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Unique Inhibitory Action of the Synthetic Compound 2-[*N*-(2-Aminoethyl)-*N*-(5-isoquinolinesulfonyl)] amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine (CKA-1306) against Calcium/Calmodulin-dependent Protein Kinase I

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ABSTRACT. A newly synthesized compound, 2-[*N*-(2-aminoethyl)-*N*-(5-isoquinolinesulfonyl)]amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine (CKA-1306), was found to inhibit cyclic AMP-dependent protein kinase (PKA) and Ca^{2+}/calmodulin-dependent protein kinase I (CaMK I) with IC_{50} values of 1.6 ± 0.14 and 2.5 ± 0.16 μM, respectively. In contrast, the established PKA inhibitors H-8 and H-89 inhibited CaMK I with relatively high IC_{50} values of >100 and 24.4 ± 3.2 μM, respectively. An additional inhibitor, KN-62, against Ca^{2+}/calmodulin-dependent protein kinase II (CaMK II) did not inhibit either PKA or CaMK I at the concentrations tested. In our library of many isoquinolinesulfonamide derivatives, only CKA-1306 inhibited CaMK I to a satisfactory degree, suggesting a unique mode of action. Indeed, the inhibition of CaMK I by CKA-1306 was competitive in every respect to Mg^{2+}/ATP, peptide substrate (syntide-2), and Ca^{2+}/calmodulin. This phenomenon may be understood from the context of the recently determined structure of the enzyme in its autoinhibited state. Such kinetic analysis was also extended to cases using a phosphorylated and activated enzyme at Thr177 or a constitutively active, COOH-terminal truncated mutant at Gln293. CKA-1306 still competed with Mg^{2+}/ATP for the two enzymes, but it no longer achieved any competitive advantage over syntide-2. These results may reflect some differences in the active conformation of CaMK I. However, the compound should be constant in its recognition of an Mg^{2+}/ATP-binding site of the enzyme. Though CKA-1306 is not specific to CaMK I, the compound will be useful in studying the enzyme further under limited conditions. BIOCHEM PHARMACOL **56**;3:329–334, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. calmodulin; protein kinase; pharmacogenesis; kinetic analysis

Ca^{2+} is widely recognized as an essential intracellular second messenger in eukaryotic systems, regulating processes such as muscle contraction, neurotransmitter release, gene expression, and cell proliferation [1]. In a number of cases, the effects of Ca^{2+} are believed to be mediated by the ubiquitously distributed Ca^{2+} receptor CaM‖ [2]. Strong evidence, in turn, indicates that the effects of Ca^{2+}/CaM are often achieved through the regulation of protein phosphorylation [3–5]. A family of CaMKs has been identified; phosphorylase kinase, myosin light chain kinase, and EF-2 kinase are enzymes that have highly specific functions, while CaMK I/V, II, and IV are multifunctional [5].

CaMK I was first identified in rat brain based on its ability to phosphorylate synapsin I, a synaptic vesicle-associated protein [6]. It has since been purified and characterized from bovine brain [7] and rat brain [8, 9]. Our recent work has uncovered the structural and functional diversity of rat CaMK I isoforms by cDNA cloning, and the isoforms were named CaMK Iα, Iβ1, Iβ2, and Iγ [10, 11]. Based on the analysis of tissue distribution of the isoforms by reverse transcription–polymerase chain reaction protocols, CaMK Iα and Iβ1 are expressed in a variety of tissues such as brain, lung, and kidney, whereas CaMK Iβ2 and Iγ are specific to the brain [10, 11]. Although CaMK I is known to phosphorylate *in vitro* several exogenous substrates, including CREB, a cAMP-response element-binding protein [12], CF-2, a portion of the R-domain of the cystic fibrosis transmembrane conductance regulator [13], and others [14], no significant physiological or pathological substrates for CaMK I have been reported.

We previously purified CaMK V from rat brain [15], which was later found to be the same enzyme or one closely

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‖*Abbreviations:* CaM, calmodulin; CaMK, Ca^{2+}/CaM-dependent protein kinase; and PKA, cyclic AMP-dependent protein kinase.

