Biochemical Pharmacology, Vol. 39, No. 1, pp. 49–57, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + .00

N-MYRISTYL-Lys-Arg-Thr-Leu-Arg: A NOVEL PROTEIN KINASE C INHIBITOR*

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(*Received* 3 *February* 1989; *accepted* 16 *June* 1989)

Abstract—In view of the critical role that the Ca^{2+}- and phospholipid-dependent enzyme protein kinase C (PKC) plays in mediating proliferative responses to a number of growth factors, hormones, and tumor promoters, it is thought that selective PKC inhibitors may provide a new class of antiproliferative drugs. Established PKC inhibitors include three major classes of agents: agents that compete with the substrate ATP, agents that compete with the protein substrate, and agents that both compete with ATP and interact with the cofactor phosphatidylserine (PS). In this report, we have characterized the interactions between PKC and *N*-myristyl-Lys-Arg-Thr-Leu-Arg, a myristylated analogue of a synthetic peptide substrate of PKC. We determined that the myristylated peptide was a novel PKC inhibitor that interacted with PS as well as competed with the protein substrate of PKC. The inhibitory activity of the peptide was conferred by myristylation. We found that the myristylated peptide antagonized Ca^{2+}- and PS-activated PKC with an IC_{50} of 75 μm, whereas the nonmyristylated peptide lacked this inhibitory activity. A fully active, Ca^{2+}- and PS-independent catalytic fragment of PKC can be generated by limited proteolysis. Although the myristylated peptide was a very poor PKC substrate, this peptide inhibited the catalytic fragment of PKC by apparent competition with the phosphoacceptor substrate histone IIIS with an IC_{50} of 200 μM, whereas the nonmyristylated peptide showed no inhibitory activity against the catalytic fragment. Thus, the myristylated peptide may serve as a model for the development of selective PKC inhibitors, because its inhibitory mechanism exploits the substrate specificity of PKC, as well as the novel regulation of the enzyme. Furthermore, since endogenous PKC substrates include acylated proteins, the observations that we report here concerning a myristylated synthetic peptide suggest that acylation of proteins may be important in the regulation of PKC activity *in vivo*.

Protein kinase C (PKC‖) is comprised of a family of isozymes that play a critical role in mediating the actions of a number of growth factors and hormones [1–3]. The importance of PKC in cell growth is indicated not only by the direct activation of PKC by tumor-promoting phorbol esters [4] and by diacylglycerol, a second messenger of certain growth factors and related agents [5], but also by the loss of growth control mechanisms in fibroblasts that specifically overexpress PKC [6, 7]. Evidence for a critical role for PKC in proliferation has provided an impetus for studies of PKC inhibitors. It is thought that inhibitors selective for PKC or one of its isozymes may provide a new class of antiproliferative drugs [8].

One major class of PKC inhibitors, which includes indole carbazoles such as staurosporine [9, 10] and isoquinolinesulfonamides such as H-7 [11], inhibits PKC simply by competing with the substrate ATP. A second major class of PKC inhibitors, which includes the triphenylethylene tamoxifen [12–15], the naphthalenesulfonamide W7 [16, 17], and the aminoacridine dye acridine orange [18], inhibits PKC by both competing with the substrate ATP and interfering with the activation of the enzyme by Ca^{2+} and PS. A third major class of PKC inhibitors, represented by the pseudosubstrate octadecapeptide synthesized by House and Kemp [19], inhibits PKC simply by competing with its phosphoacceptor substrate. In this article we report that *N*-myristyl-Lys-Arg-Thr-Leu-Arg* is a novel PKC inhibitor that competes with the phosphoacceptor substrate of PKC and interacts with the phospholipid cofactor of the enzyme.

MATERIALS AND METHODS

Chemicals

Histone IIIS, PS, ATP, Tris–HCl, PMSF, leupeptin, soybean trypsin inhibitor type I, kemptide, purified catalytic subunit of bovine heart cAMP-dependent protein kinase (36 nM units/mg protein), and *N*-tosyl-L-phenylalanyl chloromethyl ketone-treated trypsin from bovine pancreas (sp. act. = 10,000–13,000 *N*-α-benzoyl-L-arginine ethyl ester

* "Myristyl" is a commonly used abbreviated form of "myristoyl".

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‖ Abbreviations: Boc, *tert*-butyloxycarbonyl; BSA, bovine serum albumin; Bzl, benzyl; EGTA, ethyleneglycolbis(aminoethylether)tetra-acetate; H-7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; HOBt, hydroxybenzotriazole; Mbs, 4-methoxybenzenesulfonyl; NMR, nuclear magnetic resonance; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; PS, phosphatidylserine; TCA, trichloroacetic acid; TFA, trifluoracetic acid; TMS, tetramethylsilane; W7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; and Z, benzyloxycarbonyl.

units/mg protein) were purchased from the Sigma Chemical Co. (St Louis, MO). Frozen rat brains were purchased from Charles River Breeding Laboratories (Wilmington, MA). [γ-^{32}P]ATP was purchased from the Amersham Corp. (Arlington Heights, IL). Phosphocellulose paper, grade p81, was purchased from Fisher Scientific (Houston, TX). The pentapeptide Lys-Arg-Thr-Leu-Arg and the phosphopeptide Lys-Arg-Thr(P)-Leu-Arg were synthesized and purified as previously reported [20].

Synthesis of N-*myristyl-Lys-Arg-Thr-Leu-Arg*

N-Myristyl-Lys-Arg-Thr-Leu-Arg was synthesized as follows. The Boc-group of protected Lys-Arg-Thr-Leu-Arg, i.e. Boc-Lys(Z)-Arg(Mbs)-Thr(Bzl)-Leu-Arg(Mbs)-OMe (118.5 mg, 82 μmol), was removed by dissolution in 50% trifluoracetic acid in dichloromethane and stirring for 1 hr. Subsequently the solvent was removed by evaporation, and the residue solidified upon the addition of dry ether. The solid was washed twice with dry ether and dried *in vacuo* over KOH. Myristylation was carried out by reaction of the thus obtained trifluoracetic acid salt with the hydroxybenzotriazole ester (HOBt) of myristic acid in the presence of 4-ethylmorpholine and was analogous to the procedure described [20] for the coupling of amino acids. To ensure completion of the reaction, a total of four equivalents of the myristyl-OBt ester was added. The product was purified by gel filtration on Sephadex LH-20 (eluant $MeOH/CH_2Cl_2$ 1:1, v/v) to afford the homogeneous (according to thin-layer chromatography on Merck precoated silica gel 60 F254 0.25-mm plates, R_f $MeOH/CH_2Cl_2$ 1:9 (v/v) = 0.56) protected myristyl pentapeptide in 86% yield. The product was characterized by ^{1}H and ^{13}C NMR (200 MHz) spectroscopy.

