Inhibition of Protein Kinase C by *N*-Myristoylated Peptide Substrate Analogs†

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ABSTRACT: Protein kinase C (PKC) is a family of closely related phospholipid-dependent protein kinases. A fully active, phospholipid-independent catalytic fragment of PKC is produced by limited proteolysis of the enzyme. The catalytic fragment allows a simplified assay system for the analysis of PKC inhibitors that interact with the catalytic domain. Recently, we reported that *N*-myristoylation of the synthetic peptide substrate Arg-Lys-Arg-Thr-Leu-Arg-Arg-Leu (RKRTLRRL) transformed a peptide that completely lacked inhibitory activity against the histone kinase reactions of PKC and its catalytic fragment into a peptide that potently inhibited both of these reactions. *N*-Myristoylation did not alter the potency of the peptide as a PKC substrate, and the basis for the acquisition of inhibitory activity against the catalytic fragment by *N*-myristoylation of the peptide remained unclear. In this report, we propose a mechanism for catalytic fragment inhibition by the *N*-myristoylated peptide that is based on a comparison of the inhibitory potencies of several nonphosphorylatable analogs of *N*-myristoyl-RKRTLRRL, a kinetic analysis of the inhibition of the histone kinase activity of the catalytic fragment by nonphosphorylatable *N*-myristoyl-RKRTLRRL analogs, and an analysis of the inhibitory effects of the *N*-myristoylated peptide series on the intrinsic ATPase activity of PKC. Our results support a mechanism in which the *N*-myristoylated peptides inhibit the catalytic fragment by binding to PKC_{free}, but not to the complex PKC-ATP, at the protein-substrate binding site. The ability to bind PKC_{free} distinguishes the *N*-myristoylated peptides from histone substrate and dead-end synthetic peptide inhibitors, because the latter agents appear to bind only to PKC–ATP. A serious limitation observed with inhibitory oligopeptide substrate analogs of protein kinases is that they often compete with protein substrates only weakly, if at all. *N*-Myristoylation of oligopeptide substrate analogs of PKC may overcome this limitation by allowing the inhibitory peptides to bind to a form of PKC that does not bind protein substrates such as histone, so that inhibition can be achieved without direct competition with the protein substrate. Thus, *N*-myristoylation of oligopeptide substrate analogs is a promising approach for the development of potent PKC inhibitors that exploit the substrate selectivity of the enzyme.

Protein kinase C (PKC) is a family of at least 10 closely related phospholipid-dependent isozymes that can be divided into Ca^{2+}-dependent (conventional) and Ca^{2+}-independent (nonconventional) subfamilies (Nishizuka, 1992; Parker, 1992). Most PKC isozymes are activated by the second-messenger *sn*-1,2-diacylglycerol. PKC activation has been implicated in various biological and pathogenic processes, e.g., muscle contraction, neurotransmission, and carcinogenesis (Kuo *et al.*, 1989; Nishizuka, 1992; Rotenberg & Weinstein, 1991). Specific, cell-permeable inhibitors of the PKC isozyme family and of its individual members are needed to define the role of this isozyme family in these various processes (Huang, 1989; Bottega & Epand, 1992; Gescher, 1992; O'Brian *et al.*, 1988). In addition, there is increasing evidence that selective PKC inhibitors could be of value as reversal agents for the multidrug resistance (MDR) phenotype of tumor cells (Chambers *et al.*, 1993; O'Brian *et al.*, 1991).

The substrate selectivity of PKC has been subjected to detailed analysis through the use of synthetic peptide substrates (Noland *et al.*, 1989; Turner *et al.*, 1985; Chen *et al.*, 1993). Among the most selective PKC inhibitors reported to date are synthetic peptide substrate analogs of the enzyme (House & Kemp, 1987; Huang, 1989; Gescher, 1992). Potent inhibitory peptide–substrate analogs of PKC include synthetic peptides containing sequences that occur in the pseudosubstrate domains of PKC isozymes (House & Kemp, 1987; Pears *et al.*, 1990) and sequences based on major PKC phosphorylation sites in myelin basic protein (Su *et al.*, 1986) and MARCKS protein (Graff *et al*, 1991). In view of distinctions observed among substrate selectivities of PKC isozymes (Marais & Parker, 1989), it is conceivable that isozyme-selective PKC inhibitors could be developed from peptide substrate analogs. Unfortunately, the inhibitory peptides are generally unable to enter cells, so that studies of their inhibitory activity against cellular PKC has required the use of permeabilized cell systems (Alexander *et al.*, 1989; Eichholtz *et al.*, 1990).

The catalytic fragment of PKC is a fully active Ca^{2+}- and phospholipid-independent protein kinase produced by limited proteolysis of PKC that represents the catalytic domain of the enzyme (Hannun & Bell, 1990). Recently, we reported that *N*-myristoylation of the synthetic peptide PKC substrates Lys-Arg-Thr-Leu-Arg(KRTLR) and Arg-Lys-Arg-Thr-Leu-Arg-Arg-Leu(RKRTLRRL) transformed peptides that completely lacked inhibitory activity against the histone kinase reactions of PKC and its catalytic fragment into peptides with potent inhibitory activity against both of these reactions. Importantly, *N*-myristoylation also conferred on the peptides the ability to partially reverse the multidrug resistance (MDR) phenotype of a tumor cell line (O'Brian *et al.*, 1991) and to antagonize activation of a T-lymphoblastoid cell line (Ioannides *et al.*,

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1990), providing evidence for inhibition of cellular PKC by the *N*-myristoylated peptides in intact cells. Moreover, an *N*-myristoylated nonapeptide containing a sequence that occurs in the pseudosubstrate domain of cPKC-α inhibited phosphorylation of the PKC substrate MARCKS in intact fibroblasts (Eichholtz *et al.*, 1993). The inhibition of the Ca^{2+}- and phospholipid-dependent histone kinase activity of PKC by *N*-myristoyl-KRTLR and *N*-myristoyl-RKRTLRRL was shown to involve interactions between the phospholipid cofactor and the inhibitory peptides (O'Brian *et al.*, 1990a, 1991). *N*-Myristoylation did not improve the potencies of KRTLR and RKRTLRRL as PKC substrates, and the inhibitory mechanism of the *N*-myristoylated peptides against the catalytic fragment remained unclear (O'Brian *et al.*, 1990a, 1991).

