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Bioorganic & Medicinal Chemistry Letters 17 (2007) 147–151
Bioorganic & Medicinal Chemistry Letters

ATP-conjugated peptide inhibitors for calmodulin-dependent protein kinase II

Dae-Ro Ahn, Ki-Cheol Han, Hyuk Sung Kwon and Eun Gyeong Yang*

Life Sciences Division, Korea Institute of Science and Technology, PO Box 131, Cheongryang, Seoul 130-650, Republic of Korea

Received 20 July 2006; revised 21 September 2006; accepted 23 September 2006
Available online 10 October 2006

Abstract—Substrate analog peptides of CaMKII with varying degrees of the inhibitory potency were linked to ATPγS either by considering a phosphoryl transfer mechanism or simply by using a relatively long flexible linker. The latter bisubstrate inhibitors showed relatively little effects while the former ones improved inhibitory potency to different levels depending on the binding affinities of the peptide moieties. One of the mechanism-based bisubstrate inhibitors was then utilized to demonstrate an ATP-competitive but peptide substrate-uncompetitive inhibition, supporting an ordered binding mechanism for CaMKII.

Calmodulin (CaM)-dependent protein kinase II (CaMKII) modulates many cellular functions in response to intracellular Ca^{2+} levels.[1] The CaMKII holoenzyme assembles into a dodecamer and each subunit consists of three main parts; catalytic, regulatory, and association domains. The regulatory domain located on the C-terminal side of the catalytic domain interacts with the intrinsically active catalytic domain, exerting an autoinhibitory effect. Removal of the autoinhibitory segment located in the regulatory domain by Ca^{2+}/CaM activates the enzyme by enabling the binding of ATP and peptide/protein substrates to the catalytic domain. In addition, the activated CaMKII undergoes an autophosphorylation on Thr286 in the inhibitory segment by a neighboring kinase domain within the oligomeric holoenzyme, which keeps the enzyme active even without Ca^{2+}/CaM. While extensive biochemical and biophysical studies have suggested these molecular architecture and regulatory mechanisms of CaMKII,[1] a very recent report on the crystal structure of the autoinhibited CaMKII demonstrated a unique dimeric organization by the CaM-responsive regulatory segments.[2] Although this detailed structural information provide a molecular basis for understanding CaMKII inhibition and activation, use of properly designed inhibitors would allow the elucidation of more mechanistic features of the kinase, particularly in the course of the reaction.

Linking two substrate analogs into a single molecule is an effective strategy to generate inhibitors with enhanced specificity for bisubstrate enzymes such as protein kinases.[3] In general, bisubstrate analogs for protein kinases are designed to mimic the phosphate donor ATP and the acceptor peptides. They can be used in investigating the relation between the structures and the catalytic mechanisms of kinases since they are expected to interact with the well-conserved ATP-binding sites of kinases as well as the relatively diverse substrate recognition sites. Recent attempts to design and synthesize bisubstrate analog inhibitors for protein kinase A (PKA) and insulin receptor protein tyrosine kinase have been successful in achieving improved inhibitory effects on the corresponding kinases.[4] On the other hand, the bisubstrate analog-based approach has not been attempted for CaMKII although several monotopic inhibitors including KN-62, KN-93, and autocamtide-2-related inhibitory peptide (AIP) were reported previously.[5] AIP-1 (KKALRRQEAVDAL) derived from the CaMKII substrate autocamtide-2 with Ala substitution for Thr9 has been shown to inhibit CaMKII competitively with respect to autocamtide-2 but noncompetitively with respect to ATP.[6] The importance of the individual amino acid residues of AIP-1 has recently been investigated by replacing each amino acid residue with other residues.[7] Whereas AIP-2 in which Ala3 and Val10 were replaced with Lys and

Keywords: Bisubstrate analog inhibitors; ATP-conjugated peptide inhibitors; Calmodulin-dependent protein kinase II.
* Corresponding author. Tel.: +82 2 958 5178; fax: +82 2 958 5090; e-mail: eunyang@kist.re.kr

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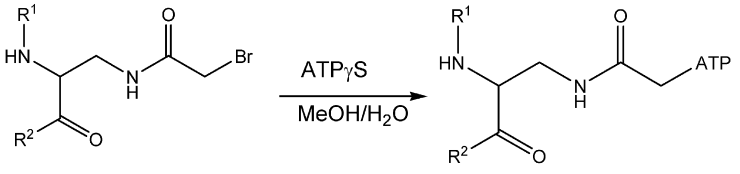
doi:10.1016/j.bmcl.2006.09.070