Final deprotection to *N*-myristyl-Lys-Arg-Thr-Leu-Arg was achieved by acidolysis with trifluoromethane sulfonic acid in trifluoracetic acid to remove the Z, Mbs, and Bzl groups, followed by saponification of the methyl ester as described previously [20]. The product was purified by preparative reverse-phase chromatography using a Pharmacia FPLC equipped with a PepRPC HR 16/10 column. An appropriate gradient, from 0% to 100% acetonitrile in water containing 0.1% TFA, was used. *N*-Myristyl-Lys-Arg-Thr-Leu-Arg was pure, according to analytical reverse-phase FPLC. The peptide was obtained in 50% yield and fully characterized by two-dimensional ^{13}C–^{1}H NMR [21] on a Bruker MSL 400-MHz apparatus interfaced with an ASPECT 3000 computer (see Fig. 1). The solvent was CD_3OD, with TMS as an internal reference. Shifts are indicated in ppm relative to TMS. The numbering is according to IUPAC recommendations [22].

NMR assignments. ^{13}C: Lys-C^1, both Arg-C^1, Thr-C^1, Leu-C^1 and myristyl-C^1 176.83, 174.92, 174.78, 174.04, 172.39; Lys-C^2 55.13; Arg-C^2 and Leu-C^2 53.56; Thr-C^2 60.06; Arg-C^2 54.61; Lys-C^3 32.15; both Arg-C^3 29.70 and 29.54; Thr-C^3 68.50; Leu-C^3 41.62; Lys-C^4 23.87; both Arg-C^4 26.27; Thr-C^4 20.17; Leu-C^4 25.81; Lys-C^5 28.14; both Arg-C^5 41.98 (two signals); Leu-C^5 and Leu-C^6 23.49 and 21.95; both Arg-C^6 158.72; Lys-C^6 40.72; Myristyl-C^2 36.74; myristyl-C^3 to C^{13} 33.04, 32.15, 30.73, 30.62, 30.43, 26.89, 23.69; myristyl-C^{14} 14.41. ^{1}H: Lys-C$^2\underline{H}$ 4.26 (q); Arg-C$^2\underline{H}$ 4.42; Leu-C$^2\underline{H}$ 4.42; Thr-C$^2\underline{H}$ 4.36 (d); Arg-C$^2\underline{H}$ 4.40; Lys-C$^3\underline{H}_2$ 1.79; both Arg-C$^3\underline{H}_2$ 1.92; Thr-C$^3\underline{H}$ 4.21 (8 lines); Leu-C$^3\underline{H}_2$ 1.64; Lys-C$^4\underline{H}_2$ 1.48; both Arg-C$^4\underline{H}_2$ 1.66; Thr-C$^4\underline{H}_3$ 1.19 (d); Leu-C$^4\underline{H}$ 1.74; Lys-C$^5\underline{H}_2$ 1.69; both Arg-C$^5\underline{H}_2$ 3.20; Leu-C$^5\underline{H}_3$ and Leu-C$^6\underline{H}_3$ 0.96 (d) and 0.92 (d); Lys-C$^6\underline{H}_2$ 2.93 (t); myristyl-C$^2\underline{H}_2$ 2.27 (t); myristyl-C$^3\underline{H}_2$ to C$^{13}\underline{H}_2$ 1.60, 1.31, 1.30, 1.28, 1.26; C$^{14}\underline{H}_3$ 0.89 (t).

Purification of rat brain PKC

Rat brain PKC was purified to near homogeneity by a procedure that entailed elution of the enzyme from melittin agarose with MgATP [23]. The PKC preparation was fully autophosphorylated as a result of its exposure to MgATP during its elution from melittin agarose, and it did not incorporate detectable amounts of ^{32}P upon exposure to Mg[γ-^{32}P]ATP under standard assay conditions in the absence of histone, in assays terminated by TCA precipitation. The PKC preparation was stimulated 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.

A fully active catalytic fragment of PKC was generated by incubating equal volumes of 1300 units/ml trypsin in 20 mM Tris–HCl, pH 7.5, and PKC (10–20 nmol ^{32}P/min/ml) together for 30 min at 4°, as previously described [15, 17]. Proteolysis was terminated with PMSF (final concentration, 1 mM). The phosphotransferase activity of the proteolyzed enzyme was stimulated less than 2-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.

PKC activity was assayed as previously described [24]. Reaction mixtures 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 IIIS, and 1–4 μg isolated PKC. Reactions were initiated by the addition of PKC and proceeded from 5 to 10 min at 30°, which is within the linear phase of the time course. Reactions were terminated by pipetting a 40-μl aliquot of the reaction mixture onto phosphocellulose paper, and the radioactivity incorporated into histone was measured as previously described [24]. In indicated experiments, reactions were terminated by TCA precipitation using 20% TCA–1% PP_i, as previously described [24].