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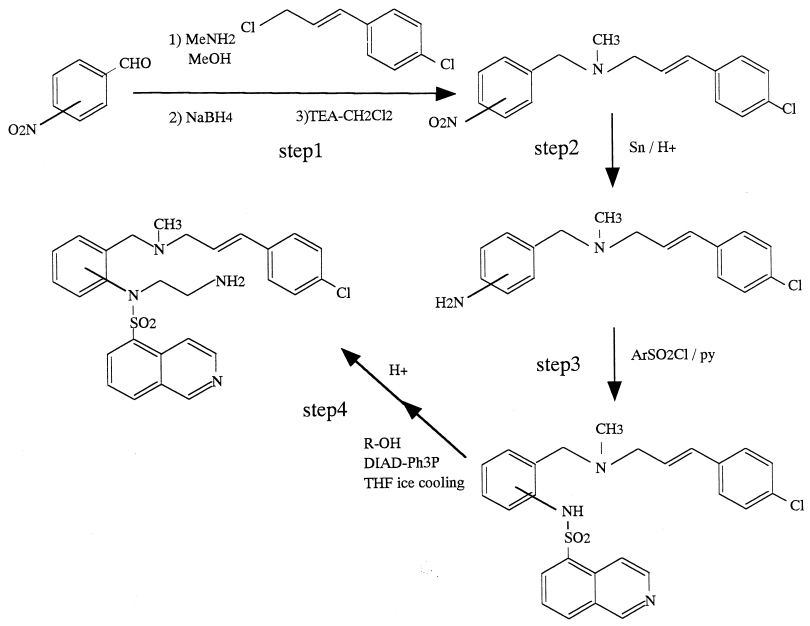

FIG. 1. Process for synthesizing CKA-1306. TEA = triethanolamine; and THF = tetrahydrofuran.

related to CaM kinase Iα [10, 16]. Using a specific antibody that reacts with CaMK Iα/V, we found the isoform to be expressed at much higher levels in gastric carcinomas than in normal gastric mucosa [17]. It is unclear yet how CaMK Iα is associated with neoplasms, and reliable pharmacological tools are needed to elucidate its pathological significance. We have to date picked up through random screening from our library of isoquinolinesulfonamide derivatives many protein kinase inhibitors such as H-8¶ (H-89) and KN-62 against PKA and CaMK II, respectively [18–20]. We tried, therefore, to screen the library in search of new potent inhibitors against CaMK I.

In this report, we introduce the synthetic compound 2-[*N*-(2-aminoethyl)-*N*-(5-isoquinolinesulfonyl)]amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine (CKA-1306), which was prominent among the isoquinolinesulfonamide derivatives tested to inhibit CaMK I. Its effects on other protein kinases are also included to describe the potential pharmacological activity of the compound. In addition, the molecular basis by which CKA-1306 inhibits CaMK I was solved by kinetic analysis, using three enzyme preparations in active states, separate from one another.

MATERIALS AND METHODS

Materials

Compounds. CKA-1306 was synthesized at the Fuji Yakuhin Co., Ltd. by the procedures illustrated in Fig. 1. H-8, H-89, KN-62, and other H-series protein kinase inhibitors were synthesized by a modification of the method of Hidaka *et al.* [18]. Staurosporine was obtained from Sigma, and K252a and Calphostin C were purchased from Calbiochem.

Protein Preparation. The catalytic subunit of protein kinase A was isolated from bovine heart according to Beavo *et al.* [21]. CaM was purified from bovine brain by the method of Yazawa *et al.* [22]. CaMK Iα and its COOH-terminal truncated mutant (residues 1–293) were prepared as described [23]. CaMK Iβ1 and Iβ2 were expressed in a baculovirus expression system (Invitrogen) and purified as described [11]. CaM kinase IIα, of which cDNA was a gift from the Nihon Shinyaku Co., Ltd., was also similarly expressed in the BAC-TO-BAC Baculovirus Expression System (Life Technologies) and purified. CaMK Iα phosphorylated and activated by CaMK kinase [24] was prepared as follows [11]: 1.5 μM CaMK Iα was incubated with 21 nM CaMK kinase at 30° for 10 min in 35 mM HEPES-NaOH (pH 8.0), 10 mM $MgCl_2$, 0.01% (v/v) Tween 20, 1 mM dithiothreitol (DTT), 1 mM $CaCl_2$, 1.5 μM CaM, and 100 μM ATP. The reaction was terminated by a 15-fold dilution with a solution containing 35 mM HEPES-NaOH (pH 8.0), 2 mg/mL of BSA, 10% glycerol, and 1 mM EDTA.

