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SYNTHETIC MYELIN BASIC PROTEIN PEPTIDE ANALOGS ARE SPECIFIC INHIBITORS OF PHOSPHOLIPID/CALCIUM-DEPENDENT PROTEIN KINASE (PROTEIN KINASE C)

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SUMMARY: Synthetic peptide analogs of the bovine myelin basic protein (MBP) corresponding to residues 104-118 were found to specifically inhibit phospholipid/Ca^{2+}-dependent protein kinase (protein kinase C). The peptides [Ala^{107}]MBP(104-118) and [Ala^{113}]MBP(104-118) inhibited protein phosphorylation of intact MBP, histone H1 and peptide phosphorylation with MBP(104-123), MBP(104-118) or [Ala^{105}]MBP(104-118) as substrates. The inhibitor peptides [Ala^{107}]MBP(104-118) and [Ala^{113}]MBP(104-118), containing alanine in place of the arginine recognition sites, apparently inhibited the enzyme noncompetitively with respect to substrates, with IC_{50} values ranging from 46-145 and 28-62 μM, respectively. These peptide analogs did not inhibit cyclic AMP-dependent protein kinase or myosin light chain kinase but inhibited phospholipid/Ca^{2+}-dependent phosphorylation of endogenous proteins in the total, solubilized fraction of rat brain. © 1986 Academic Press, Inc.

Although phospholipid/Ca^{2+}-dependent protein kinase (protein kinase C) has been shown to phosphorylate a number of purified proteins and unidentified, endogenous proteins in various tissues, the precise structural determinants for its substrate specificity are less well understood than other protein kinases (1-4). Myelin basic protein (MBP), a major endogenous substrate for the enzyme in brain (2,5), is by far the best substrate protein for the enzyme, as judged by its low K_m, high V_{max} and multiple phosphorylation sites (3). Because the amino acid sequence of bovine MBP is known (6), and serine 115 in bovine MBP is the primary site of phosphorylation by protein kinase C (7), we investigated the minimum amino acid sequence around serine 115 required for substrates of the enzyme. Of several synthetic peptides tested, we found that MBP(104-118) and MBP(104-123) are effective substrates, with K_m values of 18 and 10 μM, respectively (8). Arginine 107 and arginine 113, but not lysine 105, are essential for the substrate activity of these peptides.

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O'Brian et al. (9) reported that the histone H1 peptide Arg-Arg-Lys-Ala-Ser-Gly-Pro-Pro-Val is a moderately good substrate for the protein kinase C, with a K_m of 130 μM. It was found that when serine was substituted with alanine the peptide is a very weak inhibitor, with only 30% inhibition at 3.5 mM. These results are similar to those obtained earlier with the alanine analogs of substrate peptides for the cyclic AMP-dependent protein kinase (reviewed in Ref. 10). In the present studies, we investigated analogs of a substrate peptide MBP(104-118) in which the specificity determinants arginine 107 or arginine 113, instead of the phosphorylated serine 115, were replaced by alanine. We found that these analogs were unexpectedly specific and relatively potent inhibitors of protein kinase C.

MATERIALS AND METHODS

Materials: Phosphatidylserine (bovine brain) and histone H1 (type III-S, lysine-rich histone) were purchased from Sigma. The unmodified, dephosphorylated form (component 1) of bovine MBP (11) was a kind gift of Dr. C.-H. Jen Chou and Dr. Robert F. Kibler, Department of Neurology, Emory University School of Medicine.

Methods: Bovine MBP peptides with amino acid sequences around serine 115 and their alanine-substituted analogs were synthesized by the procedure of Hodges and Merrifield (12), as described recently (8). All peptides had the expected amino acid compositions. The original numbering system for the primary sequence of bovine MBP (6) is retained.

Protein kinase C (95% homogeneous with respect to the 80-kDa native enzyme species) was purified from pig brain extracts as described recently (13). The last step of the purification was affinity chromatography on polyacrylamide in which cholesterol and phosphatidylserine were immobilized (13,14). The enzyme was also partially purified from bovine heart extracts through the Sephacryl S-200 step as described previously (15). The enzyme was assayed as described elsewhere (3,5,7,8). Briefly, the standard reaction mixtures contained, in 0.2 ml, 5 μmol of Pipes (pH 6.5), 2 μmol of $MgCl_2$, 5 μg of phosphatidylserine, 0.04 μmol of EGTA, 20 nmol of [γ-^{32}P]ATP (containing about 1 x 10^6 cpm), with or without 0.1 μmol of $CaCl_2$. Cyclic AMP-dependent protein kinase from bovine hearts was partially purified and its activity assayed as described (16). Myosin light chain kinase and myosin light chains, both from bovine hearts, were purified to apparent homogeneity and the enzyme activity was assayed as reported previously (17,18). The reactions for all protein kinases were carried out at 30° for 5 min. The phosphorylated proteins or peptides were separated from [γ-^{32}P]ATP by means of AG 1-X8 anion exchange resin (8,19). The enzyme activities were linear as a function of incubation time, amount of the enzyme or the amount of substrate. [γ-^{32}P]ATP was prepared according to Post and Sen (20) and protein was determined by the method of Bradford (21).