RESULTS

Inhibition of Ca^{2+}- *and PS-dependent PKC activity by* N-*myristyl-Lys-Arg-Thr-Leu-Arg*

The pentapeptide Lys-Arg-Thr-Leu-Arg is a phosphoacceptor substrate of PKC with an apparent K_m of 300 ± 40 μM and an apparent V_{max} equal to approximately one-tenth that of histone IIIS [20]. We compared the capacities of the pentapeptide Lys-Arg-Thr-Leu-Arg, its phosphorylated analogue Lys-Arg-Thr(P)-Leu-Arg, and its myristylated analogue *N*-myristyl-Lys-Arg-Thr-Leu-Arg (Fig. 1) to inhibit Ca^{2+}- and PS-dependent PKC-catalyzed histone phosphorylation. We found that the myristylated

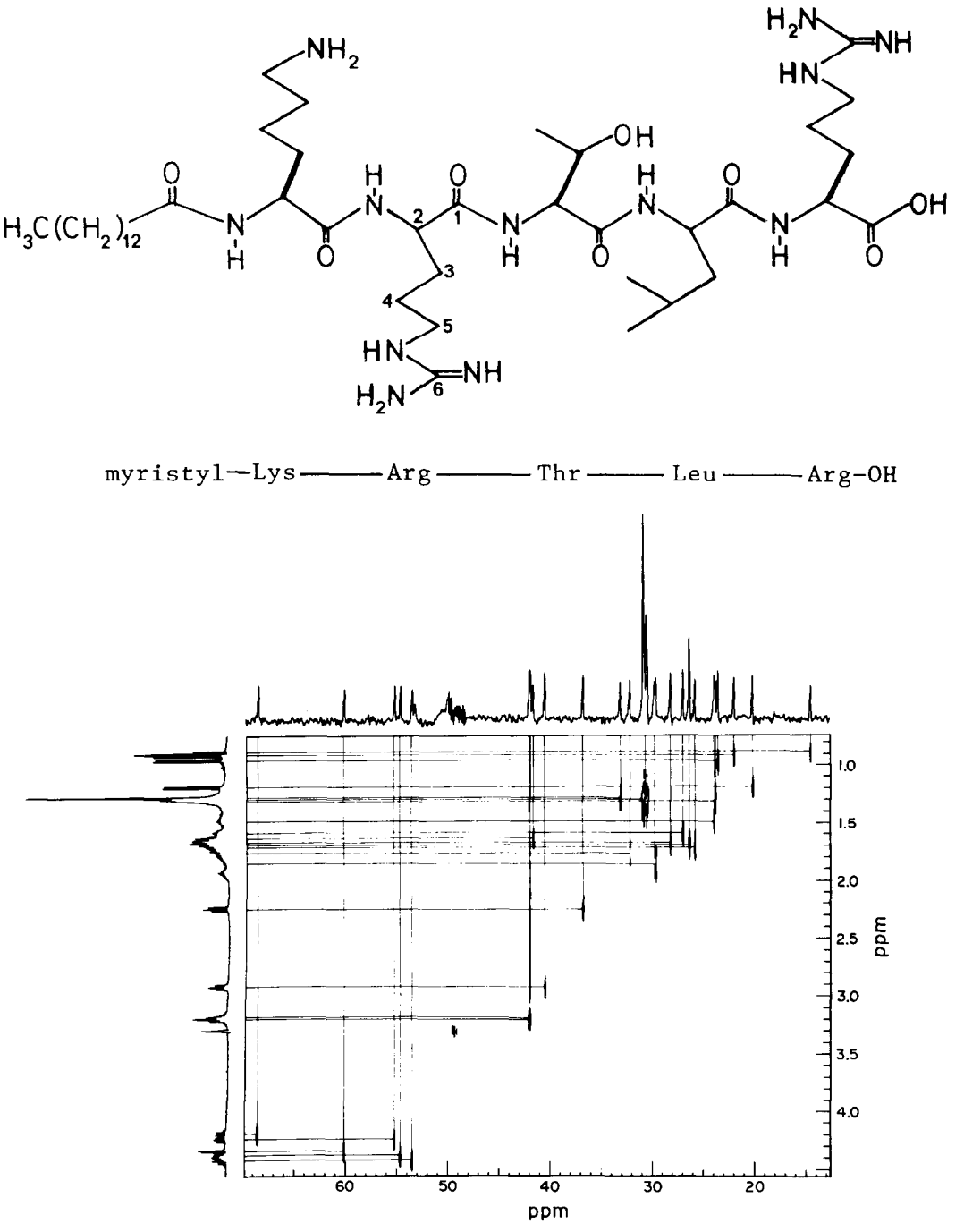

Fig. 1. Structure and NMR spectrum of *N*-myristyl-Lys-Arg-Thr-Leu-Arg. Top panel: structure of the synthetic peptide *N*-myristyl-Lys-Arg-Thr-Leu-Arg. Bottom panel: Cross-section of the 400-MHz ^{13}C–^{1}H-correlated NMR spectrum. Assignment of the resonances is described in Materials and Methods. The signals at approximately 49 ppm (^{13}C spectrum, abscissa), at approximately 3.3 ppm (^{1}H-spectrum, ordinate), and the resulting cross peaks are due to the solvent CD_3OD.

peptide inhibited this phosphorylation reaction with an IC_{50} of approximately 75 μM (Fig. 2). While the myristylated peptide was an effective inhibitor of PKC-catalyzed histone phosphorylation, it did not serve as a PKC phosphoacceptor substrate across a concentration range of 20–200 μM peptide (data not shown). This indicated that the myristylated peptide was, in fact, a PKC inhibitor rather than simply a substrate competing with histone.

We found that the parent pentapeptide Lys-Arg-Thr-Leu-Arg did not inhibit PKC-catalyzed Ca^{2+}- and PS-dependent histone phosphorylation, even at peptide concentrations as high as 600 μM (Table 1). In addition, we observed that 200 μM myristic acid

Table 1. Effects of the pentapeptide Lys-Arg-Thr-Leu-Arg and its phosphorylated analogue on PKC-catalyzed histone phosphorylation

Peptide	Ca^{2+}- and PS-dependent histone phosphorylation	
	(pmol ^{32}P/min)	% Activity
0 μM KRTLR	79 ± 7	100 ± 9
200 μM KRTLR	78 ± 6	99 ± 8
400 μM KRTLR	80 ± 3	102 ± 3
600 μM KRTLR	74 ± 2	94 ± 2
0 μM KRT(P)LR	66 ± 6	100 ± 9
200 μM KRT(P)LR	68 ± 6	103 ± 9
400 μM KRT(P)LR	66 ± 7	100 ± 11
600 μM KRT(P)LR	59 ± 8	90 ± 12

KRTLR represents Lys-Arg-Thr-Leu-Arg and KRT(P)LR represents Lys-Arg-Thr(P)-Leu-Arg. PKC activity was assayed by our standard procedure, as described in Materials and Methods. The amount of ^{32}P incorporated into Lys-Arg-Thr-Leu-Arg was negligible relative to the amount of ^{32}P incorporated into histone. Each data point is the mean ± SD of triplicate determinations.