In this report, we propose a mechanism for the inhibition of PKC and its catalytic fragment by the *N*-myristoylated oligopeptide substrate analogs. Our results provide a rationale for the design of potent, cell-permeable oligopeptide PKC inhibitors that exploit the substrate selectivity of the enzyme.

EXPERIMENTAL PROCEDURES

Materials. The oligopeptides *N*-myristoyl-RKRTLRRL, *N*-myristoyl-RKRELRRL, *N*-myristoyl-RKRCLRRL, *N*-myristoyl-RKRHypLRRL, *N*-myristoyl-RKRYLRRL, *N*-myristoyl-RKRDLRRL, *N*-myristoyl-RKRALRRL, RKRTLRRL, *N*-myristoyl-FARKGALRQ, FARKGALRQ, *N*-myristoyl-KRTLR, *N*-dimyristoylglycerol-KRTLR, and KRTLR were synthesized using the Vega Coupler 250 peptide synthesizer and purified to >98% purity by reverse-phase HPLC using a Vydac C4 column and acetonitrile gradient elution, at the M. D. Anderson Cancer Center Synthetic Antigen Facility. Rat brain PKC was purified to near-homogeneity by a procedure involving elution of PKC from melittin–agarose with MgATP (O'Brian & Ward, 1989a, 1990). The resultant preparation was fully autophosphorylated. The histone III-S kinase activity of the preparation reflected conventional PKC (cPKC) isozyme catalysis (Nishizuka, 1992), because it was stimulated approximately 10-fold by 1 mM Ca^{2+} and 30 μg/mL phosphatidylserine (PS), but was not stimulated by either Ca^{2+} or PS alone. Hydroxylapatite analysis (Ward & O'Brian, 1992a) revealed that the purified preparation contained cPKC-α and cPKC-γ, but not cPKC-β.

Ammonium molybdate tetrahydrate, dithiothreitol (DTT), ATP, PS, histone III-S, phenylmethanesulfonyl fluoride, TPCK-treated trypsin from bovine pancreas, cAMP, *Crotalus atrox* venom 5'-nucleotidase, bovine brain calmodulin, Fiske & Subbarow inorganic phosphate determination reagents, *N*,*N*-dimethylmyristamide, and Tris-HCl were purchased from Sigma Chemical Co. (St. Louis, MO). Silicotungstic acid, 2-butanol, benzene, and phosphocellulose paper (grade p81) were purchased from Fisher Scientific (Houston, TX), and bovine heart calmodulin-deficient cAMP-phosphodiesterase was from Boehringer Mannheim (Indianapolis, IN). [γ-^{32}P]-ATP was purchased from Amersham Corp. (Arlington Heights, IL), and frozen rat brains were from Pel-Freez (Rogers, AR).

Generation of a Catalytic Fragment of PKC. A fully active catalytic fragment of PKC was generated from the purified rat brain PKC preparation by limited trypsinolysis, under our previously described standard conditions (O'Brian & Ward, 1989a). Briefly, equal volumes of 1300 units/mL trypsin and 10–20 nmol of ^{32}P min^{-1} (mL of PKC)$^{-1}$ in 20 mM Tris-HCl, pH 7.5, were incubated together for 30 min at 4 °C, and then proteolysis was terminated by the addition of phenylmethanesulfonyl fluoride (final concentration = 1 mM) (O'Brian & Ward, 1989a). The histone III-S kinase activity of the resultant enzyme preparation was stimulated less than 1.5-fold by 1 mM Ca^{2+} and 30 μg/mL PS, and the yield of PKC activity was greater than 50%, indicating the generation of an active catalytic fragment of PKC. Because this method for limited proteolysis of PKC does not proteolyze cPKC-α (Ward & O'Brian, 1992b), the histone kinase activity of the catalytic fragment described in this report primarily reflects cPKC-γ catalysis. Where indicated, we purified the catalytic fragment from the regulatory fragment and residual intact PKC by DEAE chromatography using as 0.0–0.4 M NaCl gradient, as previously described (O'Brian & Ward, 1989b; Mochly-Rosen & Koshland, 1987).

Histone Kinase Assay. The histone kinase activity of PKC was assayed, as previously described, in reaction mixtures that contained 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 30 μg/mL PS, 0.67 mg/mL histone III-S, 10 μM [γ-^{32}P]ATP (3000–5000 cpm/pmol), and purified PKC in a volume of 120 μL (O'Brian & Ward, 1991). Where indicated, inhibitory peptides were included in the reaction mixtures; all of the peptides were completely soluble in 20 mM Tris-HCl, pH 7.5. In assays of the catalytic fragment of PKC and in assays of the basal histone kinase activity of PKC, $CaCl_2$ was replaced by 1 mM EGTA, and PS was omitted. Reactions were initiated by the addition of [γ-^{32}P]ATP and proceeded from 5 to 10 min at 30 °C with linear kinetics. Reactions were generally terminated on phosphocellulose paper (O'Brian & Ward, 1991); trichloroacetic acid (TCA) precipitation (20% TCA, 1% PP_i) was employed to terminate reactions in indicated control experiments (O'Brian *et al.*, 1984; O'Brian & Ward, 1990). ^{32}P-Labeled histone was quantitated as previously described (O'Brian & Ward, 1991; O'Brian *et al.*, 1991). All assays were performed in triplicate and expressed as the mean value ± standard deviation.

