}P/min/mg, as previously described (19). In indicated experiments, the enzyme was further purified to near homogeneity, according to silver-stained gels, either by chromatography on W7-agarose (20) or by chromatography on melittin-agarose, as described in Results. Each PKC preparation was activated from 10- to 30-fold by 1 mM Ca^{2+} plus 30 μg/ml PS. Neither Ca^{2+} nor PS alone stimulated the enzyme activity.

In order to generate the catalytic fragment of PKC, equal volumes of 1300 units/ml trypsin in 20 mM Tris·HCl, pH 7.5, and melittin-agarose-purified PKC (10–20 nmol of ^{32}P/min/ml) were incubated together for 30 min at 4°. Proteolysis was terminated with PMSF (final concentration, 1 mM). The phosphotransferase activity of the resultant enzyme was stimulated less than 2-fold by 1 mM Ca^{2+} and 30 μg/ml PS and the overall yield of activity was greater than 50% in all cases, indicating the generation of an active catalytic fragment of PKC (21, 22).

PKC assay. PKC assay reaction mixtures (120 μl) contained 20 mM Tris·HCl at pH 7.5, 5 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 1 mM $CaCl_2$ (or 1 mM EGTA), 30 μg/ml PS (or none), 20 μM [γ-^{32}P]ATP (300–600 cpm/pmol), 0.67 mg/ml histone III-S, and 1–4 μg of isolated PKC. Reactions were initiated by the addition of PKC and proceeded for 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 onto phosphocellulose paper, and the radioactivity incorporated into histone was measured as previously described (23).

Results

Binding of PKC to melittin-agarose. In order to determine the mechanism of inhibition of PKC by the toxic peptide melittin, we first examined whether melittin could bind directly to the enzyme in the absence of lipid and other cofactors. We prepared a melittin-agarose column by coupling melittin to CNBr-activated Sepharose 4B. We loaded a PKC preparation (5.4 nmol of ^{32}P/min), which had been purified to near homogeneity by a procedure entailing affinity chromatography on W7-agarose (20), onto a 1.5-ml melittin-agarose column that was equilibrated in buffer A (20 mM Tris·HCl, 4 mM EDTA, 4 mM EGTA, 0.2 M KCl, 15 mM 2-mercaptoethanol, 100 μg/ml leupeptin, and 0.4 mM PMSF, pH 8.3) and washed the column with 20 ml (13 column volumes) of buffer A. No PKC activity was detected in the wash, and 0.1% Triton X-100 eluted 60 ± 7% of the loaded PKC activity in one major peak (Fig. 1). In a control experiment, we found that PKC did not bind to CNBr-activated agarose that had been coupled with Tris·HCl (data not shown). Thus, the binding of PKC to melittin-agarose indicated the existence of direct binding interactions between melittin and PKC.