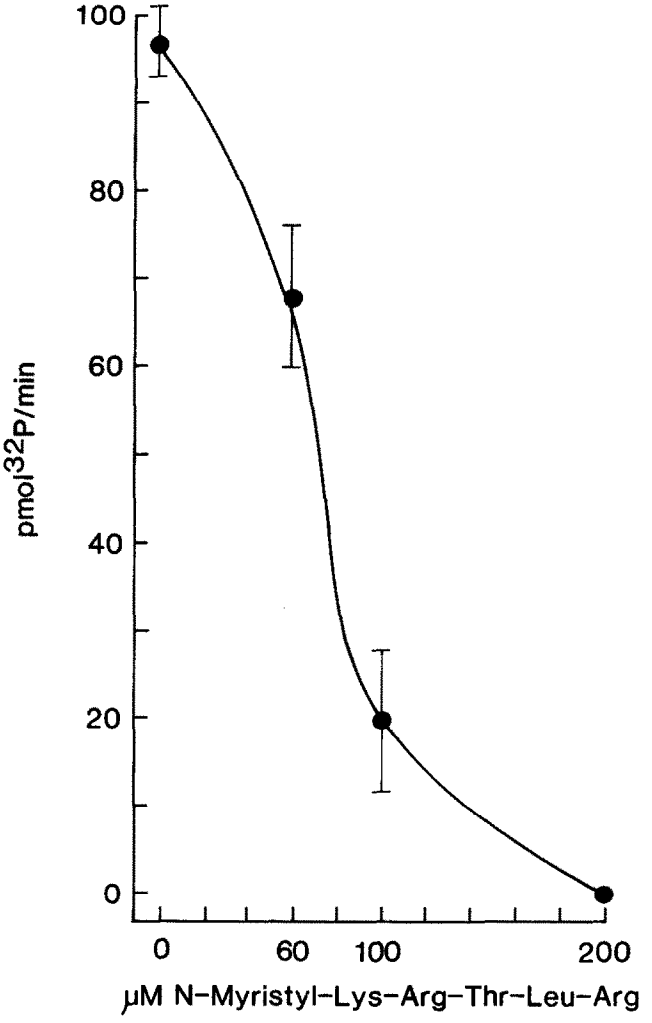

Fig. 2. Inhibition of Ca^{2+}- and PS-dependent histone phosphorylation by *N*-myristyl-Lys-Arg-Thr-Leu-Arg. PKC was assayed as described in Materials and Methods in the presence of the indicated amount of *N*-myristyl-Lys-Arg-Thr-Leu-Arg. The units on the ordinate (pmol ^{32}P/min) represent the rate of incorporation of ^{32}P into histone IIIS. Each point is the mean ± SD of triplicate determinations.

Table 2. Inhibition of Ca^{2+}- and PS-dependent PKC activity by *N*-myristyl-Lys-Arg-Thr-Leu-Arg in the presence of various PS concentrations

PS (μg/ml)	^{32}P (pmol/min) No peptide	60 μM Peptide	% Inhibition
10	63	0	100 ± 1
50	93	35	62 ± 9
100	107	111	0 ± 4
200	112	125	0 ± 3

Percent inhibition represents the percent inhibition of PKC-catalyzed, Ca^{2+}- and PS-dependent incorporation of ^{32}P into histone IIIS by 60 μM *N*-myristyl-Lys-Arg-Thr-Leu-Arg. Each value is the mean of triplicate determinations; SD values are given for the percent inhibition column.

had no effect on Ca^{2+}- and PS-dependent PKC activity (data not shown). Histone and pentapeptide phosphorylations are both measured in our standard assays by binding the histone and pentapeptide to phosphocellulose paper [20, 24]. Since the level of pentapeptide phosphorylation is very low relative to that of histone phosphorylation [20], it is unlikely that the phosphorylation of the pentapeptide would interfere with the measurement of histone phosphorylation on phosphocellulose paper. To verify that ^{32}P incorporation into the peptide did not mask an inhibitory activity of the peptide against histone phosphorylation, we conducted control studies in which reactions were terminated by TCA precipitation. Since TCA precipitates proteins but not small peptides, it allows a direct measurement of ^{32}P incorporation into peptide-free histone. The parent peptide exhibited no inhibitory activity against Ca^{2+}- and PS-dependent histone phosphorylation in assays terminated by TCA precipitation (data not shown). Furthermore, the phosphopeptide analogue, which served as a control peptide that could not be phosphorylated, also had no inhibitory activity against Ca^{2+}- and PS-dependent histone phosphorylation (Table 1).

A number of cationic amphiphiles, including tamoxifen [12, 13], W7 [16] and melittin [25], inhibit PKC with potencies that are reduced as the phospholipid cofactor concentration is increased. Since the myristylated peptide is a cationic amphiphile, we tested the effects of PS on the inhibitory potency of the myristylated peptide against PKC. Table 2 shows that the capacity of the myristylated peptide to inhibit Ca^{2+}- and PS-dependent PKC activity was diminished as the PS cofactor concentration was elevated. Thus, the myristylated peptide, like other cationic amphiphilic PKC inhibitors, inhibited Ca^{2+}- and PS-dependent PKC activity with a potency that was dependent on the lipid cofactor concentration.

Inhibition of the activity of the catalytic fragment of PKC by N-*myristyl-Lys-Arg-Thr-Leu-Arg*

A fully active, Ca^{2+}- and PS-independent catalytic fragment of PKC can be liberated from the enzyme by limited proteolysis [26]. We determined the inhibitory effects of Lys-Arg-Thr-Leu-Arg and its phosphorylated and myristylated derivatives against a catalytic fragment of PKC generated by limited proteolysis. At concentrations as high as 600 μM, neither the parent pentapeptide nor its phosphorylated analogue (which served as a nonphosphorylatable control peptide) inhibited the Ca^{2+}- and PS-independent phosphorylation of histone IIIS catalyzed by the catalytic fragment of PKC (data not shown). In addition, we found that 200 μM myristic acid had no effect on the activity of the catalytic fragment. In contrast, the myristylated peptide inhibited the histone kinase activity of the catalytic fragment of PKC with an IC_{50} of approximately 200 μM (Fig. 3).

