Vol. 212, No. 3, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 26, 1995 Pages 806-812

A NOVEL HIGHLY SPECIFIC AND POTENT INHIBITOR OF CALMODULIN-DEPENDENT PROTEIN KINASE II

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Received June 17, 1995

SUMMARY: A novel synthetic peptide AIP (autocamtide-2-related inhibitory peptide), a nonphosphorylatable analog of autocamtide-2, was found to be a highly specific and potent inhibitor of calmodulin-dependent protein kinase II (CaM-kinase II). It was 50 and 500 times more potent than CaMK-(281-302Ala286) and KN-93, respectively, under the assay conditions used. The inhibition was unaffected by the presence or absence of Ca^{2+}/calmodulin, and it was competitive with autocamtide-2 and noncompetitive with syntide-2. AIP (1 μM) completely inhibited CaM-kinase II activity, but did not affect cyclic AMP-dependent protein kinase, protein kinase C, calmodulin-dependent protein kinase IV, and unidentified protein kinases occurring in a rat brain extract. These results indicate that AIP is a useful tool for studying the physiological roles of CaM-kinase II.

Calmodulin-dependent protein kinase II (CaM-kinase II) occurs very abundantly in the brain and has a broad substrate specificity (1-4). It is thought to play a number of roles in the functioning of the central nervous system in response to intracellular Ca^{2+} (reviewed in Refs. 1-3). The possible involvement of CaM-kinase II in the regulation of neuronal functions such as neurotransmitter synthesis (4-6), neurotransmitter release (7,8), long-term potentiation (9-11), and the formation of spatial learning (12) has so far been suggested. For studying the physiological roles of CaM-kinase II, a selective and potent inhibitor of the enzyme is a useful tool. Among a variety of inhibitors so far developed, KN-62, KN-93, and synthetic peptides corresponding to the autoinhibitory domain of CaM-kinase II have been used extensively to investigate the physiological roles of CaM-kinase II (2,9,10,13-18). However, the inhibition by KN-62 and KN-93 are competitive with respect to calmodulin (13,14), suggesting that these inhibitors may also inhibit other calmodulin-dependent enzymes. In fact, KN-62 has been reported to inhibit other calmodulin-dependent protein kinases (19,20). The

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Abbreviations: CaM-kinase II, Calmodulin-dependent protein kinase II; CaM-kinase IV, Calmodulin-dependent protein kinase IV; PKA, cyclic AMP-dependent protein kinase; PKC, protein kinase C.

0006-291X/95 $12.00

synthetic peptide inhibitors modeled after the autoinhibitory domain of CaM-kinase II so far used for studying the specific roles of CaM-kinase II have also been demonstrated to be not sufficiently specific for CaM-kinase II (15,21). Thus, the development of selective inhibitors of CaM-kinase II is of urgent necessity for exploring the specific roles of this enzyme. Since we recently found that a novel synthetic peptide, AIP (autocamtide-2-related inhibitory peptide), which is made by the substitution of Ala for the phosphorylation site of autocamtide-2, Thr9, is a potent inhibitor with the lowest Ki value (2 to 8 nM) for the constitutively active 30-kDa proteolytic fragment of CaM-kinase II so far reported (22), the specificity of the inhibition by AIP was examined in the present study.

MATERIALS AND METHODS

Materials

ATP and 1,2-dioleoyl-rac-glycerol were purchased from Sigma. Phosphatidylserine was from Serdary Research Laboratories, Inc. [γ-^{32}P]ATP (5000 Ci/mmol) was from Amersham International. KN-93 was obtained from Seikagaku Kogyo (Tokyo). Anti-CaM-kinase II monoclonal antibodies, CBα-2 and CBβ-1, were from GIBCO BRL. Synthetic peptide substrates, syntide-2 (23) and autocamtide-2 (24), were synthesized by American Peptide Company, Inc. AIP (KKALRRQEAVDAL) (22) was synthesized by Accord Co. Ltd. CaMK-(281-289) (MHRQETVDC) (25) was synthesized by a Shimadzu PSSM-8 automated peptide synthesizer. CaMK-(281-302Ala286) (MHRQEAVDCLKKFNARRKLKGA) (16,17) was purchased from Research Biochemicals International.