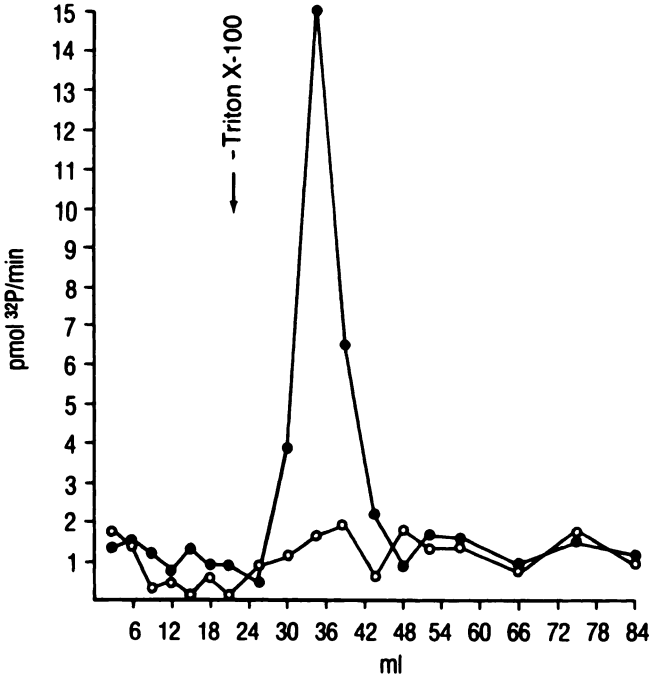


Fig. 1. Binding of PKC to melittin-agarose. Melittin-agarose was prepared as described in Experimental Procedures. PKC was loaded onto melittin-agarose, and the column was washed (see Results for details). PKC activity was eluted with 0.1% Triton X-100 in buffer A and was assayed as described in Experimental Procedures. *pmol ^{32}P/min* represents pmol of ^{32}P transferred from [γ-^{32}P]ATP to histone III-S/min. ●, Protein kinase activity observed in the presence of Ca^{2+} and PS; ○, Ca^{2+}- and PS-independent protein kinase activity.

In order to define the nature of the binding interactions between PKC and melittin, we attempted to elute PKC from melittin-agarose with MgATP. According to cDNA sequence analysis, PKC has an ATP-binding region in its active site and a homologous region outside of its active site, which may code for a second ATP-binding site (1, 24). We found that 10 mM $MgCl_2$ and 1 mM ATP could elute PKC (17.3 nmol of ^{32}P/min) from melittin-agarose in a single peak, with a yield of 56 ± 6%. In the absence of ATP, 10 mM $MgCl_2$ did not elute the enzyme (data not shown). In order to measure the specificity of the binding interactions between PKC and melittin, we loaded partially purified PKC (approximately 10% pure) (19) onto melittin-agarose and eluted the enzyme with $MgCl_2$ and ATP. We observed a 5-fold enhancement of the specific activity of the PKC preparation as a consequence of chromatography on melittin-agarose. We subjected the loaded and eluted PKC samples to sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (25), using a silver stain (26). It is apparent in Fig. 2 that PKC, the 82,000-Da doublet in the melittin-agarose-eluted PKC sample (Fig. 2, *lane 1*), was greatly enriched compared with PKC in the preparation that was loaded onto the melittin-agarose column (Fig. 2, *lane 2*). Thus, we found that the binding interactions between melittin and PKC were quite specific.

The ATP-binding region of the active site of PKC and the second potential ATP-binding region of PKC that is inferred from cDNA analysis are both located in the catalytic domain

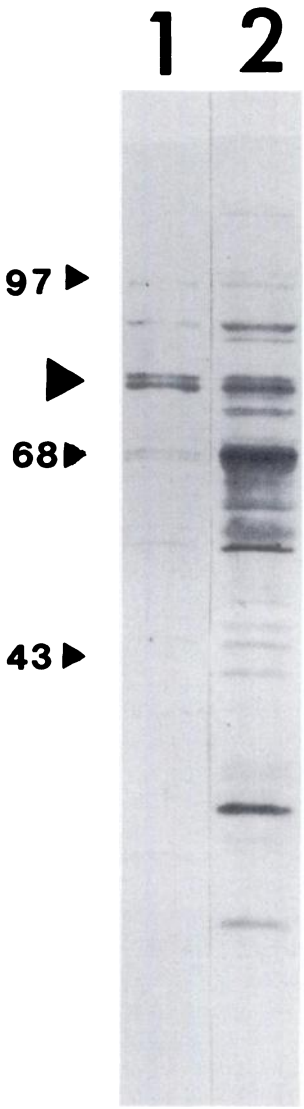


Fig. 2. Sodium dodecyl-sulfate-polyacrylamide gel electrophoresis analysis of PKC-containing samples. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis was employed to assess the enrichment of a PKC preparation by chromatography on melittin-agarose. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining of the gels (7.5%) were done by standard procedures (25, 26). *Lane 1*, the pooled PKC-containing fractions eluted from melittin-agarose with MgATP. *Lane 2*, the PKC preparation (19) that was loaded onto melittin-agarose. The *arrow* indicates the 82,000-Da doublet in our PKC preparations, which we previously identified as PKC (3).