RESULTS AND DISCUSSION

We reported recently that the synthetic peptide MBP(104-118), (NH_2)-Gly^{104}-Lys^{105}-Gly-Arg^{107}-Gly-Leu-Ser-Leu-Ser-Arg^{113}-Phe-Ser^{115}-Trp^{116}-Gly-Ala^{118}(COOH), is a good substrate for protein kinase C, and that certain alanine-substituted analogs

TABLE I

Summary of IC_{50} values of protein kinase C inhibition by peptide inhibitors

Substrate	App. K_m[a] (μM)	IC_{50} (μM)	
		[Ala^{107}]MBP(104-118)	[Ala^{113}]MBP(104-118)
MBP(104-118)	18	72	35
MBP(104-123)	10	63	28
[Ala^{105}]MBP(104-118)	22	46	32
MBP(1-170) (intact)	1	145	62
Histone H1	3	126	45

[a]Taken from Turner et al. (8).

of the peptides are poor substrates for the enzyme (8). Because alanine substitutions affect primarily the V_{max} but not the K_m values, these analogs could potentially inhibit phosphorylation of the parent peptide or even other peptide and protein substrates. In the present studies, we found that [Ala^{107}]MBP(104-118) and [Ala^{113}]MBP(104-118) (hereinafter referred to as inhibitor peptides) indeed inhibited phosphorylation of MBP(104-118), MBP(104-123), [Ala^{105}]MBP(104-118), MBP(1-170) (i.e. intact MBP) and histone H1. The IC_{50} values (concentrations causing a 50% inhibition) for [Ala^{107}]MBP(104-118) (i.e. 46-145 μM) and [Ala^{113}]MBP(104-118) (i.e. 28-62 μM) in inhibiting the protein kinase C-catalyzed phosphorylation of various substrates are summarized (Table 1).

Next, we examined the mode of action of the inhibitor peptides. It was found that their inhibition was not overcome by increasing concentrations of the parent peptide MBP(104-118) (Fig. 1), suggesting a noncompetitive nature of the inhibition with respect to the substate. The apparent noncompetitive inhibition by the inhibitor peptides was also noted when [Ala^{105}]MBP(104-118), intact MBP and histone H1 instead were used as substrates (data not shown).

Because [Ala^{107}]MBP(104-118) and [Ala^{113}]MBP(104-118) markedly inhibited phosphorylation of MBP and histone H1, in addition to that of MBP peptides, it was clear that they inhibited phosphorylation of other sites besides serine 115 in MBP. Consistent with this notion were the findings that they similarly inhibited the phospholipid/Ca^{2+}-dependent phosphorylation of various endogenous substrate proteins from rat brains, notably the species of 97-, 84-, 67-, 50- and 14-kDa (small MBP)

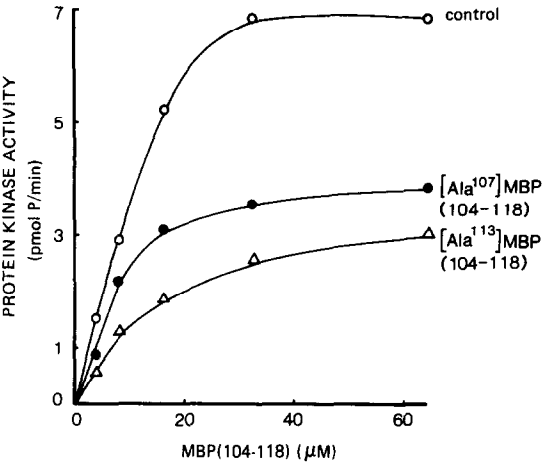

Fig. 1. Inhibition of protein kinase C phosphorylation of MBP(104-118) by [Ala^{107}]MBP(104-118) and [Ala^{113}]MBP(104-118). The concentration of the inhibitor peptides used was 50 μM. The small intrinsic substrate activity (< 10%) of the inhibitor peptides, relative to the substrate peptide, were subtracted from the phosphorylation values for calculations. The purified brain protein kinase C (0.05 μg) was used for the assays.

proteins (Fig. 2). The phospholipid/Ca^{2+}-independent phosphorylation, notably that of the 54-kDa protein, in comparison, was only slightly affected.

Because of the noncompetitive nature of the inhibition, we examined whether the MBP peptide analogs acted as inhibitors of other protein kinases. The ability of [Ala^{107}]MBP(104-118) to inhibit phosphorylation of certain proteins and peptides by three different protein kinases was investigated. It was found that [Ala^{107}]MBP-(104-118), while inhibiting protein kinase C partially purified from pig heart as shown above for the purified brain enzyme, was without effect on cyclic AMP-dependent protein kinase or myosin light chain kinase (Table 2). Similar results were also noted for [Ala^{113}]MBP(104-118) (data not shown).