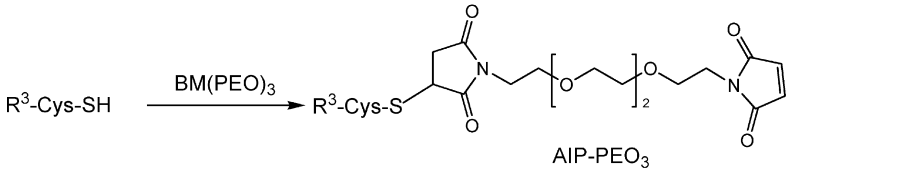
Phe, respectively, showed the most significant decrease in the IC_{50}, AIP-3 with the substitution of Gly for Val10 increased the IC_{50} value dramatically. Considering the relatively high K_m values of ATP in kinase reactions,[1] we have selected these three inhibitor peptides to synthesize ATP-conjugated AIP analogs employing two different linkers between ATP and peptides (Scheme 1) and to examine the effects of their conjugation with ATP on CaMKII inhibition.

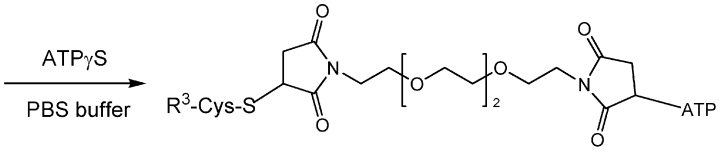
Taking advantage of the previously reported mechanism-based design of the bisubstrate analogs for other kinases,[4] we used an amide linkage branching at the phosphorylation site of the peptides. Aminoalanine was introduced as a substitute for the Thr residue phosphorylatable by CaMKII, followed by bromoacetylation of the amino group, through which ATPγS was coupled to the peptides. Purification of the crude products using reverse-phase HPLC yielded the desired bisubstrate analog inhibitors, AIP-amide-ATP analogs (Scheme 1). In addition, we utilized another linker containing a triethyleneglycol linker flanked by two maleic imide groups reactive with sulfhydryl functional groups, in order to investigate the importance of the site to which the linker is attached and of the linker length. A Cys residue inserted at the C-terminus of the peptide was first coupled to a maleic imide at one end of the linker while leaving the other maleic imide unmodified. The intermediate purified using reverse-phase HPLC was then used to conjugate with ATPγS through the unreacted maleic imide group, followed by reverse-phase HPLC purification to produce another series of

AIP-1	KKALRRQEAVDAL
AIP-2	KKKLRRQEAFDAL
AIP-3	KKALRRQEAGDAL

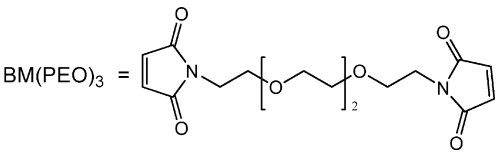


R^1 = biotin-KKALRRQE, AIP-1-amide-ATP
biotin-KKKLRRQE, AIP-2-amide-ATP
biotin-KKALRRQE, AIP-3-amide-ATP
R^2 = VDAL, AIP-1-amide-ATP
FDAL, AIP-2-amide-ATP
GDAL, AIP-3-amide-ATP





R^3 = biotin-KKALRRQEAVDAL, AIP-1-PEO_3-ATP
biotin-KKKLRRQEAFDAL, AIP-2-PEO_3-ATP
biotin-KKALRRQEAGDAL, AIP-3-PEO_3-ATP



Scheme 1. Amino acid sequences of AIP series and synthetic schemes for the preparation of their ATP-conjugated bisubstrate analog inhibitors.

bisubstrate analog inhibitors, AIP-PEO_3-ATP analogs, as presented in Scheme 1.

To evaluate the influence of conjugating ATP with AIP series on the inhibition of kinase activities, recombinant CaMKII expressed in COS-7 cells[8] was reacted with a fluorescently labeled autocamtide-3 (F-AC-3), which was monitored by a fluorescence polarization (FP)-based assay.[9] Nano-structured metal beads that bind specifically to phosphorylated F-AC-3 were employed to induce changes in FP upon phosphorylation reaction. Inhibition curves for the synthesized bisubstrate analog inhibitors as well as for the AIP peptide inhibitors were obtained as shown in Figure. 1.