To examine whether the reduced potency of the myristylated peptide against PKC in the presence of elevated PS concentrations might reflect the sequestration of the peptide by PS, we examined the effect of PS on the inhibition of the Ca^{2+}- and PS-independent catalytic fragment of PKC by the peptide. We found that 300 μM myristylated peptide inhibited the catalytic fragment 100 ± 1% in the absence of PS, 75 ± 15% with 50 μg/ml PS, 1 ± 5% with 100 μg/ml PS and 0 ± 9% with 200 μg/ml PS. Thus, the reduced inhibition of PKC in the presence of elevated PS appears to result, at least in part, from the sequestration of the peptide inhibitor by PS.

Kinetics of inhibition of the catalytic fragment of PKC by N-*myristyl-Lys-Arg-Thr-Leu-Arg*

We recently observed that the cationic amphiphilic PKC inhibitors tamoxifen [15] and W7 [17] inhibit PKC by the dual mechanisms of interaction with the phospholipid cofactor and competition with the substrate ATP. Therefore, we tested whether the cationic and amphiphilic myristylated peptide inhibited the catalytic fragment of PKC by competitive inhibition with respect to ATP. We first determined that the myristylated peptide was a very weak substrate of the catalytic fragment of the enzyme. At a concentration of 300 μM, the myristylated peptide was phosphorylated to 2.0 ± 0.5% of the extent that histone IIIS was phosphorylated (38 ± 2 pmol ^{32}P/min) under identical conditions. Thus, the incorporation of ^{32}P into the myristylated peptide was negligible in measurements of the incorporation of ^{32}P into histone IIIS. Figure 4 shows that the myristylated peptide, unlike other cationic amphiphilic PKC inhibitors that we have studied, inhibited the catalytic fragment of PKC noncompetitively with respect to ATP, according to Lineweaver–Burk analysis. Consistent with noncompetitive kinetics, the apparent V_{max} for histone phosphorylation was altered significantly by the peptide. In the absence of peptide, the apparent V_{max} was 48 pmol ^{32}P/min, whereas it was 33 pmol ^{32}P/min in the presence of 150 μM peptide and 17 pmol ^{32}P/min in the presence of 250 μM peptide. Noncompetitive mechanisms include cases where the apparent K_m is changed and cases where it is not altered. In our study, the apparent K_m for ATP was 14 μM in the absence of peptide, 12 μM with 150 μM peptide, and 35 μM with 250 μM peptide. This kinetic result was consistent with our observation that the myristylated peptide could serve as a phosphoacceptor substrate of PKC.

We next examined the kinetics of inhibition with

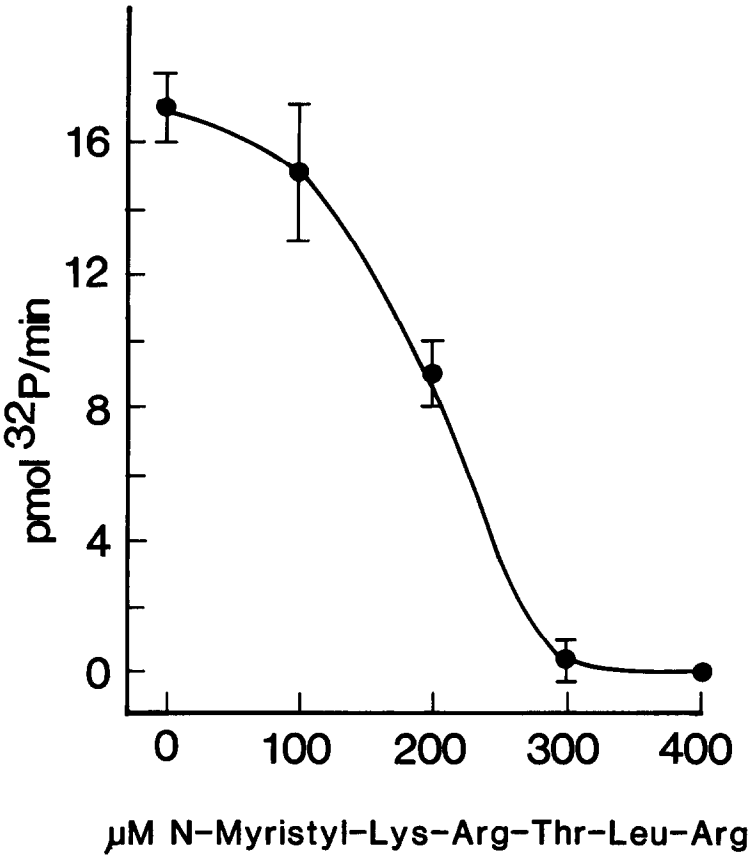

Fig. 3. Inhibition of histone phosphorylation catalyzed by the catalytic fragment of PKC. The Ca^{2+}- and PS-independent incorporation of ^{32}P into histone IIIS catalyzed by the catalytic fragment of PKC was measured in the presence of indicated concentrations of *N*-myristyl-Lys-Arg-Thr-Leu-Arg, as described in Materials and Methods. See the legend to Fig. 2 for further details.

respect to histone IIIS. Consistent with other studies [27], we found that the kinetics observed when histone concentrations were varied were complicated and could not be subjected to Lineweaver–Burk analysis. However, a simple comparison of the inhibitory potency of the myristylated peptide with the concentration of the substrate histone IIIS reveals that the inhibition of the activity of the catalytic fragment by the peptide was reduced and even overcome as histone concentrations were increased (Fig. 5). Taken together with the sequence specificity of the phosphoacceptor substrate binding region of PKC, the data presented here provide strong evidence that the myristylated peptide inhibits the catalytic fragment of the enzyme by binding to the phosphoacceptor substrate binding region of the active site of the enzyme.