Other Materials. The CREB peptide (LSRRPSYRKILNDL) and syntide-2 (PLARTLSVAGLPGKK) were synthesized with a model 431A peptide synthesizer (Applied Biosystems, Inc.). P-81 paper was purchased from Whatman (Maidstone). [γ-^{32}P]ATP (>5000 Ci/mmol) was obtained from the Amersham. All other chemicals were reagent grade or better.

¶ *Chemical names:* CKI-7, *N*-(2-aminoethyl)-5-chloroisoquinoline-8-sulfonamide; H-7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; H-8, *N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide; H-9, *N*-(2-aminoethyl)-5-isoquinolinesulfonamide; H-89, *N*-[2-*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide; HA-1077, 1-(5-isoquinolinesulfonyl)-homopiperazine; KN-62, 1-[*N*,*O*-bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine; and ML-9, 1-(5-chloronaphthalenesulfonyl)-1*H*-hexahydro-1,4-diazepine.

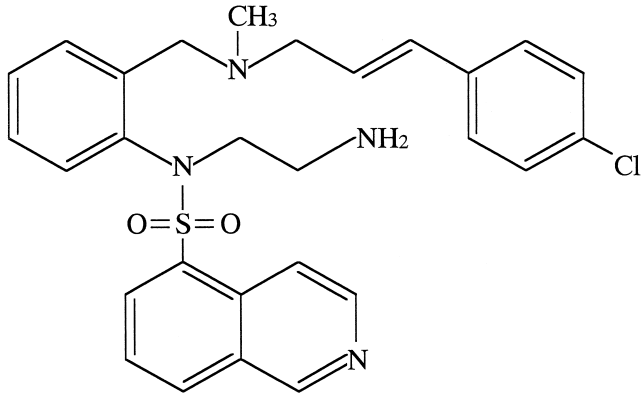

FIG. 2. Structural formula of 2-[N-(2-aminoethyl)-N-(isoquinolinesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine (CKA-1306).

Protein Kinase Assays

All isoforms of CaMK I, its COOH-terminal truncated mutant, and phosphorylated and activated CaMK Iα were assayed at 30° for 10 min in a 50-μL mixture containing 35 mM HEPES-NaOH (pH 8.0), 10 mM $MgCl_2$, 0.01% (v/v) Tween 20, 1 mM DTT, 50 μM syntide-2, 100 μM [γ-^{32}P]ATP (50–90 cpm/pmol), various amounts of test compound as indicated, and either 1 mM EGTA or a set of 0.5 mM $CaCl_2$ and 100 nM CaM. CaMK IIα was assayed at 30° for 5 min in a 50-μL mixture containing 35 mM HEPES-NaOH (pH 8.0), 10 mM $MgCl_2$, 50 μM syntide-2, 10 μM [γ-^{32}P]ATP (500–900 cpm/pmol), various amounts of test compound as indicated, and either 1 mM EGTA or 2 mM $CaCl_2$, 50 nM CaM. PKA was assayed at 30° for 5 min in a 50-μL mixture containing 25 mM Tris–HCl (pH 7.6), 10 mM $MgCl_2$, 2 mM EGTA, 50 μM CREB, 10 μM [γ-^{32}P]ATP (500–900 cpm/pmol), and various amounts of test compound as indicated. Reactions were initiated by the addition of ATP, and incorporation of ^{32}P into these substrates was measured according to the method of Roskoski [25]. At the end of the incubation, 25 μL of the reaction mixture was spotted onto P-81 phosphocellulose paper followed by washing with 75 mM phosphoric acid. The paper was dried, and its radioactivity was counted by Cerenkov counting.