Protein Preparations

CaM-kinase II was purified from rat cerebral cortex as described previously (26). Catalytic subunit of cyclic AMP-dependent protein kinase (PKA) was prepared from bovine heart as described previously (27). Protein kinase C (PKC) was purified from rat cerebral cortex essentially according to the method of Woodgett and Hunter (28). Calmodulin-dependent protein kinase IV (CaM-kinase IV) was prepared as described previously (29). An active 30-kDa proteolytic fragment of CaM-kinase II was prepared according to the method of Yamagata et al. (30) with slight modifications (22,26). Calmodulin was purified from Escherichia coli transformed with expression vector pET11d carrying a cDNA encoding chicken brain calmodulin (26). "CaM-kinase II-depleted" preparations of a rat brain extract were obtained as follows. The 100,000 x g supernatant of a rat brain extract was applied to a phosphocellulose column as described previously (26), and the flow-through fraction of the column was pooled and then dialyzed against 50 mM Tris-HCl (pH 7.6) containing 1 mM dithiothreitol, 0.1 mM EDTA, 0.2 M NaCl, and 0.05% Tween 40 for 2 hr with two changes of buffer. The dialysate concentrated by Ultrafree CL(UFC4LGC25) (Millipore) was used as "P-through" preparation. The 100,000 x g supernatant was subjected to precipitation with ammonium sulfate at 60% saturation and the resultant precipitate was dissolved and applied to a calmodulin-Sepharose 4B column as described previously (26). The flow-through fraction was pooled, dialyzed, and concentrated as described above, and it was used as "CaM-through" preparation. CaM-kinase II was not detected in both preparations by immunoblotting with anti-CaM-kinase II monoclonal antibodies, CBα-2 and CBβ-1.

Assay of Protein Kinases

All of the following assays were carried out at 30°C. The activity of CaM-kinase II was measured as described previously (26), except that the concentrations of Ca^{2+} and calmodulin were 0.3 mM and 1 μM, respectively.

The activity of the active 30-kDa fragment of CaM-kinase II was measured as described previously (26). CaM-kinase IV activity was determined in the presence of 0.2 mM $CaCl_2$ with 100 μM syntide-2 as a substrate as described previously (29), except that 40 mM Hepes-NaOH (pH 8.0) was used instead of 50 mM Hepes-NaOH (pH 7.0). PKA and PKC activities were determined by phosphate incorporation into syntide-2 . The assay mixture for PKA consisted of 40 mM Mes-NaOH (pH 7.0), 5 mM magnesium acetate, 50 μM [γ-^{32}P]ATP, 0.01% Tween 20, 100 μM syntide-2, and a suitable amount of catalytic subunit of PKA. The assay mixture for PKC contained 40 mM Hepes-NaOH (pH 8.0), 10 mM magnesium acetate, 50 μM [γ-^{32}P]ATP, 0.1 mM EGTA, 0.35 mM $CaCl_2$, 0.01% Tween 20, 2.0 μg/ml 1,2-dioleoyl-rac-glycerol, 20.2 μg/ml phosphatidylserine, 100 μM syntide-2, and a suitable amount of PKC. The reaction was initiated by the addition of catalytic subunit of PKA or PKC, and the activity was determined by the phosphocellulose paper method (31).

Other Analytical Procedures

SDS-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (32). The concentrations of CaM-kinase II and calmodulin were determined spectrophotometrically as described previously (26). The concentrations of CaM-kinase IV was determined as described previously (29). Other proteins were determined by the method of Lowry et al. (33) as modified by Peterson (34) with bovine serum albumin as a standard. The concentrations of synthetic peptides were determined by amino acid analysis.

RESULTS AND DISCUSSION

Our recent finding (22) that a novel synthetic peptide, AIP, is a potent inhibitor of the 30-kDa fragment of CaM-kinase II, which is active in the presence or absence of Ca^{2+}/calmodulin (30), led us to examine the possibility that AIP is a selective inhibitor of CaM-kinase II. Fig. 1A