of the enzyme (1, 24). A fully active Ca^{2+}- and phospholipid-independent catalytic fragment of PKC can be generated by limited trypsinolysis of PKC (21, 22). In order to further address whether melittin was binding directly to an ATP-binding site of PKC, we chromatographed proteolyzed PKC on melittin-agarose that was equilibrated in buffer A. We found that the catalytic fragment of PKC bound to melittin-agarose and Triton X-100 coeluted the catalytic fragment of PKC from melittin-agarose with the residual intact enzyme, with a yield of 60% (data not shown), indicating that the binding sites for melittin were located in the catalytic domain of PKC, and thereby, providing further evidence that melittin bound directly to an ATP-binding site on PKC.

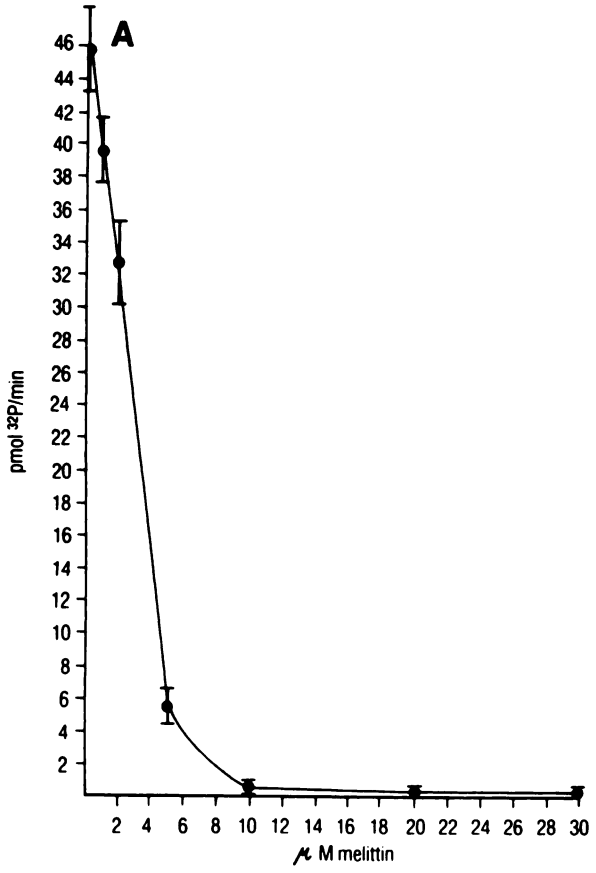
Inhibition of PKC activity by melittin. Our observation that melittin could compete with MgATP for binding to PKC suggested that melittin might inhibit PKC by directly binding to the active site of the enzyme. In order to test this possibility, we compared the capacity of melittin to inhibit PKC with its capacity to inhibit the catalytic fragment of PKC. We found that melittin (>97% pure) inhibited the Ca^{2+}- and PS-dependent phosphotransferase activity of PKC, with an IC_{50} of 3 μM (Fig. 3A). This was consistent with the previously reported IC_{50} value of 4 μM (10). In addition, we found that melittin could inhibit the Ca^{2+}- and PS-independent phosphotransferase activity of the catalytic fragment of PKC. The IC_{50} of melittin (>97% pure) against this activity was 25 μM (Fig. 3B).

Because the inhibition of the catalytic fragment of PKC by melittin and the disruption of the binding of melittin to PKC by MgATP provided evidence that melittin could inhibit the enzyme by competition with the substrate ATP, we analyzed the kinetics of inhibition of the catalytic fragment of PKC by melittin. Fig. 4 shows that the kinetics of inhibition of the catalytic fragment of PKC by melittin were consistent with noncompetitive inhibition with respect to ATP, according to Lineweaver-Burk analysis. Melittin at 60 μM changed the $V_{max_{app}}$ of the catalytic fragment of PKC from 7.6 to 3.0 pmol of ^{32}P/min, and it changed the $K_{m_{app}}$ from 5 to 20 μM. These data provided evidence that melittin did not bind to the ATP-binding region located in the active site of PKC. In order to test the possibility that melittin competed with phosphoacceptor substrates of PKC, we attempted to elute PKC from melittin-agarose with 25 μM histone III-S and with 1 μM Arg-Phe-Ala-Arg-Lys-Gly-Ser-Leu-Arg-Gln-Lys-Asn-Val [pseudosubstrate analog [ser^{25}]PKC(19–31)]. The $K_{m_{app}}$ values of the PKC substrates histone III-S and [ser-^{25}]PKC(19–31) are 20 and 0.2 μm, respectively (23, 27). We found that, under conditions in which MgATP eluted PKC with a yield of greater than 50%, neither histone nor the peptide substrate could elute detectable amounts of PKC activity (data not shown).