RESULTS

Comparison of IC_{50} Values of CKA-1306, H-89, and H-8 Against CaMK Iα, CaMK II, and PKA

The structural formula of CKA-1306, a new isoquinolinesulfonamide derivative, is given in Fig. 2. CKA-1306, H-89, and H-8 were compared for their IC_{50} values against CaMK Iα, CaMK II, and PKA in Table 1. CKA-1306 inhibited all of them to a comparable degree with IC_{50} values of <3.5 μM. H-89, a potent and selective inhibitor against PKA [19], did not inhibit CaMK Iα as strongly as CKA-1306. Moreover, H-8, another PKA inhibitor [18], did not exert any effect on the enzyme even at concentration >100 μM.

TABLE 1. Comparison of IC_{50} values for CKA-1306, H-89, and H-8 against CaMK Iα CaMK II, and PKA

	IC_{50} (μM)		
	CKA-1306	H-89	H-8
CaMK Iα	2.5 ± 0.16	24.4 ± 3.2	>100
CaMK II	3.1 ± 0.59	29.7 ± 8.1	68.3 ± 0.59
PKA	1.6 ± 0.14	0.034 ± 0.002	2.6 ± 0.21

CaMK Iα, CaMK II, and PKA were assayed as described in Materials and Methods. CKA-1306, H-89, and H-8 were added at various concentrations. The IC_{50} values represent the concentrations at which the inhibitors caused 50% inhibition of the protein kinase activity. The data were derived from three independent experiments, each performed in duplicate, and expressed as mean ± SEM values.

Similarly, both H-89 and H-8 inhibited CaMK II to a much lesser degree than they inhibited PKA.

Effects of Other Representative H-Series Protein Kinase Inhibitors on CaMK Iα

The inhibition rates (IC_{50} values) obtained with other representative H-series protein kinase inhibitors against CaMK Iα were compared at the same concentration (30 μM) (Table 2). Because KN-62 [20] was not sufficiently soluble in 2% DMSO at concentrations greater than 10 μM, its effect at 10 μM was shown. H-9 [26], ML-9 [27], and CKI-7 [28] slightly inhibited CaMK Iα with at the most ~25% of control activity (set at 100%), while H-7 [18], HA-1077 [29], and KN-62 were almost without any effects. Thus, CKA-1306 should be marked by its ability to inhibit CaMK Iα. In addition, after a random screening from our library of isoquinolinesulfonamide derivatives in search of CaMK I inhibitors, we have not yet obtained a compound better than CKA-1306 (data not shown).

Kinetic Analysis of Inhibition of Ca^{2+}/CaMK Iα by CKA-1306

To elucidate the mechanisms involved in its inhibition of CaMK Iα, we first examined CKA-1306 for its ability to compete with Ca^{2+}/CaM, ATP, or syntide-2. Figure 3a

TABLE 2. Inhibitory effects of other protein kinase inhibitors against CaMK Iα

	CaMK Iα (%)	Target enzyme K_i (μM)
H-7	0.6	3.0 [18]
H-9	13.1	1.9 [26]
HA-1077	2.8	1.6 [29]
ML-9	22.8	3.8 [27]
CKI-7	14.5	8.5 [28]
KN-62	0	0.9 [20]
CKA-1306	97.6	

The K_i values for the target protein of the respective inhibitors are indicated on the right, and the inhibitory rates for CaMK Iα at a concentration of 30 μM in the center. The data were derived from two independent experiments, each performed in duplicate, and expressed as the mean values. The K_i values are from the literature, referred to by the number in brackets.