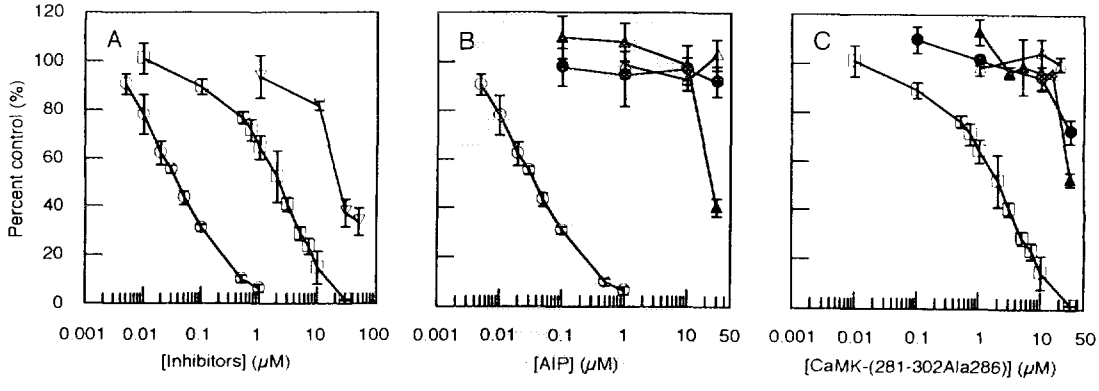

Fig. 1. Inhibition of protein kinases by CaM-kinase II inhibitors. (A) The activity of CaM-kinase II was measured in the presence of the indicated concentrations of AIP (○), CaMK-(281-302Ala286) (□), and KN-93 (▽). (B) The activities of CaM-kinase II (○), CaM-kinase IV (●), PKA (△), and PKC (▲) were measured in the presence of the indicated concentrations of AIP. (C) The activities of CaM-kinase II (□), CaM-kinase IV (●), PKA (△), and PKC (▲) were measured in the presence of the indicated concentrations of CaMK-(281-302Ala286). The activities are expressed as percentages of the activities determined in the absence of the inhibitors. The data represent the means ± S.E.M. of triplicates.

shows effect of varying concentration of AIP on the activity of CaM-kinase II, compared with CaMK-(281-302Ala286) and KN-93, which have been widely used as CaM-kinase II-specific inhibitors to study the physiological roles of CaM-kinase II. The IC_{50} value of AIP was 40 nM, whereas those of CaMK-(281-302Ala286) and KN-93 were 2 μM and 20 μM, respectively, indicating that AIP was 50 and 500 times more potent than CaMK-(281-302Ala286) and KN-93, respectively, in inhibiting the activity of CaM-kinase II under the assay conditions used. Kinetic analysis of the inhibition of CaM-kinase II by AIP revealed that the inhibition was noncompetitive with respect to syntide-2 (Ki = 18 nM) and competitive with respect to autocamtide-2 (Ki = 320 nM) and CaMK-(281-289) (Ki = 10 nM) (data not shown). This is consistent with our previous observation (22) that the AIP inhibition of the 30-kDa fragment is noncompetitive with respect to syntide-2 and ATP but competitive with respect to autocamtide-2 and CaMK-(281-289), suggesting that AIP inhibits the enzyme by binding to the substrate-binding site for autophosphorylation site, which is distinct from that for the exogenous substrate such as syntide-2. As expected, AIP potently inhibited autophosphorylation of CaM-kinase II (IC_{50} = 100 nM) as shown in Fig. 2.

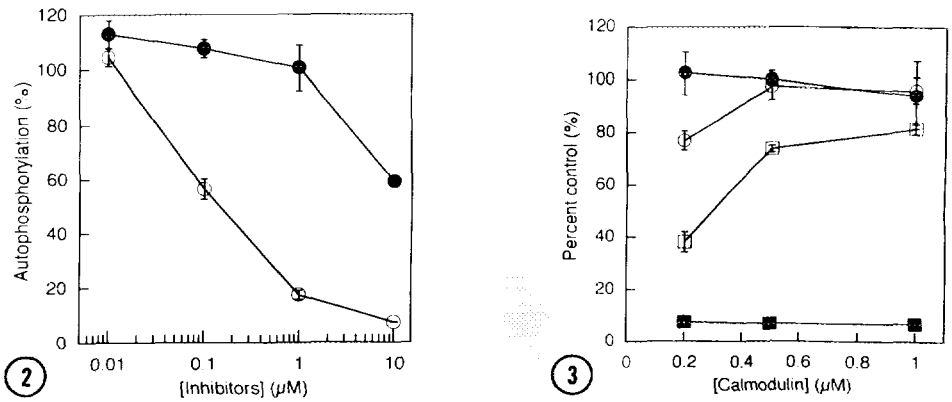

Fig. 2. Effect of AIP and CaMK-(281-302Ala286) on the autophosphorylation of CaM-kinase II. CaM-kinase II (1.1 μg/ml) was incubated at 30°C for 10 min in the reaction mixture containing 40 mM Hepes-NaOH (pH 8.0), 5 mM magnesium acetate, 0.2 mM $CaCl_2$, 0.1 mM EGTA, 0.01% Tween 20, 1 μM calmodulin, and 50 μM [γ-^{32}P]ATP with the indicated concentrations of AIP (○) or CaMK-(281-302Ala286) (●), followed by the addition of 22.7 mM EDTA to stop the autophosphorylation reaction, and then the incorporation of [^{32}P]phosphate into the kinase was determined by the phosphocellulose paper method (31). The results are expressed as percentages of the phosphate incorporation in the absence of the inhibitors. The data represent the means ± S.E.M. of triplicates.

Fig. 3. Inhibition of CaM-kinase II and CaM-kinase IV by AIP and KN-93 in the presence of various concentrations of calmodulin. The activities of CaM-kinase II (□, ■) and CaM-kinase IV (○, ●) were determined in the assay mixture containing 1 μM AIP (■, ●) or 10 μM KN-93 (□, ○) in the presence of the indicated concentrations of calmodulin. The activities are expressed as percentages of the activities determined in the absence of the inhibitors. The data represent the means ± S.E.M. of triplicates.