Considerable homology is observed among protein kinases in the ATP-binding regions of their active sites. Of the known protein kinases, the cyclic nucleotide-dependent protein kinases are the most homologous to PKC in their catalytic domains (28). Therefore, in order to assess the specificity of melittin in its inhibition of PKC, we tested the capacity of melittin to inhibit the phosphorylation of the heptapeptide kemptide that is catalyzed by the catalytic subunit of PKA (29). We found that melittin, at concentrations up to 67 μM, had no effect on PKA activity (data not shown), indicating that melittin inhibited the catalytic fragment of PKC selectively.

Discussion

In this report, we found that melittin bound to PKC in the absence of enzyme cofactors, and MgATP completely disrupted the binding interactions between PKC and melittin, whereas phosphoacceptor PKC substrates did not. The catalytic fragment of PKC, which contains two potential ATP-binding sites according to sequence analysis of PKC-encoding cDNAs (1, 24), also bound to melittin. Kinetic analysis of the inhibition of the catalytic fragment of PKC by melittin was consistent with a noncompetitive mechanism with respect to the substrate ATP, providing evidence that melittin binds to the catalytic domain at a site other than the ATP-binding region of the active site. It is possible that melittin binds to the putative ATP-binding site located outside of the active site of PKC that



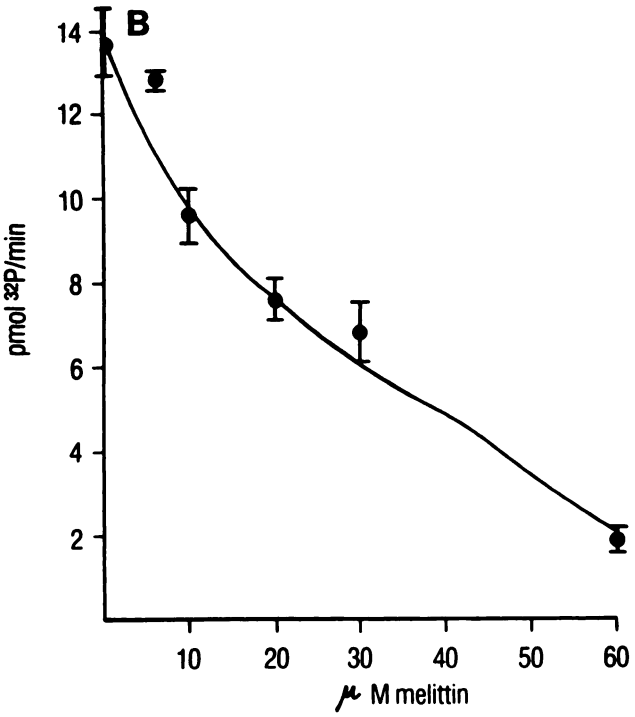


Fig. 3. Inhibition of PKC by melittin. A, The inhibition of the Ca^{2+}- and PS-dependent phosphotransferase activity of PKC by melittin was measured by standard PKC assays (see Experimental Procedures), using PKC that had been purified to near homogeneity by MgATP elution from melittin-agarose (see Experimental Procedures and Fig. 2). *pmol ^{32}P/min* represents the pmol of ^{32}P transferred from [γ-^{32}P]ATP to histone III-S/min, in a Ca^{2+}- and PS-dependent manner. B, The inhibition of the Ca^{2+}- and PS-independent phosphotransferase activity of the catalytic fragment of PKC by melittin was measured using PKC that was purified by chromatography on melittin-agarose (see Fig. 2) and then subjected to limited proteolysis (see Experimental Procedures). *pmol ^{32}P/min* represents the pmol of ^{32}P transferred from [γ-^{32}P]ATP to histone III-S/min in the presence of EGTA.