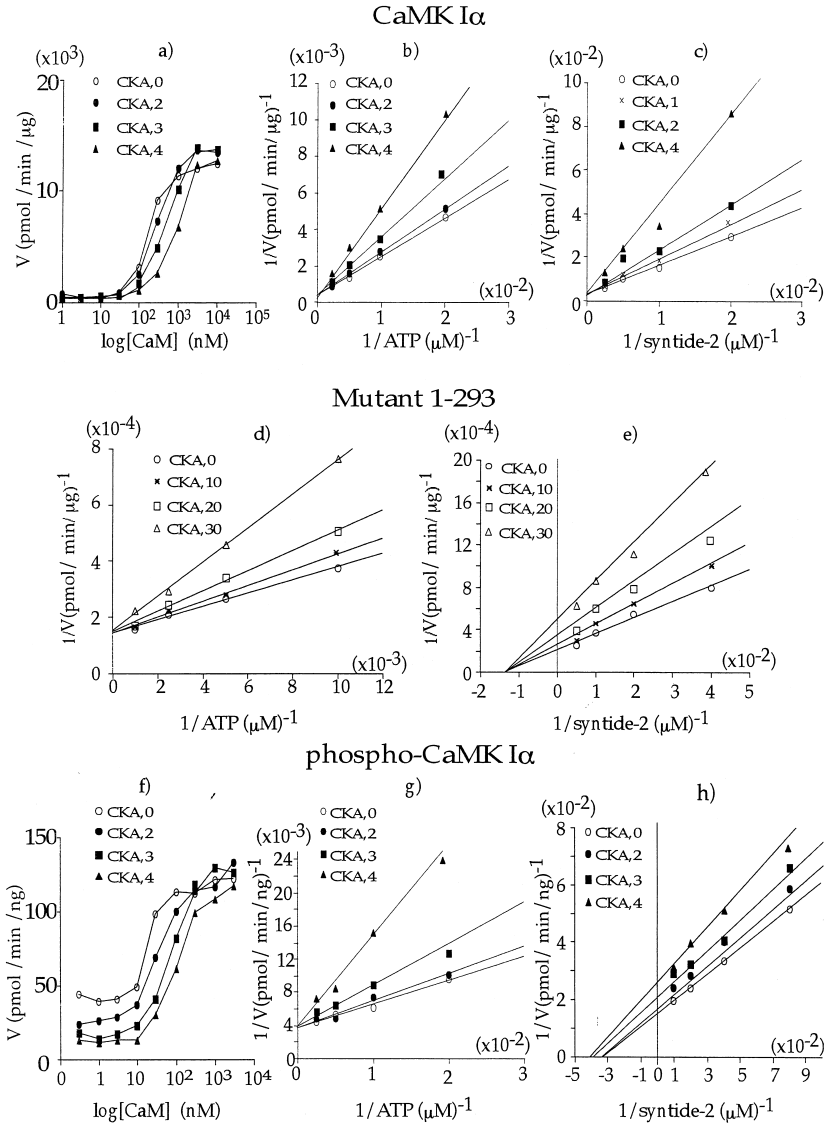

FIG. 3. Inhibition of CaMK Iα, Mutant 1-293, and phospho-CaMK Iα by CKA-1306. Panels a and f: CaMK Iα (0.5 μg) or phospho-CaMK Iα (6.8 ng) was incubated (30°, 12 min) with 0 μM (○), 2.0 μM (●), 3.0 μM (■), or 4.0 μM (▲) CKA-1306 using 1–10000 nM or 0.3–3000 nM CaM. Panels b, d, and g: double-reciprocal plots of inhibition of these three enzymes by CKA-1306. CaMK Iα (0.5 μg), Mutant 1-293 (0.2 μg), or phospho-CaMK Iα (6.8 ng) was incubated (30°, 12 min) with various concentrations of CKA-1306 using 50–1000 μM [γ-^{32}P]ATP. Panels c, e, or h: under the same assay conditions as mentioned above, using 12.5 to 400 μM syntide-2. The ^{32}P incorporation into syntide-2 was determined as described in Materials and Methods. The results are representative of three independent experiments, each performed in duplicate. Phospho-CaMK Iα activity (about 40 pmol/min/ng) was observed at concentrations of CaM < 10 nM (Fig. 3f). This was attributed to the presence of CaM derived from the preparation of phospho-CaMK Iα, because the activity was inhibited by EGTA.

shows the inhibition data obtained when CaM concentrations were varied in the absence or presence of 2.0, 3.0, and 4.0 μM CKA-1306. The inhibitory action of CKA-1306 was abolished by the addition of excess CaM, and this pattern denotes a competitive manner with respect to Ca^{2+}/CaM. CKA-1306 also inhibited the enzyme competitively with respect to ATP and syntide-2 (Fig. 3, b and c). Then we investigated the kinetic action of CKA-1306 against COOH-terminal truncated mutant 1-293 (Mutant 1-293) to confirm that CKA-1306 was not a CaM antagonist but could directly affect CaMK Iα. Mutant 1-293 is a mutant that removes amino acids 294–321 from wild-type enzyme, resulting in a constitutively active enzyme that can no longer bind Ca^{2+}/CaM. The action against Mutant 1-293 was the same as that against wild type with respect to ATP, but not to syntide-2 (Fig. 3, d and e). These results suggest that CKA-1306 can directly affect CaMK Iα.