It is intriguing that alanine substitutions of one of the arginine recognition sites in MBP(104-118) produced noncompetitive inhibitors of protein kinase C. The phosphate accepting serine residues in substrate peptides have been conventionally replaced by alanine for earlier studies on inhibitor peptides. For example, the alanine-substituted Kemptide Leu-Arg-Arg-Ala-Ala-Leu-Gly is a competitive and relatively selective inhibitor for cyclic AMP-dependent protein kinase, with an apparent K_i of about 300 μM (19,22,23). The alanine-substituted histone H2B peptide Arg-Lys-Arg-Ala-Arg-Lys-Glu has been shown to be a competitive and rather selective

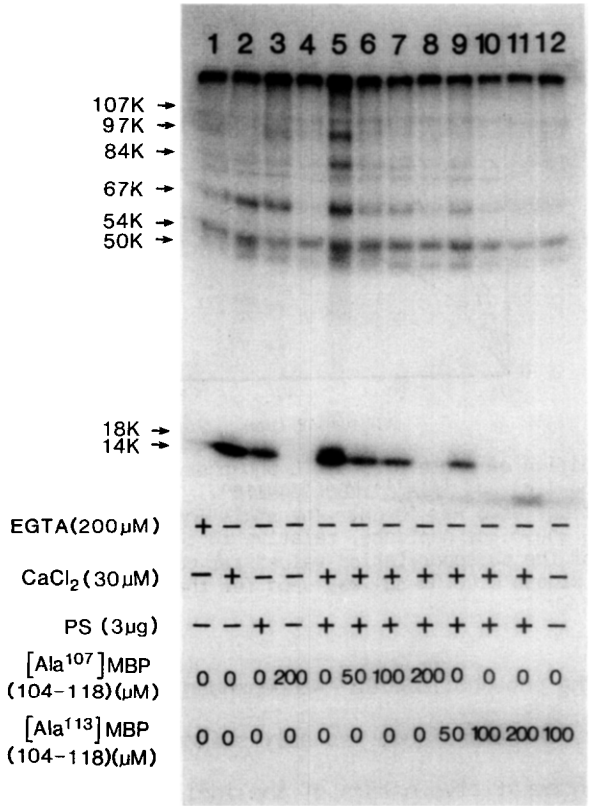

Fig. 2. Autoradiograph showing inhibition by [Ala^{107}]MBP(104-118) and [Ala^{113}]MBP(104-118) of the phospholipid/Ca^{2+}-dependent phosphorylation of endogenous proteins from rat brain. The total, solubilized fraction of rat brains, prepared as described previously (2,5), were phosphorylated under various conditions as indicated.

TABLE II

Specificity of protein kinase C inhibition by [Ala^{107}]MBP(104-118)

Substrate	Protein kinase activity (pmol/min)					
	Cyclic AMP-dependent		Myosin light chain kinase		Protein kinase C	
	- I	+ I	- I	+ I	- I	+ I
MBP(104-118)	2.5	2.5	0.5	0.5	7.5	0.2
MBP(104-123)	2.5	2.4			7.1	0.6
[Ala^{105}]MBP(104-118)	1.5	1.4			7.9	2.8
MBP(1-170)[a]	2.7	2.7			17.4	5.3
Histone H1	4.8	4.4			20.0	8.5
Myosin light chain[b]			7.9	7.1		

The enzymes, all from bovine heart, were assayed in the presence of various substrates (50 μM), with or without the inhibitor peptide (I), [Ala^{107}]MBP(104-(100 μM), as indicated. The concentration of [γ-^{32}P]ATP was 100 μM for all cases.

[a]The concentration of MBP was 3 μM; higher concentrations were inhibitory.

[b]The concentration of myosin light chain was 25 μM.

inhibitor for cyclic GMP-dependent protein kinase, with an apparent K_i of about 90 μM (23). It is conceivable that the alanine substitution of serine 115 (the phosphorylation site) in MBP(104-118) could also yield a competitive inhibitor peptide for protein kinase C. Although we cannot predict the type of inhibition, it is likely that alanine substitutions of one of the two recognition sites (arginine 107 or 113) as well as phosphorylated serine 115 in MBP(104-118) could yield highly potent and specific inhibitors for protein kinase C.

Kishimoto et al. (24) reported recently that serine 115 in bovine MBP is preferentially phosphorylated by cyclic AMP-dependent protein kinase, in contrast to our earlier findings that it is phosphorylated initially and preferentially by protein kinase C (7), the enzyme endogenous to myelin membranes (5). The discrepancy between the two laboratories is likely due to the following possibilities. (a) Their MBP preparation is not the dephosphorylated, unmodified species and therefore is already partially phosphorylated particulately at serine 115 as suggested by a low phosphorylation stoichiometry, (b) the high MBP concentration used is inhibitory to protein kinase C but not to the cyclic AMP enzyme, thus resulting in an apparently preferential phosphorylation of serine 115 by the latter enzyme, and (c) they used the exhaustively phosphorylated MBP and therefore the sites determined are not necessarily the initial and preferred phosphorylation sites.

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