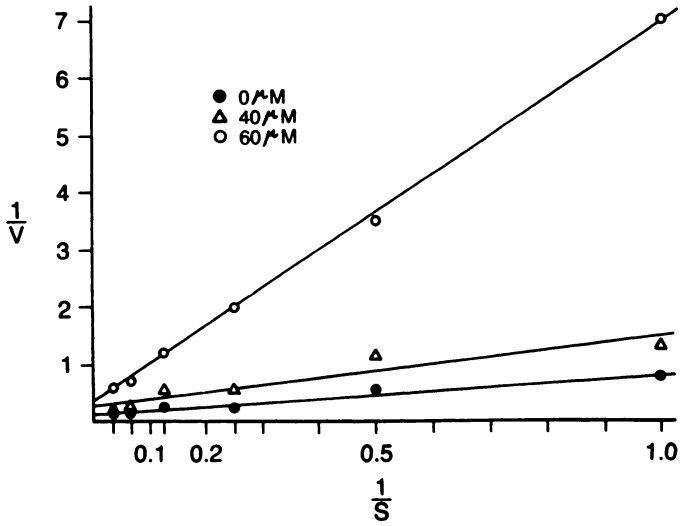


Fig. 4. Inhibition of the catalytic fragment of PKC by melittin. Phosphotransferase activity of the catalytic fragment of PKC was measured as described in the legend to Fig. 3B. *V* is pmol of ^{32}P/min; *S* is μM ATP.

is inferred from cDNA analysis. Melittin appeared to bind to the catalytic domain of PKC quite selectively, because melittin did not inhibit PKA, a protein kinase closely related to PKC (28), and chromatography of partially purified PKC on melittin-agarose purified the enzyme severalfold, according to specific activity measurements and silver-stained polyacrylamide gels. Our results suggest that the binding of melittin and MgATP to PKC may induce mutually exclusive conformations of the enzyme.

Previously, melittin was demonstrated to inhibit the Ca^{2+}- and PS-dependent activity of PKC with an inhibitory potency that was reduced as the phospholipid cofactor concentration was increased (10). In this paper, we determined that melittin could inhibit PKC with two distinct potencies. We found that melittin could inhibit the activation of PKC by Ca^{2+} and PS with an IC_{50} of 4 μM. In addition, when the enzyme activity was released from regulation of Ca^{2+} and PS by limited proteolysis, melittin inhibited the enzyme activity with an IC_{50} of 25 μM.

The indole carbazole staurosporine (12, 30), the isoquinolinesulfonamide H7 (30, 31), and the nucleoside analog sangivamycin (15) are PKC inhibitors that compete with the substrate ATP. In addition, we recently determined that the triphenylethylene *N*-desmethyltamoxifen (32) and the naphthalenesulfonamide W7 (33) can inhibit PKC not only by antagonizing its activation by Ca^{2+} and PS but also by competing with ATP, albeit with low potencies; IC_{50} values are 130 and 260 μM, respectively. Aminoacridines inhibit PKC by an analogous mixed mechanism, achieving 50% inhibition by competition with ATP at concentrations of 110 μM (acridine yellow) and 170 μM (acridine orange) (34). Of the amphiphilic PKC inhibitors, melittin is a particularly potent inhibitor of the catalytic fragment of PKC. The primary structure of melittin is established, and its secondary structure has been well defined

and essentially consists of an amphiphilic α-helical domain and a cationic tail (17, 18). Considering the defined structure of melittin, its potency and selectivity in binding to the catalytic domain of PKC, and the facility innate in modeling synthetic peptides, melittin and structural analogs of this hexacosapeptide should prove to be of value as probes of the structure and function of the catalytic domains of PKC isozymes.

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