ATPase Assay. The intrinsic ATPase activity of PKC was assayed by our standard procedure (O'Brian & Ward, 1990, 1991; Ward & O'Brian, 1992a) in reaction mixtures containing 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM $CaCl_2$ (or 1 mM EGTA), 30 μg/mL PS (or none), 10 μM [γ-^{32}P]ATP (6000-10 000 cpm/pmol), and purified rat brain PKC. Where indicated, Tris-buffered stock solutions of inhibitory peptides were added as a component of the final reaction mixture. Reactions were initiated by the addition of [γ-^{32}P]ATP and proceeded with linear kinetics for 20 min at 30 °C. Reactions were terminated on ice by the addition of 20 mM EDTA (final concentration). [^{32}P]P_i was extracted from the reaction mixture into an organic phase by the formation of a phosphomolybdate complex, and the extracted radioactivity was quantitated by previously described procedures (O'Brian & Ward, 1990, 1991; Ward & O'Brian, 1992a). All assays were done in triplicate and expressed as the mean value ± standard deviation.

Phosphodiesterase Assay. Calmodulin-dependent and basal activities of bovine heart cAMP-phosphodiesterase were assayed as previously described (O'Brian *et al.*, 1990b). The phosphodiesterase activity was stimulated 10–20-fold by 100 μM $CaCl_2$ and 1–5 units of calmodulin.

Kinetic Analysis. Lineweaver–Burk plots were generated by least-squares regression analysis. K_i values were obtained from secondary plots of the data (Palmer, 1985) that were generated by least-squares regression analysis.

Table I: Inhibitory Potencies of N-Myristoyl-RKRTLRRL and Nonphosphorylatable Analogs of the Peptide against the Histone Kinase Reactions of Purified Rat Brain PKC and Its Catalytic Fragment

peptide[a]	IC_{50}(catalytic fragment)[b] (μM)	IC_{50}(intact PKC)[b] (μM)	nature of residue substituted for Thr[c]
N-m-RKRCLRRL	2.7 ± 0.2	6.9 ± 0.3	polar uncharged, nonphosphorylatable
N-m-RKRCLRRL + DTT[d]	31 ± 5	5.8 ± 1.1	polar uncharged, nonphosphorylatable
N-m-RKRHypLRRL	41 ± 2	3.6 ● 0.2	polar uncharged, nonphosphorylatable
N-m-RKRYLRRL	55 ± 3	4.1 ± 0.4	polar uncharged, nonphosphorylatable
N-m-RKRTLRRL	80 ± 5[e]	5.0 ± 0.5[e]	polar uncharged, phosphorylatable
N-m-RKRDLRRL	103 ● 6	7.8 ± 0.5	polar charged, nonphosphorylatable
N-m-RKRELRRL	108 ± 9	11.4 ± 1.0	polar charged, nonphosphorylatable
N-m-RKRALRRL	117 ± 6	4.6 ± 0.8	nonpolar, nonphosphorylatable
RKRTLRRL	≫200[e]	≫50[e]	polar uncharged, phosphorylatable

[a] Peptide nomenclature includes the following abbreviations: N-myristoyl (N-m), Cys (C), hydroxyproline (Hyp), Tyr (Y), Thr (T), Asp (D), Ala (A), Glu (E), Arg (R), Lys (K), and Leu (L). [b] Rat brain PKC and its catalytic fragment were assayed for histone kinase activity as described under Experimental Procedures. Inhibition of the Ca^{2+}- and PS-independent activity of the catalytic fragment and the Ca^{2+}- and PS-dependent activity of intact PKC by the peptides was measured. The 100% activity values for PKC and its catalytic fragment were 14 ± 1 and 10 ± 1 pmol of ^{32}P/min, respectively. Inhibitory constants (IC_{50}'s) were generated for the peptides by constructing inhibitory curves that contained at least five data points. The results shown were obtained from assays terminated on phosphocellulose paper; in control experiments, virtually identical results were obtained by terminating assays by TCA precipitation. In additional control experiments, we achieved essentially identical results in assays that employed a catalytic fragment that had been chromatographically purified from intact PKC and the regulatory domain fragment (see Experimental Procedures). [c] Residues described as charged are typically charged at neutral pH. Peptides with residues substituted for Thr described as nonphosphorylatable are not PKC substrates (data not shown). [d] Where indicated, DTT is present at a concentration of 0.5 mM. [e] Taken from O'Brian et al. (1991).

RESULTS

The catalytic fragment of PKC provides a convenient assay system for defining the effects of amphiphilic PKC inhibitors on the catalytic domain of the enzyme, because the absence of lipid cofactor in catalytic fragment assay mixtures eliminates interactions between the lipid cofactor and the inhibitor from the analysis of inhibition (Hannun & Bell, 1990). With the objective of defining the mechanism of catalytic fragment inhibition by N-myristoyl-RKRTLRRL, we first determined the relative inhibitory potencies of a series of nonphosphorylatable N-myristoylated octapeptide analogs of N-myristoyl-RKRTLRRL that contained single amino acid substitutions at Thr. From the IC_{50} values shown in Table I for inhibition of the catalytic fragment of PKC, it is evident that the inhibitory potencies of N-myristoyl-RKRTLRRL analogs are influenced by the nature of the amino acid that replaces the phosphoacceptor residue. In fact, a comparison of the inhibitory potencies of the N-myristoylated peptides against the histone kinase activity of the catalytic fragment shows that replacement of Thr with polar, uncharged residues generally results in more inhibitory activity than does replacement with either nonpolar or acidic residues (Table I). Most potent of all is N-myristoyl-RKRCLRRL in the absence of DTT (Table I), although the marked reduction in the inhibitory potency of N-myristoyl-RKRCLRRL by DTT indicates that the observed inhibition (IC_{50} = 2.7 μM) includes a major irreversible component. The fact that the nonphosphorylatable peptides shown in Table I that most closely resemble PKC substrates (N-myristoyl-RKRCLRRL + DTT and N-myristoyl-RKRHypLRRL) are the most potent inhibitors of the catalytic fragment and that the peptides most closely resembling PKC products (N-myristoyl-RKRDLRRL and N-myristoyl-RKRELRRL) are among the weakest provides evidence that the peptides function by binding the protein substrate binding region of the catalytic fragment (see Discussion).