However, with respect to syntide-2, the inhibitory action against the mutant was different from that against the wild type. Moreover, we were interested in an inhibitory action against phosphorylated CaMK Iα (phospho-CaMK Iα). The enzyme is phosphorylated at Thr177 by CaMK kinase, one of the Ca^{2+}/CaM kinase family. In this case, the compound inhibited the enzyme competitively with respect to Ca^{2+}/CaM and ATP, but not to syntide-2 (Fig. 3, f, g, and h).

Comparison of IC_{50} Values of CKA-1306, H-89, and H-8 against CaMK I Isoforms

To evaluate the difference in inhibitory effects on CaMK I isoforms, the IC_{50} values of CKA-1306, H-8, and H-89 were determined (Table 3). These compounds showed similar inhibitory action against CaMK Iα and Iβ1 but less against CaMK Iβ2.

TABLE 3. Comparison of IC_{50} values for CKA-1306, H-89, and H-8 against CaMK I isoforms

	IC_{50} (μM)		
	CKA-1306	**H-89**	**H-8**
CaMK Iα	2.5 ± 0.16	24.4 ± 3.2	>100
Phosphorylated CaMK Iα	3.3 ± 0.33		
Mutant 1-293	19.5 ± 1.27	>100 [23]	
CaMK Iβ1	4.0 ± 0.44	26.5 ± 0.96	>100
CaMK Iβ2	12.5 ± 0.57	65.3 ± 4.64	>100

CaMK Iα, phosphorylated CaMK Iα, Iβ1, Iβ2, and Mutant 1-293 were assayed as described in Materials and Methods. CKA-1306, H-89, and H-8 were added at various concentrations. The data were derived from three independent experiments, each performed in duplicate, and expressed as mean ± SEM values.

DISCUSSION

CaMK I is a relatively new enzyme whose physiological or pathological significance remains unclear. The enzyme was identified originally in rat brain extracts due to its phosphorylation on site 1 of the neuronal protein synapsin I [6]. The present study showed that, whereas our previous protein kinase inhibitors H-7, H-8, H-9, H-89, KN-62, HA-1077, ML-9, CKI-7, and KN-62 failed to inhibit CaMK Iα to a satisfactory degree, CKA-1306 was very potent with an IC_{50} value of 2.5 ± 0.16 μM (Tables 1 and 2). Interestingly, the compound also potently inhibited PKA and CaMK II (Table 1). Because CKA-1306 proved to be distinct from the other H-series compounds tested, it is of great interest to determine precisely how the compound exerts its inhibitory potential, in particular against CaMK Iα.

CKA-1306 was found to inhibit CaMK Iα in a competitive manner with respect to Ca^{2+}/CaM, Mg^{2+}/ATP, and syntide-2 (Fig. 3). This may be understood in the context of the recently determined structure of the enzyme in its autoinhibited state [30]. The COOH-terminal regulatory region extends across the catalytic core and interacts with the Mg^{2+}/ATP-anchoring glycine flap in the NH_2-terminal lobe, resulting in a deformation of the active site. It is believed that Ca^{2+}/CaM binds to the regulatory region [23], which is probably followed by release of its interaction with the glycine flap and a resultant rearrangement into an open shape. Indeed, this may be responsible for the activation of the enzyme itself. One molecule of CKA-1306 may bind to the active site of the enzyme and interfere concomitantly with the binding of Ca^{2+}/CaM, Mg^{2+}/ATP, and syntide-2 to the enzyme. However, it is also possible that multiple molecules of CKA-1306 bind to the Ca^{2+}/CaM-CaMK I system, and that they separately affect those binding to the enzyme. As shown in Table 1, CKA-1306 inhibited the non-Ca^{2+}/CaM-dependent protein kinase PKA, with an IC_{50} value of 1.6 ± 0.14 μM. That inhibition was competitive with respect to Mg^{2+}/ATP and noncompetitive with respect to the peptide substrate CREB (data not shown). Engh *et al.* [31] recently resolved the structure of the catalytic subunit of PKA complexed with H-7, H-8, or H-89 by x-ray crystallography. In these complexes, the insoquinoline ring, which is also used in CKA-1306, was placed on an adenosine pocket of the active site of the enzyme. Part of the inhibition of CaMK Iα by CKA-1306 should be achieved by binding of the compound to the active site of the enzyme.