Inhibition of PKA by N-*myristyl-Lys-Arg-Thr-Leu-Arg*

To assess the selectivity of the myristylated peptide in its inhibitory activity against PKC, we determined its inhibitory potency against PKA, since the catalytic domains of cyclic nucleotide-dependent protein kinases bear the greatest homology to those of PKC isozymes, when the established primary structures of protein kinases are compared [28]. We first tested the myristylated peptide as a PKA substrate at peptide concentrations from 100 to 400 μM, and we found that the peptide was not phosphorylated by the catalytic subunit PKA (data not shown). We then examined the capacity of the myristylated peptide to inhibit the phosphorylation of the heptapeptide kemptide [29] catalyzed by the catalytic subunit of PKA. We found that the myrisylated peptide inhibited PKA with an IC_{50} greater than 400 μM (Table 3).

DISCUSSION

In this report, we show that myristylation of the synthetic pentapeptide PKC substrate Lys-Arg-Thr-Leu-Arg at its N terminus greatly enhances the affinity of the peptide for an activated PKC complex. Myristylation endowed the peptide with an inhibitory activity against Ca^{2+}- and PS-activated PKC (IC_{50} = 75 μM) that the nonmyristylated peptide lacked, and myristylation greatly enhanced the capacity of the peptide to bind to the phosphoacceptor substrate binding region of a catalytic fragment of PKC, as evidenced by its apparent competition at this site with the substrate histone IIIS (IC_{50} = 200 μM). Our results suggest that myristylation may facilitate the recognition of certain endogenous PKC substrates by

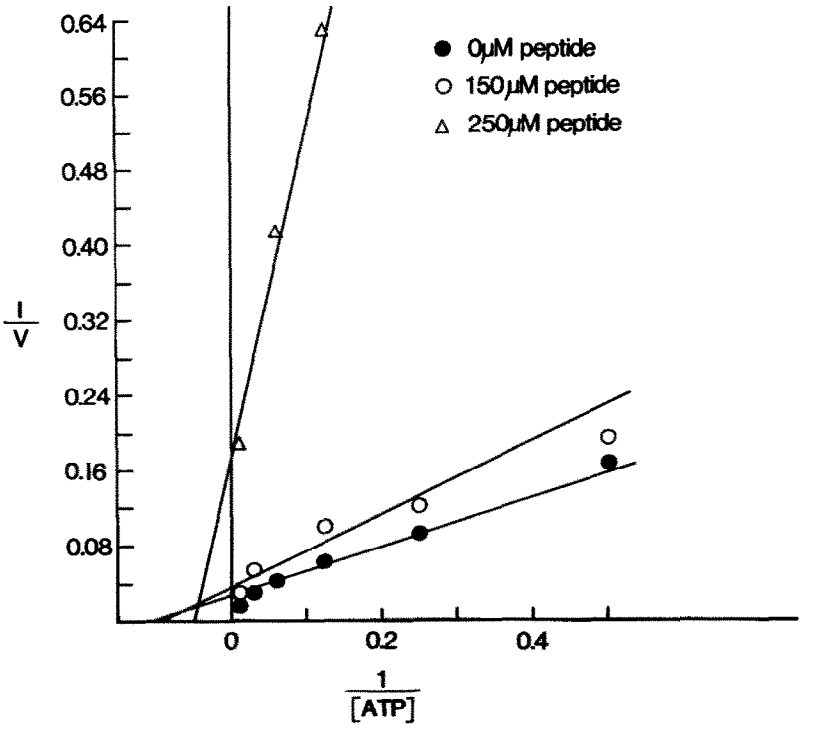

Fig. 4. Inhibition of the catalytic fragment of PKC by *N*-myristyl-Lys-Arg-Thr-Leu-Arg in the presence of various ATP concentrations. See legend to Figure 2 and Materials and Methods for experimental details. The linear correlation coefficients were 0.998 (0 μM peptide), 0.990 (150 μM peptide), and 0.890 (250 μM peptide). Peptide = *N*-myristyl-Lys-Arg-Thr-Leu-Arg; v = pmol ^{32}P/min.

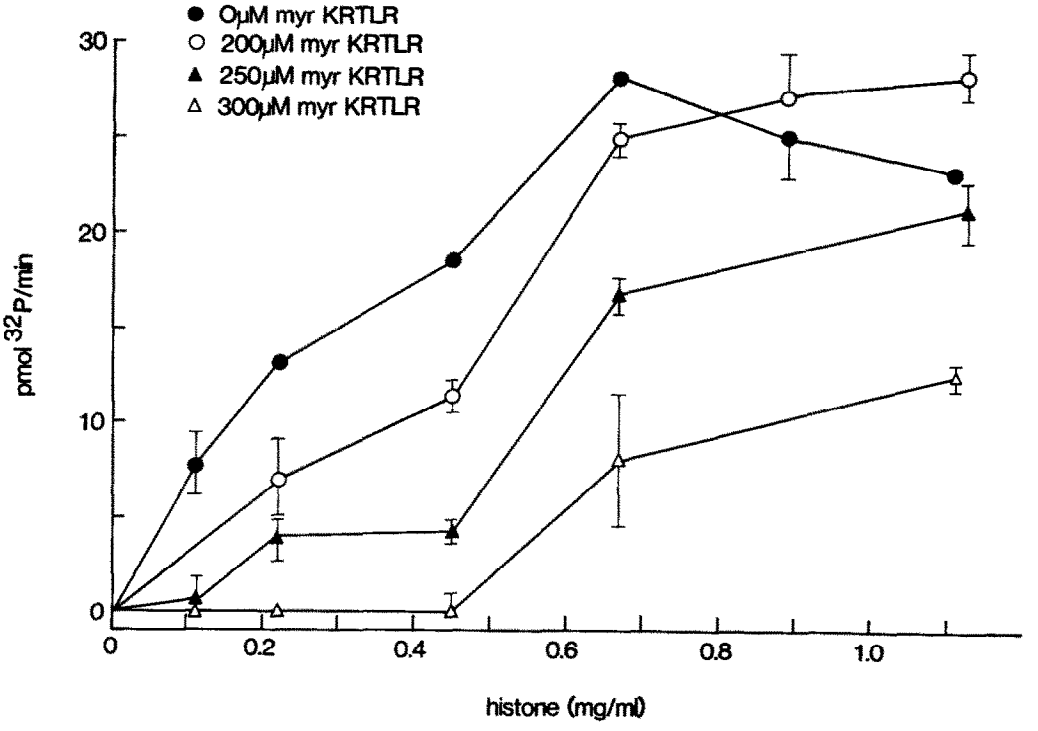

Fig. 5. Inhibition of the catalytic fragment of PKC by *N*-myristyl-Lys-Arg-Thr-Leu-Arg in the presence of various histone IIIS concentrations. See legend to Figure 2 and Materials and Methods for experimental details. MyrKRTLR = *N*-myristyl-Lys-Arg-Thr-Leu-Arg.