PKC has an intrinsic Ca^{2+}- and PS-dependent ATPase activity that is catalyzed at the active site of the enzyme and appears to represent the bond-breaking step of the protein kinase reaction (O'Brian & Ward, 1990, 1991; Ward & O'Brian, 1992a). Because approximately half of the ATPase activity of PKC is Ca^{2+}- and PS-independent (O'Brian & Ward, 1990), the basal component of the ATPase activity can be used to measure the effects of PKC inhibitors on the catalytic activity of PKC in the absence of lipid cofactor and protein substrate. [The catalytic fragment is not suitable for this purpose, because it catalyzes the ATPase reaction only in the presence of protein substrate (O'Brian & Ward 1990, 1991).] The basal ATPase activity of PKC is inhibited by the pseudosubstrate peptide PKC(19–36) and by histone III-S (O'Brian & Ward, 1990, 1991). Table II shows that each of the nonphosphorylatable analogs of N-myristoyl-RKRTLRRL in our study inhibited the basal ATPase activity of PKC. As in the case of histone III-S and PKC(19–36), the maximal

Table II: Effects of Octapeptide Analogs of N-Myristoyl-RKRTLRRL on the Basal ATPase Activity of PKC

peptide[a]	peptide concn	% inhibition[b]
N-m-RKRCLRRL	1 μM	0 ± 9
	10 μM	61 ± 7
	50 μM	63 ● 10
N-m-RKRCLRRL plus DTT[e]	1 μM	0 ± 5
	10 μM	22 ± 8
	50 μM	43 ± 4
N-m-RKRHypLRRL	1 μM	7 ± 17
	10 μM	34 ± 6
	50 μM	44 ± 8
N-m-RKRYLRRL	1 μM	0 ± 15
	10 μM	59 ± 6
	50 μM	62 ± 3
N-m-RKRDLRRL	1 μM	0 ± 10
	10 μM	42 ± 1
	50 μM	69 ± 11
N-m-RKRELRRL	1 μM	0 ± 5
	10 μM	9 ± 17
	50 μM	59 ± 6
N-m-RKRALRRL	1 μM	0 ± 9
	10 μM	61 ± 4
	50 μM	73 ● 3
RKRTLRRL	1 μM	<5
	10 μM	<5
	50 μM	<5
pseudosubstrate PKC(19–36)	0.1 μM	18 ± 6
	0.5 μM	55 ● 8[c]
histone III-S	10 μg/mL	43 ± 5[d]
	100 μg/mL	40 ± 10[d]

[a] For definition of abbreviations, see legend to Table I. [b] Percent inhibition of the Ca^{2+}- and PS-independent ATPase activity of PKC by the peptides is shown. 100% activity is 0.73 ± 0.08 pmol of $[^{32}P]P_i$/min. For assay methods and reaction conditions, see Experimental Procedures. [c] Taken from O'Brian and Ward (1990). [d] Taken from O'Brian and Ward (1991). [e] Where indicated, DTT was present at 0.5 mM.

inhibition observed with each *N*-myristoylated peptide was less than 75% (Table II). Thus, analogous effects on the basal ATPase activity of PKC were obtained with nonphosphorylatable *N*-myristoyl-RKRTLRRL analogs and with a protein substrate and an inhibitor peptide known to compete with peptide substrates of PKC (House & Kemp, 1987). The nonmyristoylated peptide RKRTLRRL failed to antagonize either the histone kinase activity of the catalytic fragment of PKC (Table I) (O'Brian *et al.*, 1991) or the basal ATPase activity of PKC (Table II). The results shown in Table II indicate that the *N*-myristoylated inhibitory peptide series interferes with the formation of products (ADP + P_i) from the complex PKC–MgATP. In addition, these results provide evidence that the *N*-myristoyl-RKRTLRRL peptide series and an inhibitory peptide that binds to the protein substrate binding site of PKC [PKC(19–36)] affect the bond-breaking step of PKC catalysis similarly, in the absence of lipid cofactor.

To define the inhibitory mechanism of the *N*-myristoyl-RKRTLRRL peptide series against lipid cofactor-independent PKC activity, we determined the kinetics of inhibition of the histone kinase reaction of the catalytic fragment of PKC by *N*-myristoyl-RKRCLRRL + DTT and by *N*-myristoyl-RKRALRRL. The Cys peptide was chosen because it was the most potent inhibitor of the catalytic fragment in our study (Table I), the Ala peptide was of particular interest because Ala is present instead of a phosphoacceptor residue in pseudosubstrate sequences of PKC isozymes (Parker, 1992; House & Kemp, 1987), and histone was employed as the substrate because the mechanism of PKC-catalyzed histone phosphorylation has been subjected to detailed kinetic analysis (Leventhal & Bertics, 1991; Hannun & Bell, 1990). The catalytic fragment of rat brain PKC has a $K_{m,app}$(histone) value of 117 μg/mL (Hannun & Bell, 1990). Our kinetic analysis of catalytic fragment inhibition at varying histone III-S concentrations included a histone concentration very close to the $K_{m,app}$ value (100 μg/mL) and a range of histone concentrations distributed equivalently above and below the $K_{m,app}$ (25, 50, 200, and 400 μg/mL). Figure 1 shows that inhibition of the catalytic fragment of PKC by *N*-myristoyl-RKRCLRRL(A) and by *N*-myristoyl-RKRALRRL (B) is noncompetitive with respect to histone III-S. The noncompetitive kinetics provide evidence that histone does not affect the binding of the *N*-myristoylated peptide inhibitors to the catalytic domain of PKC (Palmer, 1985). The K_i for *N*-myristoyl-RKRCLRRL was 50 ± 2 μM, and the K_i for *N*-myristoyl-RKRALRRL was 108 ± 11 μM, according to secondary plot analyses (Palmer, 1985) of the data shown in Figure 1 (data not shown).