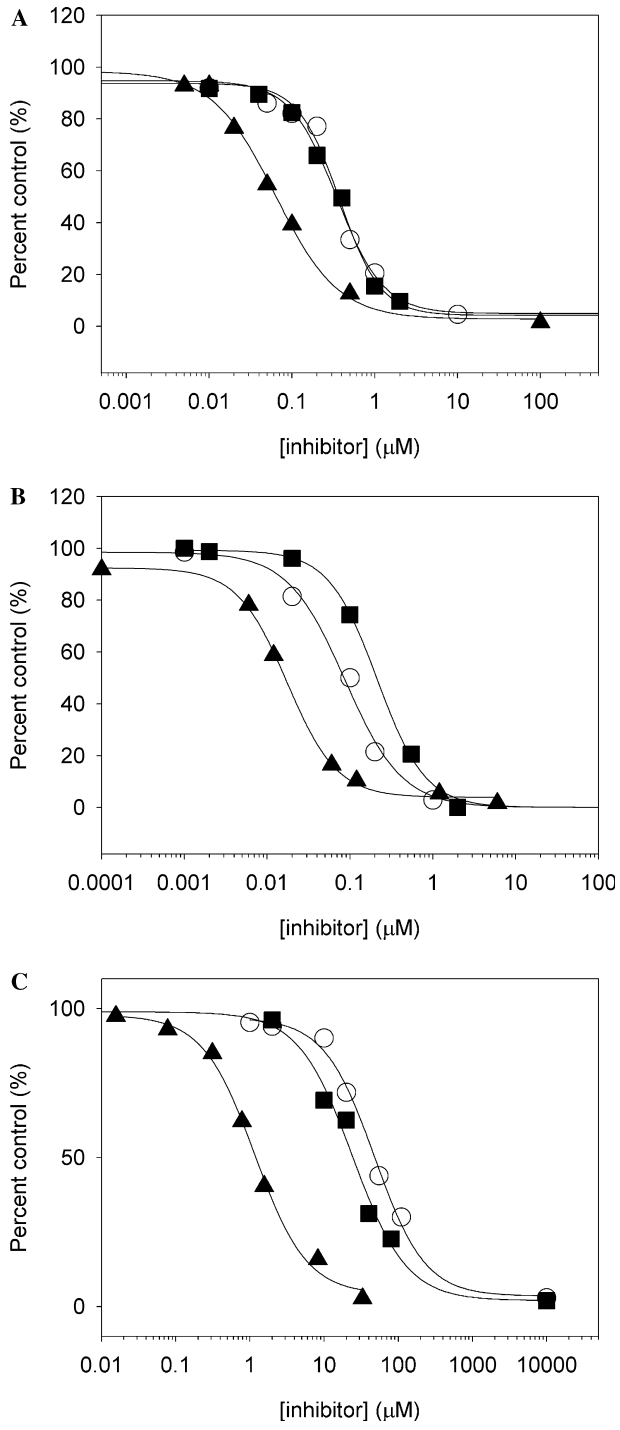


Figure 1. Inhibitory effects of AIP-derived analogs on CaMKII activity. The activities were determined in the presence of (A) AIP-1, (B) AIP-2, and (C) AIP-3 series with unmodified peptides (○), and amide-linked (▲) or PEO_3-linked bisubstrate analog inhibitors (■). Detailed experimental procedures are described in Supplementary data.

Compared with the AIP inhibitors, conjugation of AIP with ATP via the amide linkage obviously shifted the inhibition curves to the left, indicating higher inhibition potency, whereas the bisubstrate analog inhibitors with the PEO_3 linkage showed much less significant changes in the inhibition curves (Fig. 1). A similar increase in inhibitory potency has been observed in the ATP-kemptide analog conjugated using the same amide linker[4] in spite of the very weak affinity of the kemptide analog to PKA.[10] While the amide linker was designed based on the position in which phosphorylation takes place and the distance between the nucleophilic oxygen and the γ-phosphorus of ATP (ca. 5–6 Å) in a dissociative transition state,[4] the relatively long PEO_3 linker (ca. 18 Å) connects the C-terminus of the peptide instead of its phosphorylation site to ATP. Thus, these results demonstrated the importance of maintaining a certain distance between the two substrate analog components in achieving potent inhibition. Furthermore, the observed enhancement of inhibitory potency of the amide-linked bisubstrate analogs reconfirms the rationality of such mechanism-directed approaches in developing potent inhibitors for protein kinases.[3]