Table 3. Inhibition of PKA-catalyzed kemptide phosphorylation by *N*-myristyl-Lys-Arg-Thr-Leu-Arg

MyrKRTLR concn (μM)	^{32}P (pmol/min)	% Activity
0	146 ± 8	100 ± 5
100	177 ± 4	121 ± 3
200	129 ± 11	88 ± 8
400	96 ± 12	66 ± 8

MyrKRTLR represents *N*-myristyl-Lys-Arg-Thr-Leu-Arg; pmol ^{32}P/min represents the picomoles of ^{32}P transferred to kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) [29] by PKA catalysis per minute. PKA activity was measured by the procedure employed in the assay of PKC with the following modifications: 40 μM kemptide was used in place of histone IIIS; Ca^{2+} and PS were omitted; 4 mg/ml dithiothreitol (DTT) was used in place of 2-mercaptoethanol; 0.1 mg/ml BSA was present in all reaction mixtures; and 4 units of the catalytic subunit of bovine heart PKA was employed to catalyze the phosphotransferase reaction. In control experiments, we determined that PKA did not catalyze the phosphorylation of *N*-myristyl-Lys-Arg-Thr-Leu-Arg. Values are means ± SD of triplicate determinations.

the enzyme *in vivo*. In fact, at least three endogenous PKC substrates are modified by lipids. pp60$^{v\text{-}src}$ is myristylated at glycine 2 [30] and phosphorylated by PKC at a residue nearby, serine 12 [31]. cKi-*ras* p21 protein is phosphorylated by PKC, apparently at serine 181 [32], and the site of palmitylation of v-Ha-*ras* p21 protein has been tentatively mapped at cysteine 186, very near the putative PKC-catalyzed phosphorylation site of cKi-*ras* p21 protein [33]. Finally, vinculin is also a PKC substrate [34] that contains covalently attached lipid [35].

The peptide *N*-myristyl-Lys-Arg-Thr-Leu-Arg is a novel PKC inhibitor, insofar as it functions by interacting with the PS cofactor of PKC and by competing with the phosphoacceptor substrate at the active site of the enzyme. The myristylated peptide is a cationic and amphiphilic compound, and it clearly interacts with the lipid cofactor of the enzyme, as do several cationic amphiphilic PKC inhibitors [8, 12, 13, 16, 25, 36]. In this study, we show that the cofactor PS reduces the potency of a cationic amphiphilic inhibitor against both PS-dependent and PS-independent activities of the enzyme, providing evidence that reduced potencies of at least some cationic amphiphilic inhibitors in the presence of elevated PS concentrations involve sequestration of the inhibitor by PS. A number of cationic amphiphilic PKC inhibitors also compete with ATP at the active site of PKC [15, 17]. The myristylated peptide is unique as a cationic amphiphilic PKC inhibitor that competes with the phosphoacceptor substrate of PKC. The myristylated peptide is much more potent as an active site inhibitor of PKC than as a PKC substrate, suggesting that myristylation of the peptide causes it to bind to the active site of PKC in a nonproductive position, i.e. in a position that does not allow the threonine residue of the peptide to be readily phosphorylated. Thus, its half-life as an inhibitor is prolonged by its inefficiency as a PKC substrate.

The critical role of PKC in proliferation suggests that selective antagonists of PKC or particular PKC isozymes may be useful as antiproliferative agents [8]. However, established PKC inhibitors lack specificity [8]. We consider *N*-myristyl-Lys-Arg-Thr-Leu-Arg to be an important model in the search for more selective PKC inhibitors, since it represents a new class of PKC inhibitors that exploits not only the remarkable discrimination of the phosphoacceptor binding sites of protein kinases but also the differences in the lipid cofactor requirements of PKC isozymes [2, 3].

Acknowledgements—We thank C. Erkelens for recording the two-dimensional ^{13}C–^{1}H NMR spectrum. This work was supported by Grant G-1141-01 from the Robert A. Welch Foundation (C.A.O.), Biomedical Research Support Grant RR5511-25 from the University of Texas M.D. Anderson Cancer Center (C.A.O.), NCI Core Grant CA16672 to The University of Texas M.D. Anderson Cancer Center (C.A.O), and The Netherlands Cancer Foundation (Koningin Wilhelmina Fonds) (R.M.L.).

REFERENCES

1. Nishizuka Y, Studies and perspectives of protein kinase C. *Science* **233**: 305–312, 1986.
2. Nishizuka Y, The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature* **334**: 661–665, 1988.
3. Jaken S and Leach KL, Isozymes of protein kinase C. *Annu Rep Medic Chem* **23**: 243–252, 1988.
4. Castagna M, Takai Y, Kaibuchi K, Sano K, Kikkawa U and Nishizuka Y, Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor promoting phorbol esters. *J Biol Chem* **257**: 7847–7851, 1982.
5. Kishimoto A, Takai Y, Mori T, Kikkawa U and Nishizuka Y, Activation of calcium and phospholipid-dependent protein kinase by diacylglycerol, its possible relation to phosphatidylinositol turnover. *J Biol Chem* **255**: 2273–2276, 1980.
6. Housey GM, Johnson MD, Hsiao WLW, O'Brian CA, Murphy JP, Kirschmeier P and Weinstein IB, Overproduction of protein kinase C causes disordered growth control in rat fibroblasts. *Cell* **52**: 343–354, 1988.
7. Persons DA, Wilkison WO, Bell RM and Finn OJ, Altered growth regulation and enhanced tumorigenicity of NIH 3T3 fibroblasts transfected with protein kinase C-1 cDNA. *Cell* **52**: 447–458, 1988.
8. O'Brian CA, Housey GM, Johnson MD, Kirschmeier P, Liskamp RM and Weinstein IB, The mechanism of action of protein kinase C: New insights through the study of inhibitors and gene cloning. In: *Tumor Promoters: Biological Approaches for Mechanistic Studies and Assay Systems* (Eds. Langenbach R, Elmore E and Barrett JC), pp. 343–356. Raven Press, New York, 1988.
9. Kase H, Iwahashi K, Nakanishi S, Matsuda Y, Yamada K, Takahashi M, Murakata C, Sato 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.
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. Hidaka H, Inagaki M, Kawamoto S and Sasaki Y, Isoquinolinesulfonamides, novel and potent inhibitors