Figure 2 shows the kinetics of inhibition of the histone kinase reaction of the catalytic fragment by the Cys and Ala peptides when the concentration of ATP was varied. Mixed competitive/noncompetitive kinetics were observed with *N*-myristoyl-RKRCLRRL(A), and a somewhat more complex mixed inhibitory pattern with substantial noncompetitive character was observed with *N*-myristoyl-RKRALRRL (B). These results provide kinetic evidence that the substrate ATP affects the binding of the inhibitory peptides to PKC, although the peptides do not compete with the nucleotide substrate (Palmer, 1985). An interpretation of the inhibitory kinetics (Figures 1 and 2) is provided in the Discussion.

The pentapeptide KRTLR is a weak PKC substrate ($K_{m,app}$ = 300 μM) compared with RKRTLRRL ($K_{m,app}$ = 20 μM) (O'Brian *et al.*, 1990a, 1991), and the *N*-myristoylated octapeptides (Table I) are 2–7-fold more potent than the truncated analog *N*-myristoyl-KRTLR (IC_{50} = 200 μM) in

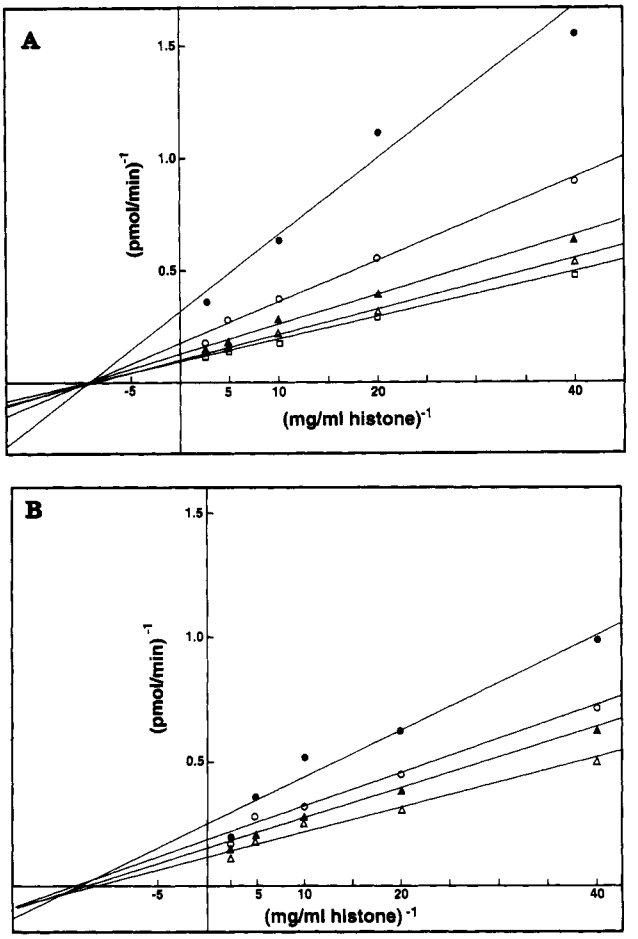

FIGURE 1: Kinetics of inhibition of the histone kinase activity of the catalytic fragment of PKC by *N*-myristoyl-RKRCLRRL and *N*-myristoyl-RKRALRRL in the presence of varying histone III-S concentrations. Lineweaver–Burk analyses of the inhibition of the catalytic fragment by *N*-myristoyl-RKRCLRRL in the presence of 0.5 mM DTT (A) and by *N*-myristoyl-RKRALRRL (B) are shown. For assay conditions, see Experimental Procedures. The label pmol/min represents the rate of transfer of ^{32}P from [γ-^{32}P]ATP to histone III-S. Each point represents a mean of triplicate assays, and lines were determined by least-squares regression analysis. In (A), (●) = 100 μM, (○) = 50 μM, (▲) = 25 μM, (△) = 10 μM, and (□) = 0 μM *N*-myristoyl-RKRCLRRL. Lines corresponding to 100, 50, 25, 10, and 0 μM *N*-myristoyl-RKRCLRRL had correlation coefficients of 0.9833, 0.9968, 0.9937, 0.9979, and 0.9987, respectively. In B, (●) = 100 μM, (○) = 80 μM, (▲) = 60 μM, and (△) = 0 μM *N*-myristoyl-RKRALRRL. Lines corresponding to 100, 80, 60, and 0 μM *N*-myristoyl-RKRALRRL had correlation coefficients of 0.9754, 0.9935, 0.9963, and 0.9843, respectively. The inhibitory patterns shown were obtained reproducibly.

the inhibition of the catalytic fragment. Like RKRTLRRL, the pentapeptide KRTLR does not inhibit PKC (O'Brian *et al.*, 1990a, 1991). While these data provide another correlation between protein substrate binding site recognition and catalytic fragment inhibition by *N*-myristoylated peptide substrate analogs, they also indicate a correlation between the overall positive charge of the *N*-myristoylated peptides and their inhibitory potencies against the catalytic fragment. We next examined the ability of *N*-myristoyl-FARKGALRQ to inhibit the catalytic fragment. *N*-Myristoyl-FARKGALRQ contains the pseudosubstrate sequence of PKC-α and has the same number of basic residues as *N*-myristoyl-KRTLR. The *N*-myristoylated pseudosubstrate peptide is a potent inhibitor of PKC-mediated events in intact cells (Eicholtz et al., 1993), but its effects on isolated PKC have not been reported. We found that *N*-myristoylated-FARKGALRQ was 5 times more