Fig. 1B shows that AIP did not significantly affect the activities of other second-messenger-responsive multifunctional protein kinases such as PKA, PKC, and CaM-kinase IV at a concentration (10 μM) more than two orders of magnitude higher than the IC_{50} for CaM-kinase II, indicating that AIP is a selective inhibitor of CaM-kinase II. In contrast, CaMK-(281-302Ala286) significantly inhibited the activities of PKC and CaM-kinase IV at a concentration (30 μM) required for complete inhibition of CaM-kinase II, as shown in Fig. 1C, in agreement with the previous report (21).

KN-93 significantly inhibited the activity of CaM-kinase II (Fig. 1A), but did not significantly inhibit the activity of the 30-kDa fragment at 50 μM (data not shown), consistent with the previous report (14) that the inhibition by KN-93 is competitive with respect to calmodulin. Fig. 3 shows effect of varying the concentration of calmodulin on the inhibitions of CaM-kinase II and CaM-kinase IV by KN-93 and AIP. KN-93 (10 μM) significantly inhibited the activities of both CaM-kinase II and CaM-kinase IV at a low concentration of calmodulin, but the inhibitions decreased with increasing the concentration of calmodulin. In contrast, AIP (1 μM) completely inhibited the activity of CaM-kinase II and the inhibition was not affected by the presence of calmodulin at all, indicating that the inhibition of CaM-kinase II by AIP was independent on Ca^{2+}/calmodulin.

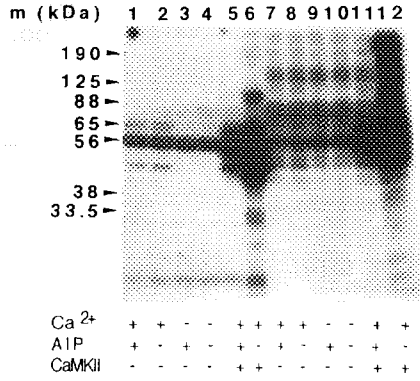

Fig. 4. Effect of AIP on the endogenous phosphorylation of the rat brain extract which was depleted of CaM-kinase II. Two "CaM-kinase II-depleted" preparations of a rat brain extract, "P-through" preparation (0.80 mg/ml, lanes 1-6) and "CaM-through" preparation (0.26 mg/ml, lanes 7-12), which were obtained as described in "Materials and Methods", were incubated at 30°C for 1 min in the mixture consisting of 40 mM Hepes-NaOH (pH 8.0), 5 mM magnesium acetate, 0.1 mM EGTA, 1 μM calmodulin, 0.01% Tween20, and 50 μM [γ-^{32}P]ATP, containing 1 mM $CaCl_2$ (lanes 1,2,5-8,11,12), 1 μM AIP (lanes 1,3,5,7,9,11) or 0.96 μg/ml of CaM-kinase II (lanes 5,6,11,12). After incubation, 22.7 mM EDTA was added to stop the kinase reaction, and aliquots were subjected to SDS-polyacrylamide gel electrophoresis on a 10% polyacrylamide gel. The gel was visualized by autoradiography.

Since only CaM-kinase II, among the well-known second-messenger-responsive multifunctional protein kinases, was highly susceptible to the inhibition by AIP, susceptibility of endogenous protein phosphorylation in the rat brain crude extract was examined with two "CaM-kinase II-depleted" preparations, "P-through" and "CaM-through" preparations, which were obtained by two distinct procedures as described in "Materials and Methods" (Fig. 4). AIP (1 μM) did not affect the endogenous phosphorylation of both preparations (lanes, 1-4 and 7-10), irrespective of the presence or absence of Ca^{2+}. However, phosphorylation of the two preparations caused by the addition of purified CaM-kinase II in the presence of Ca^{2+}/calmodulin was markedly inhibited by 1 μM AIP (lanes 5,6,11,12). These results indicate that 1 μM AIP had no effect on the activities of protein kinases other than CaM-kinase II in the brain extract, whereas it markedly inhibited the phosphorylation of the endogenous proteins by CaM-kinase II, suggesting a high degree of the specificity of the inhibition by AIP. Thus, AIP is the most potent and most selective inhibitor of CaM-kinase II so far reported, and therefore it will be a useful tool for studying the physiological roles of CaM-kinase II.

ACKNOWLEDGMENTS

This work was supported by grants-in-aid for scientific research from the Ministry of Education, Science, and Culture of Japan, and by grants from the Smoking Research Foundation, and the Byotai Taisha Research Foundation, and the Uehara Memorial Foundation.

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