of cyclic nucleotide dependent protein kinase and protein kinase C. *Biochemistry* **23**: 5036–5041, 1984.
12. O'Brian CA, Liskamp RM, Solomon DH and Weinstein IB, Inhibition of protein kinase C by tamoxifen. *Cancer Res* **45**: 2462–2465, 1985.
13. Su H-D, Mazzei GJ, Vogler WR and Kuo JF, Effects of tamoxifen, a nonsteroidal antiestrogen, on phospholipid/calcium-dependent protein kinase and phosphorylation of its endogenous substrate proteins from the rat brain and ovary. *Biochem Pharmacol* **34**: 3649–3653, 1985.
14. O'Brian CA, Housey GM and Weinstein IB, Specific and direct binding of protein kinase C to an immobilized tamoxifen analogue. *Cancer Res* **48**: 3626–3629, 1988.
15. O'Brian CA, Ward NE and Anderson BW, Role of specific interactions between protein kinase C and triphenylethylenes in inhibition of the enzyme. *J Natl Cancer Inst* **80**: 1628–1633, 1988.
16. Tanaka T, Ohmura T, Yamakado T and Hidaka H, Two types of calcium-dependent protein phosphorylations modulated by calmodulin antagonists. *Mol Pharmacol* **22**: 408–412, 1982.
17. O'Brian CA and Ward NE, Binding of protein kinase C to *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide through its ATP binding site. *Biochem Pharmacol* **38**: 1737–1742, 1989.
18. Hannun YA and Bell RM, Aminoacridines, potent inhibitors of protein kinase C. *J Biol Chem* **263**: 5124–5131, 1988.
19. House C and Kemp BE, Protein kinase C contains a pseudosubstrate prototope in its regulatory domain. *Science* **238**: 1726–1728, 1987.
20. de Bont HBA, Liskamp RMJ, O'Brian CA, Erkelens C, Veeneman GH and van Boom JH, Synthesis of a substrate of protein kinase C and its corresponding phosphopeptide. *Int J Pept Protein Res* **33**: 115–123, 1989.
21. Bax A, Broadband homonuclear decoupling in heteronuclear shift correlation NMR spectroscopy. *J Magnet Resonance* **53**: 517–520, 1983.
22. IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN), Nomenclature and symbolism for amino acids and peptides. Recommendations 1983. *J Biol Chem* **260**: 14–42, 1985.
23. O'Brian CA and Ward NE, ATP-sensitive binding of melittin to the catalytic domain of protein kinase C. *Mol Pharmacol*, in press.
24. O'Brian CA, Lawrence DS, Kaiser ET and Weinstein IB, Protein kinase C phosphorylates the synthetic peptide Arg-Arg-Lys-Ala-Ser-Gly-Pro-Pro-Val in the presence of phospholipid plus either Ca^{2+} or a phorbol ester tumor promoter. *Biochem Biophys Res Commun* **124**: 296–302, 1984.
25. Katoh N, Raynor RL, Wise BC, Schatzman RC, Turner RS, Helfman DM, Fain JN and Kuo JF, Inhibition by melittin of phospholipid-sensitive and calmodulin-sensitive Ca^{2+}-dependent protein kinases. *Biochem J* **202**: 217–224, 1982.
26. Lee M-H and Bell RM, The lipid binding, regulatory domain of protein kinase C. *J Biol Chem* **261**: 14867–14870, 1986.
27. Charp PA, Rice WG, Raynor RL, Reimund E, Kinkade JM, Ganz T, Selsted ME, Lehrer RI and Kuo JF, Inhibition of protein kinase C by defensins, antibiotic peptides from human neutrophils. *Biochem Pharmacol* **37**: 951–956, 1988.
28. Hanks SK, Quinn AM and Hunter T, The protein kinase family: Conserved features and deduced phylogeny of the catalytic domains. *Science* **241**: 42–52, 1988.
29. Kemp BE, Graves DJ, Benjamini E and Krebs EG, Role of multiple basic residues in determining the substrate specificity of cAMP-dependent protein kinase. *J Biol Chem* **252**: 4888–4894, 1977.
30. Kaplan JM, Mardon G, Bishop JM and Varmus HE, The first seven amino acids encoded by the v-*src* oncogene act as a myristylation signal: lysine 7 is a critical determinant. *Mol Cell Biol* **8**: 2435–2441, 1988.
31. Gould KL, Woodgett JR, Cooper JA, Buss JE, Shalloway D and Hunter T, PKC phosphorylates $pp60^{src}$ at a novel site. *Cell* **42**: 849–857, 1985.
32. Ballester R, Furth ME and Rosen OM, Phorbol ester- and protein kinase C-mediated phosphorylation of the cellular kirsten *ras* gene product. *J Biol Chem* **262**: 2688–2695, 1987.
33. Buss JE and Sefton BM, Direct identification of palmitic acid as the lipid attached to $p21^{ras}$. *Mol Cell Biol* **6**: 116–122, 1986.
34. Werth DK, Niedel JE and Pastan I, Vinculin, a cytoskeletal substrate of protein kinase C. *J Biol Chem* **258**: 11423–11426, 1983.
35. Burn P and Burger MM, The cytoskeletal protein vinculin contains transformation-sensitive, covalently bound lipid. *Science* **235**: 476–479, 1987.
36. O'Brian CA and Weinstein IB, *In vitro* inhibition of rat brain protein kinase C by rhodamine 6G: Profound effects of the lipid cofactor on the inhibition of the enzyme. *Biochem Pharmacol* **36**: 1231–1235, 1987.