The direct binding of CKA-1306 to CaMK Iα was supported by the experiments using Mutant 1-293 and phospho-CaMK Iα. Mutant 1-293 is devoid of the regulatory domain and can phosphorylate syntide-2 even in the absence of Ca^{2+}/CaM, which has twice the activity of CaMK Iα in the presence of Ca^{2+}/CaM [23]. Some structural change in the active site may be caused by the truncation, resulting in the difference between the activity of CaMK Iα and Mutant 1-293. CKA-1306 inhibited Mutant 1-293 eight-fold less potently than CaMK Iα, and this inhibition was competitive with respect to Mg^{2+}/ATP, but not with respect to syntide-2. We have also determined the potency of CKA-1306 on phospho-CaMK Iα, which is phosphorylated at Thr177 by CaMK kinase. The inhibitory effect of CKA-1306 on phospho-CaMK Iα was the same as that on CaMK Iα in our assay condition. However, a simple comparison of these inhibitory effects should not be made because the affinity of phospho-CaMK Iα for Ca^{2+}/CaM, Mg^{2+}/ATP, and syntide-2 was 3 to 5-fold higher than that of CaMK Iα. The specific activity of phospho-CaMK Iα was also about 300-fold higher than that of CaMK Iα. In Table 3, the inhibitory effects of CKA-1306 on CaMK Iα and phospho-CaMK Iα were compared under the same assay conditions. If the assay conditions were optimized for phospho-CaMK Iα, the IC_{50} value should change significantly. The inhibition of phospho-CaMK Iα by CKA-1306 was competitive with respect to Ca^{2+}/CaM and ATP, but not to syntide-2. Taken together with the data for CaMK Iα, the inhibitory mechanisms of CKA-1306 against Mutant 1-293 and phospho-CaMK Iα were variable, but remained consistent in their competition with respect to Mg^{2+}/ATP.

Thus far, it is obvious that CKA-1306 binds primarily to the active site of CaMK I and inhibits the enzyme activity. However, the possibility that the compound binds to Ca^{2+}/CaM as well as CaMK I cannot be excluded. In this respect, CKA-1306 resembles, in structure, HMN-709 (2-[*N*-(aminoethyl)-*N*-(4-chlorobenzenesulfonyl)]amino-*N*-(4-fluorocinnamyl)-*N*-methylbenzylamine), which was reported previously to be a potent CaM antagonist [32]. Because a benzene ring in HMN-709 is exchanged for an isoquinoline ring in CKA-1306, the latter compound may preferably bind to CaMK I.

It is clearly of interest whether other protein kinase inhibitors can influence CaMK Iα. Staurosporine and K252a, which appear to be nonspecific protein kinase inhibitors, inhibited CaMK Iα with IC_{50} values of 0.17 ± 0.01 and 0.12 ± 0.01 μM, respectively (Sakaguchi H, unpublished data). Calphostin C, which is a specific inhibitor of protein kinase C, has been found to have an IC_{50} value of 8.63 ± 0.43 μM for CaMK Iα (Sakaguchi H,

unpublished data). These inhibitory effects of the compounds should also be taken into account when they are used as pharmacological tools in bioassays.

In summary, the present study demonstrated that the newly synthesized compound CKA-1306 inhibited CaMK Iα more effectively than our previous protein kinase inhibitors, although the effects were not specific. It is hoped that this new compound may serve as a touchstone for the development of other protein kinase inhibitors, in particular for CaMK Iα.

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