To analyze the inhibition data more quantitatively, the IC_{50} values were determined from the inhibition curves (Fig. 1) and are presented in Table 1. AIP-2 was found to be ~4-fold more potent than AIP-1 whereas AIP-3 to be ~125-fold less potent, in good agreement with the previous report.[7] Conjugation of AIP-1, AIP-2, and AIP-3 to ATP via the amide linker then decreased the IC_{50} by approximately 5-fold, 4-fold, and 30-fold, respectively, indicating that the higher level of improvement in the inhibitory effect was achieved by linking ATP to the AIP peptide with low affinity for CaMKII. The K_i of AIP-3 calculated from its IC_{50} (Table 1) using the previously reported K_m (0.28 μM) of the AC-3 substrate[8] is ~8.8 μM, comparable to the K_m (~10 μM) of ATP. Therefore, the 30-fold enhancement in inhibitory potency for AIP-3-amide-ATP was presumably achieved by increased cooperativity of the peptide and ATP moieties of the bisubstrate analog inhibitor in binding with the

Table 1. IC_{50} values of AIP peptides and their ATP conjugates

IC_{50}[a] (nM)	AIP-1	AIP-2	AIP-3
Peptide	320 (12.5[b])	76 (11.8)	40000 (22.5)
Amide-linked (AIP-amide-ATP)	67 (0.8)	22 (16.2)	1325 (5.4)
PEO_3-linked (AIP-PEO_3-ATP)	305 (9.8)	181 (4.9)	18000 (33.3)

[a] Data obtained from at least duplicated experiments for each inhibitor.
[b] Error (%).

kinase. On the other hand, since AIP-2 itself has by far stronger affinity to CaMKII ($K_i \sim 17$ nM) than ATP does, binding of AIP-2-amide-ATP to the kinase appears to be driven more by the peptide moiety. This low cooperativity of the peptide and ATP moieties in binding with the kinase would result in the much less enhancement in inhibition. These results from the amide-linked bisubstrate inhibitors suggest that more cooperativity could be obtained in inhibition by an ATP-peptide conjugate as the K_i of the peptide part approaches to the K_m of ATP. Therefore, the relative affinities of the two substrate analog moieties might need to be considered in designing bisubstrate analog inhibitors.

Unlike the amide-linked bisubstrate analog inhibitors, the inhibitory potency of the PEO_3-linked ones was not always improved when compared to the corresponding parent peptide inhibitors. Conjugation of AIP-1 with ATP using the PEO_3 linker little improved the inhibitory effect whereas the IC_{50} of AIP-3-PEO_3-ATP was ~2-fold lower than that of the original peptide, AIP-3. The IC_{50} value of AIP-2-PEO_3-ATP, however, was ~2-fold higher than that of ATP-2, the strongest peptide inhibitor for CaMKII used in this report. Although the reason for these differences in inhibition effects is unclear, it could be speculated that the ATP part of the PEO_3-linked bisubstrate inhibitors contribute little on their binding to the kinase, thus resulting in insignificant effects on IC_{50}. Taken together with the above, these results imply that a large energetic advantage in binding for a bisubstrate analog inhibitor that incorporates binding motifs of two substrates within the same molecule is achieved only when the two binding motifs with similar affinities are arranged in a specific distance.

The mechanism of CaMKII inhibition by the amide-linked bisubstrate analogs was further investigated by detailed kinetic analysis using AIP-1-amide-ATP. This was performed using capillary electrophoretic separations of unphosphorylated and phosphorylated F-AC-3 of the reaction mixtures. The bisubstrate inhibitor was found to be competitive versus ATP (Fig. 2A) while uncompetitive versus the peptide substrate (Fig. 2B), which is different from the kinetic pattern of AIP-1.[6] These observations suggest an ordered binding mechanism with a preference for ATP, followed by peptide binding. CaMKII has previously been shown to have an equilibrium ordered kinetic mechanism[11] in which the K_m values of the substrate AC-3 and ATP are very close to the dissociation constants of the substrates for the enzyme.[12] When the K_m values of AC-3 (0.28 μM) and ATP (10 μM) were accordingly used as dissociation constants, the product of these yielded a much higher value than the slope inhibition constant ($K_{is} \sim 0.12$ μM derived from Fig. 2A), indicating that the simple sum of the free energies of binding of the two individual analog moieties would not account for the free energy of binding of AIP-1-amide-ATP. Rather, these kinetic data imply that the bisubstrate analog inhibitor induces some conformational change in CaMKII.