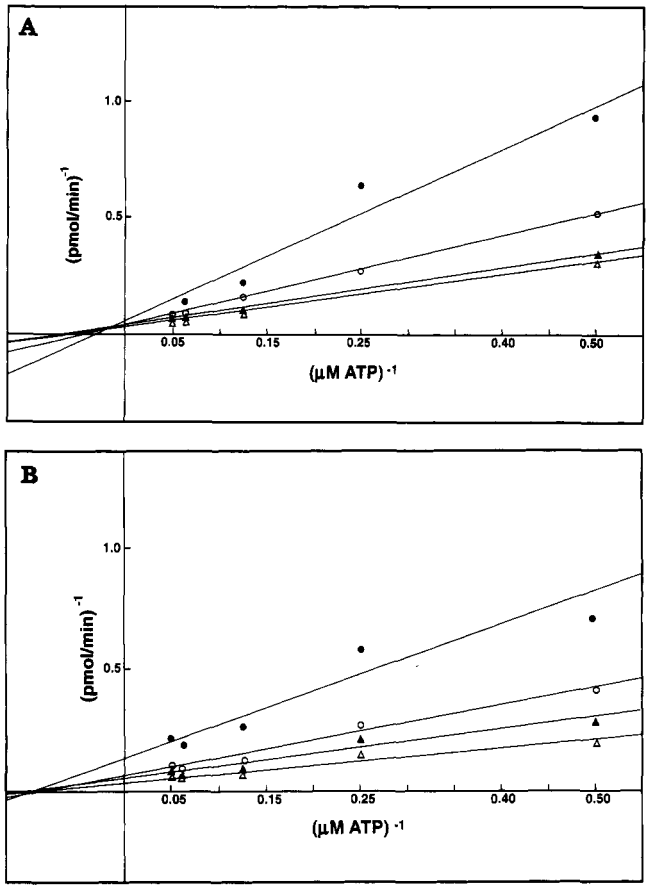

FIGURE 2: Kinetics of inhibition of the histone kinase activity of the catalytic fragment of PKC by N-myristoyl-RKRCLRRL and N-myristoyl-RKRALRRL in the presence of varying ATP concentrations. Lineweaver–Burk analyses of the inhibition of the catalytic fragment by N-myristoyl-RKRCLRRL in the presence of 0.5 mM DTT (A) and by N-myristoyl-RKRALRRL (B) are shown. The [γ-^{32}P]ATP concentration was varied as indicated, and the specific activity of the nucleotide was held constant. For other assay conditions, see Experimental Procedures. The axis labeled pmol/min represents the rate of transfer of ^{32}P from [γ-^{32}P]ATP to histone III-S. Each point represents a mean of triplicate assays, and lines were generated by least-squares regression analysis. In (A), (●) = 100 μM, (○) = 50 μM, (▲) = 10 μM, and (△) = 0 μM N-myristoyl-RKRCLRRL. Lines corresponding to 100, 50, 10, and 0 μM N-myristoyl-RKRCLRRL had correlation coefficients of 0.9724, 0.9999, 0.9999, and 0.9999, respectively. In (B), (●) = 125 μM, (○) = 100 μM, (▲) = 75 μM, and (△) = 0 μM N-myristoyl-RKRALRRL. Lines corresponding to 125, 100, 75, and 0 μM N-myristoyl-RKRALRRL had correlation coefficients of 0.9668, 0.9922, 0.9679, 0.989, respectively. The inhibitory patterns shown were obtained reproducibly.

potent than N-myristoyl-KRTLR in inhibiting the catalytic fragment (IC_{50} of N-myristoyl-FARKGALRQ = 36 ± 3 μM) and it was just as potent as the N-myristoylated octapeptide substrate analogs that contained five basic residues (Table I). The nonmyristoylated peptide FARKGALRQ was not inhibitory. These results indicate that the inhibitory potencies of the N-myristoylated peptides are not simply a function of the overall cationic character of the peptide moiety.

We next tested whether N-acylated inhibitory peptides could also be designed using an N-acyl head group other than the N-myristoyl moiety. We found that N-acylation of KRTLR with the relatively bulky head group dimyristoylglycerol resulted in an N-acylated peptide that inhibited the catalytic fragment over 30-fold more potently than the N-myristoylated pentapeptide and about an order of magnitude more potently than the reversible inhibitors in the N-myristoylated octapeptide series (IC_{50} of N-dimyristoylglycerol-KRTLR = 6.0 ± 0.5 μM). This result indicates the importance of the lipid moiety in determining the potencies of N-acylated peptide substrate analogs in the inhibition of the catalytic fragment of PKC. To test the importance of the peptide moiety in this regard, we measured the inhibitory activity of a nonpeptidic amide of myristic acid. We found that N,N-dimethylmyristamide was devoid of inhibitory activity against the catalytic fragment over a concentration range of 1–500 μM, indicating an essential role for the peptide moiety of the N-acylated peptides in their inhibition of the catalytic fragment.