In summary, we have utilized three peptide inhibitors derived from the substrate for CaMKII with varying affinities to synthesize ATP-conjugated peptide inhibitors. The levels of changes in inhibitory potency exerted by these inhibitors were different depending on affinities of the peptide moieties as well as the linker types. Analysis of the inhibition patterns for one of the bisubstrate analog inhibitors designed based on a dissociative transition state supported an ordered kinetic mechanism for CaMKII. Therefore, the systematic design of bisubstrate analog inhibitors would provide a promising tool for mechanistic studies of protein kinases as well as for development of their potent inhibitors.

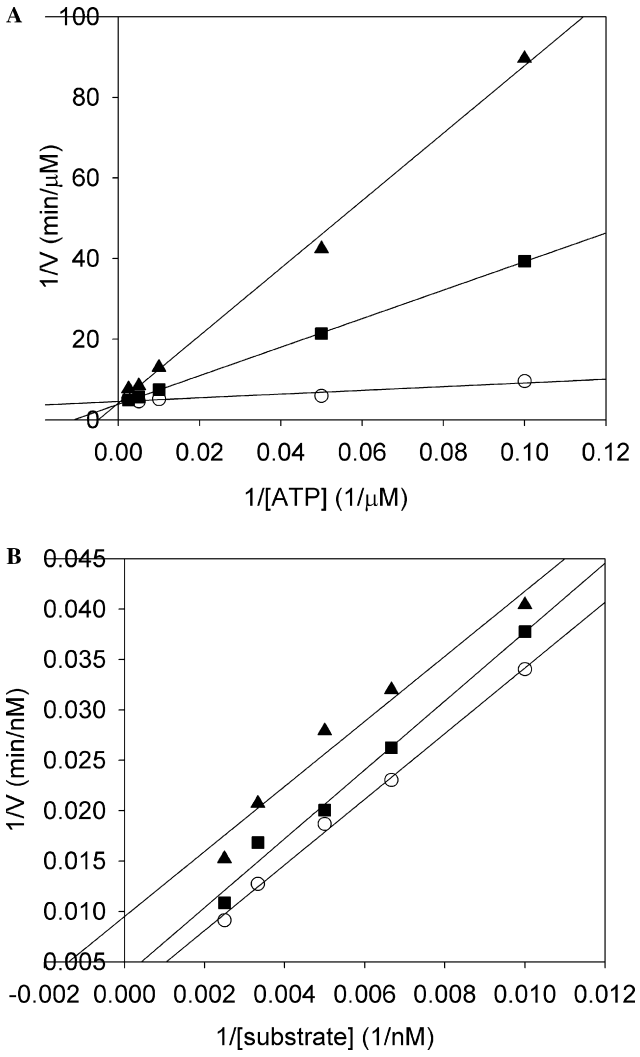


Figure 2. Kinetic analyses of the inhibition of CaMKII by AIP-1-amide-ATP. The enzyme activities were measured with varying concentrations of AIP-1-amide-ATP and the data were plotted as a double-reciprocal plot. (A) $1/V$ versus 1/[ATP] evaluated at a constant concentration of the substrate F-AC-3 (2 μM) in the presence of 0 nM (▲), 50 nM (■), and 100 nM AIP-1-amide-ATP (○). (B) $1/V$ versus 1/[substrate] evaluated at a constant concentration of ATP (100 μM) in the presence of 0 nM (▲), 25 nM (■), and 50 nM AIP-1-amide-ATP (○). Assays were performed as described in Supplementary data.

Acknowledgments

This research has been supported by the Functional Proteomics Research Center of the 21st Century Frontier

Research Program funded by the Korean Ministry of Science and Technology, by the Intelligent Microsystem Center of the 21st Century Frontier R&D Program sponsored by the Korean Ministry of Commerce, Industry and Energy, and by the KIST grant.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.09.070.

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