N-Myristoylation of RKRTLRRL results in a peptide with a cationic–amphiphilic structure (O'Brian *et al.*, 1991). Previously, we reported that N-myristoyl-RKRTLRRL inhibits the Ca^{2+}- and PS-dependent histone kinase activity of PKC at peptide concentrations that had no effect on the catalytic fragment (IC_{50} = 5 μM). The inhibitory potency of N-myristoyl-RKRTLRRL against the Ca^{2+}- and PS-dependent histone kinase activity of PKC was reduced as the amount of PS in the reaction mixture was increased (O'Brian *et al.*, 1991). Analogous inhibitory behavior has been reported for many other cationic–amphiphilic PKC inhibitors and has been ascribed to an inhibitory mechanism that involves interference with the cofactor PS in the activation of PKC (Mori *et al.*, 1980; Katoh *et al.*, 1982; Epand & Lester, 1990). A common property among cationic–amphiphilic PKC inhibitors is inhibitory activity against calmodulin (Katoh *et al.*, 1982; Mazzei *et al*, 1982; O'Brian *et al.*, 1990b). We tested the ability of N-myristoyl-RKRTLRRL to serve as a calmodulin antagonist by analyzing its effects on calmodulin-dependent cAMP-phosphodiesterase.

N-Myristoyl-RKRTLRRL potently inhibited activation of the phosphodiesterase by calmodulin (IC_{50} = 1 μM) without affecting the basal activity of the enzyme.

Table I shows that N-myristoylated peptides with similar amounts of positive charge and hydrophobic character according to their amino acid compositions (N-myristoyl-RKRTLRRL and analogs with Cys + DTT, Hydroxypro, Tyr, and Ala in place of Thr) had similar IC_{50} values for the inhibition of the Ca^{2+}- and PS-dependent histone kinase reaction of PKC (IC_{50}'s ranged from 3.6 ± 0.2 to 5.8 ± 1.1 μM). Weaker inhibitory activity was observed with the analogs containing Asp or Glu, which had less overall cationic character than the other peptides (Table I). The net positive charge of N-myristoyl-FARKGALRQ predicted from its amino acid composition is about 25% lower than the predicted values for the Asp and GLu peptides of Table I, and the pseudosubstrate peptide was somewhat weaker than the Asp and Glu peptides in inhibiting Ca^{2+}- and PS-dependent histone phosphorylation (IC_{50} of N-myristoyl-FARKGALRQ = 16 ± 1 μM) (Table I). The pentapeptide N-myristoyl-KRTLR was about an order of magnitude weaker than the N-myristoylated octapeptides (Table I) in inhibiting the Ca^{2+}- and PS-dependent histone kinase reaction (IC_{50} of N-myristoyl-KRTLR = 74 μM) (O'Brian *et al.*, 1990a), apparently due to its truncated cationic peptide domain. No inhibitory activity was observed with the nonmyristoylated peptides RKRTLRRL, KRTLR (O'Brian *et al.*, 1990a, 1991), and FARKGALRQ, which lacked a segregated hydrophobic domain. Likewise, the nonpeptidic amide N,N-dimethylmyristamide (1–500 μM) was not inhibitory. N-Dimyristoylglycerol-KRTLR was a more potent inhibitor of Ca^{2+}- and PS-dependent PKC activity (IC_{50} = 20 μM) than N-myristoyl-KRTLR, although substituting the N-myristoyl moiety with dimyristoylglycerol had a much more pronounced effect on the inhibitory potency against the catalytic fragment. Thus, the ability of the N-myristoylated

peptides to inhibit the Ca^{2+}- and PS-dependent histone kinase activity of PKC was generally insensitive to single amino acid substitutions (Table I) but was affected by changes in the overall cationic–amphiphilic character of the peptide.

DISCUSSION

Recently, Leventhal and Bertics (1991) provided evidence for a kinetic mechanism of PKC-catalyzed histone phosphorylation that was steady-state-ordered with ATP binding first (eq 1). For inhibitors that bind the catalytic domain of PKC,

$$PKC_{free} + ATP + \text{histone} \rightleftharpoons PKC\text{–}ATP + \text{histone} \rightleftharpoons PKC\text{–}ATP\text{–histone} \rightleftharpoons \text{products} \quad (1)$$

the most simple inhibitory mechanisms that are possible according to eq 1 are exclusive binding of the inhibitor (1) to PKC_{free}, (2) to PKC–ATP, and (3) to PKC–ATP–histone. Inhibitor binding to PKC–ATP–histone (where PKC denotes the catalytic fragment of the enzyme) can be ruled out as an exclusive or major inhibitory mechanism of *N*-myristoyl-RKRCLRRL and *N*-myristoyl-RKRALRRL, because it would result in an uncompetitive pattern of inhibition with respect to histone (Palmer, 1985), and the inhibition observed with each of the *N*-myristoylated peptides was noncompetitive with respect to histone. Inhibitor binding to PKC–ATP would result in uncompetitive kinetics with respect to ATP (Palmer, 1985); this type of inhibition against PKC has been observed with the pseudosubstrate peptide PKC(19–36) (House & Kemp, 1987) and the dead-end inhibitor poly(L-lysine) Leventhal & Bertics, 1991). However, inhibitor binding to PKC–ATP can be ruled out as a major or exclusive mechanism for the *N*-myristoylated peptide inhibitors because the mixed inhibitory kinetics observed with respect to ATP lacked an uncompetitive component. The mixed competitive/noncompetitive kinetics with respect to ATP that were obtained with the *N*-myristoylated peptides are consistent with the inhibitors binding to PKC_{free} in a manner that is affected by ATP, but not in direct competition with the nucleotide (Palmer, 1985). In addition, binding to PKC_{free} would be expected to result in the observed noncompetitive kinetics with respect to histone, because the inhibitor would interact with a form of the enzyme that cannot bind histone (Leventhal & Bertics, 1991; Palmer, 1985). Thus, the observed kinetics are consistent with a mechanism of inhibition by the *N*-myristoylated peptides that is described by eq 2. Equation 2 is also consistent with the

$$PKC_{free} + ATP + \text{histone} + I \rightleftharpoons PKC\text{–}ATP + PKC\text{–}I + PKC\text{–}ATP\text{–}I + \text{histone} \quad (2a)$$

$$PKC\text{–}ATP + \text{histone} \rightleftharpoons PKC\text{–}ATP\text{–histone} \rightleftharpoons \text{products} \quad (2b)$$

observed inhibition of the ATPase reaction of PKC by the *N*-myristoylated peptides (Table II), because I affects the formation of PKC–ATP and may also affect its breakdown to products, according to the equation.

The evidence that the *N*-myristoyl-RKRTLRRL peptide series shown in Table I inhibits the catalytic fragment by binding the protein substrate binding site of PKC is as follows. First, all of the peptides are close structural homologs of *N*-myristoyl-RKRTLRRL, which serves as a PKC substrate with a $K_{m,app}$ of 29 μM (O'Brian *et al.*, 1991). Second, the inhibitory effects of the nonphosphorylatable *N*-myristoyl-RKRTLRRL analogs on the basal ATPase activity of PKC (Table II) were analogous to the inhibitory effects observed with PKC(19–36), which is an inhibitory peptide substrate analog known to bind at the protein substrate binding site of PKC (House & Kemp, 1987; O'Brian & Ward, 1990). It is worthwhile to note that several inhibitory peptide substrate analogs of cAMP-dependent protein kinase (PKA) have been reported to inhibit the ATPase reaction of that enzyme (Salerno *et al.*, 1990), and that a novel heptapeptide substrate analog that inhibits the protein kinase reaction of PKA while stimulating its ATPase reaction has recently been designed and characterized (Mendelow *et al.*, 1993). Third, the nonphosphorylatable *N*-myristoyl-RKRTLRRL analogs contained single amino acid substitutions at Thr, and their inhibitory potencies against the histone kinase activity of the catalytic fragment positively correlated with the structural resemblance between Thr and the substituted residue and negatively correlated with the structural resemblance between phospho-Thr and the substituted residue (Table I). Because Thr is a polar, uncharged residue, those peptides in which Thr is replaced with a polar, uncharged residue structurally resemble the substrate more closely than peptides containing nonpolar or acidic residues in place of Thr; the peptides with polar, uncharged residues in place of Thr were the most potent *N*-myristoyl-RKRTLRRL analogs in the inhibition of the catalytic fragment (Table I). Furthermore, the Cys peptide is the closest structural homolog to the peptide substrate *N*-myristoyl-RKRTLRRL in this study, and it was also the most potent inhibitor of the catalytic fragment. Next most potent was the Hydroxypro peptide, and its resemblance to the peptide substrate is indicated by the reported phosphorylation of the residue Hydroxypro by cAMP-dependent protein kinase, which is a Ser/Thr protein kinase (Bramson *et al.*, 1984). Substitution of Ser or Thr phosphoacceptor residues with Asp or Glu results in peptide substrate analogs that resemble the phosphopeptide product (Hurley *et al.*, 1990), and replacement of the pseudophosphoacceptor residue Ala with Glu in the pseudosubstrate peptide PKC(19–31) results in a peptide with relatively weak inhibitory activity against PKC (Pears *et al.*, 1990); among the weakest *N*-myristoyl-RKRTLRRL analogs in our study were those containing Asp or Glu in place of Thr. Fourth, the inhibitory potencies of structurally diverse *N*-myristoylated peptide substrate analogs did not correlate with their overall cationic character, but did correlate with their structural resemblance to peptide substrates of PKC. The nonpeptidic analog *N*,*N*-dimethylmyristamide was not inhibitory.

The relative abilities of the peptides in the *N*-myristoyl-RKRTLRRL series to inhibit the Ca^{2+}- and PS-dependent histone kinase activity of PKC correlated with their overall cationic–amphiphilic character (Table I). Biophysical studies of the inhibition of Ca^{2+} and phospholipid-dependent PKC activity by cationic amphiphiles indicate that the properties of positive charge and membrane bilayer-stabilizing activity contribute to the potency of cationic–amphiphilic PKC inhibitors (Bottega & Epand, 1992; Senisterra & Epand, 1992; Epand & Lester, 1990). Our results show that *N*-myristoyl-RKRTLRRL falls into this category of PKC inhibitors because it has in common with them the abilities to inhibit calmodulin and to inhibit lipid-dependent PKC activity by interference with the lipid cofactor. Consideration of positive charge and membrane bilayer-stabilizing activity in the design of *N*-acylated peptide substrate analogs may facilitate the development of peptides with optimized inhibitory potencies against PKC.

In conclusion, we provide evidence that a series of *N*-myristoylated octapeptide substrate analogs directly inhibit

the catalytic domain of PKC by binding at the protein substrate binding site of PKC_{free}. The ability to bind PKC_{free} distinguishes the *N*-myristoylated octapeptides from histone substrate and the dead-end inhibitor PKC(19–36), because histone and PKC(19–36) bind only to the complex PKC–ATP (Leventhal & Bertics, 1991; House & Kemp, 1987). One of the limitations noted with inhibitory oligopeptide PKC substrate analogs is that they often compete with protein substrates only weakly, if at all (O'Brian *et al.*, 1984; Graff *et al.*, 1991; Chen *et al.*, 1993). For example, the pseudosubstrate peptide PKC(19–31) inhibits PKC-catalyzed phosphorylation of a glycogen synthase synthetic peptide substrate with an IC_{50} of 92 ± 5 nM, but does not inhibit PKC-catalyzed histone phosphorylation at all (House & Kemp, 1987). Our results suggest that *N*-myristoylation of oligopeptide PKC substrate analogs may overcome this limitation by endowing the peptides with the ability to bind at the active site of PKC_{free} and, therefore, to inhibit PKC-catalyzed phosphorylation of potent substrates such as histone without direct competition between the inhibitory peptide and the protein substrate. Thus, *N*-myristoylation of oligopeptide PKC substrate analogs is a novel and promising approach for the development of potent, cell-permeable PKC inhibitors that exploit the substrate selectivity of the enzyme.

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