KS-505a, an isoform-selective inhibitor of calmodulin-dependent cyclic nucleotide phosphodiesterase

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The effects of KS-505a, a novel microbial metabolite, on the activity of calmodulin-dependent cyclic nucleotide phosphodiesterase (CaM-PDE) were investigated. (1) KS-505a potently inhibited the purified 61 kDa isoenzyme of CaM-PDE from bovine brain and required much higher doses to inhibit the purified 59 kDa isoenzyme of CaM-PDE from bovine heart. The inhibition of both isoenzymes was observed only in the presence of calcium-activated calmodulin ($\text{Ca}^{2+}/\text{CaM}$). The IC_{50} values for the 61 and 59 kDa isoenzymes were 0.17 and 13 μM respectively with 20 μM cAMP as a substrate. (2) Kinetic analysis indicated that the inhibitory mode of KS-505a for the 61 kDa isoenzyme was competitive with respect to $\text{Ca}^{2+}/\text{CaM}$; the K_i for KS-505a was 0.089 μM . The inhibition was not competitive with respect to the substrates cAMP or cGMP. (3) KS-505a did not interfere with the interaction between $\text{Ca}^{2+}/\text{CaM}$ and *n*-phenyl-1-naphthylamine, a hydrophobic fluorescent probe, nor was it

adsorbed to CaM-conjugated gels in the presence of Ca^{2+} , thereby indicating that KS-505a does not bind to $\text{Ca}^{2+}/\text{CaM}$. (4) Trypsin-activated 61 kDa isoenzyme, which lacked the $\text{Ca}^{2+}/\text{CaM}$ -binding domain, was not inhibited by KS-505a at less than micromolar concentrations. Taken together, these results suggest that KS-505a apparently bound to a site in the $\text{Ca}^{2+}/\text{CaM}$ -binding domain of the 61 kDa isoenzyme and selectively inhibited $\text{Ca}^{2+}/\text{CaM}$ -activated 61 kDa isoenzyme activity. (5) In rat hippocampal slices, KS-505a at 10 μM increased the intracellular cAMP concentration to approximately three times the basal level, whereas in rat striatal slices it had no effect on the cAMP concentration at concentrations of 1.0–10 μM , suggesting that each CaM-PDE isoenzyme functions differentially in these regions. These results demonstrate that KS-505a is a highly potent selective inhibitor both *in vitro* and *in vivo* and distinguishes between subfamily members within the CaM-PDE family.

INTRODUCTION

Cyclic nucleotide phosphodiesterases (PDEs) (EC 3.1.4.17), which convert cyclic nucleotides into biologically inert 5'-nucleoside monophosphates, play an essential role in controlling intracellular levels of cyclic nucleotides. The existence of at least seven families of PDE isoenzymes, each composed of several isoforms and having different tissue distributions, has been described [1–3]. One large family, calmodulin-dependent phosphodiesterases (CaM-PDEs), play a critical role in intracellular signalling in that they respond to calcium-activated calmodulin ($\text{Ca}^{2+}/\text{CaM}$) and are involved in the interaction between the cyclic nucleotide and Ca^{2+} signalling system. Among the well-characterized forms of this family are the 59 kDa isoenzyme from bovine heart [4] and the 61 and 63 kDa isoenzymes isolated from bovine brain [5,6]. Partial or complete primary sequences for these CaM-PDEs have been reported [7]. The 59 and 61 kDa isoenzymes have sequence differences only in each CaM-binding site at the N-terminus, suggesting that the two isoenzymes are products of an alternative splicing [7]. A partial sequence of the 63 kDa isoenzyme suggests that it is the product of a gene different from the gene coding for the 59 and 61 kDa isoenzymes [8]. Bovine and murine cDNAs for the 61 kDa isoenzyme [9] and the 63 kDa isoenzyme [10–12] have been isolated.

Although a number of compounds are capable of inhibiting CaM-PDE, most of them lack overall selectivity and so far no inhibitor distinguishing between CaM-PDE isoenzymes has been described. The availability of such selective inhibitors should not only provide very useful tools for understanding the biological function of each isoenzyme but also open possibilities for therapeutic drug development. We have recently isolated a potent

CaM-PDE inhibitor, designated KS-505a, from *Streptomyces argenteolus* A-2 [13]. KS-505a has a unique structure with a 2-*O*-methylglucuronic acid moiety and decacyclic isoprenoid skeleton and a tautomeric aromatic γ -hydroxy- γ -lactone (Figure 1). KS-505a potently inhibits bovine brain CaM-PDEs, and much higher doses were required to inhibit bovine heart CaM-PDEs [13]. Although both enzymes were partly purified preparations, the results suggested that KS-505a could distinguish between CaM-PDE isoenzymes. We further examined the selectivity and the inhibition mode of KS-505a for the 59 and 61 kDa isoenzymes by purifying them from bovine heart and brain respectively. We have thus demonstrated that KS-505a actually distinguishes the

Figure 1 Structure of KS-505a

Abbreviations used: CaM, calmodulin; $\text{Ca}^{2+}/\text{CaM}$, calcium-activated calmodulin; PDE, phosphodiesterase; CaM-PDE, calmodulin-dependent cyclic nucleotide phosphodiesterase; NPN, *n*-phenyl-1-naphthylamine.

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61 kDa isoenzyme from the 59 kDa isoenzyme. Furthermore, from the kinetic analyses and the binding experiments with a hydrophobic fluorescent probe and CaM-conjugated gels, we have proposed that KS-505a acts by selectively binding to a specific CaM-binding domain of the 61 kDa isoenzyme. A part of the preliminary results of these experiments has been reported in abstracts [14–16]. Interestingly, we observed that KS-505a administered intraperitoneally showed anti-amnesia activity in a rat model [16].

In this study we report the precise biochemical characterization of KS-505a in terms of its potency, selectivity and mechanism of inhibition for the CaM-PDE isoenzyme. We also describe differential effects of KS-505a on the level of intracellular cAMP between striatal and hippocampal slices.

EXPERIMENTAL

Materials

cAMP, cGMP, 5'-nucleotidase (snake venom), calmodulin (bovine brain) for PDE assay and trypsin (bovine pancreas) were purchased from Sigma. DEAE-Sephadex A-25 (Pharmacia), Protein A-Sepharose CL-4B (Pharmacia), soybean trypsin inhibitor (Gibco) were purchased. Calmodulin was prepared from frozen pig brain by modified procedures adapted from those reported by Yazawa et al. [17], Kakiuchi et al. [18] and Gopalakrishna and Anderson [19]. Calmodulin affinity gel was prepared by coupling calmodulin with Affigel-15 (Bio-Rad) [20]. The CaM binding capacity of this affinity gel was 10 mg per ml of wet gel. [2,8-³H]cAMP (1.11–1.85 TBq/mmol) and [8-³H]cGMP (185–555 GBq/mmol) were obtained from New England Nuclear, and ACAP-1 monoclonal antibody from Chemicon International, Inc. Calmodulin antagonist R24571 was a gift from Janssen Pharmaceutica Co. Ltd. KS-505a was prepared at the Fermentation Division of Kyowa Hakko Laboratories. NPN (*n*-phenyl-1-naphthylamine) was purchased from Tokyo Kasei Kogyo Co. CaM (28000 units per mg of protein; 1 unit is the amount of CaM that gives rise to 50 % of the maximal enzyme activation of a standard level of activator-deficient CaM-PDE) used for fluorescence measurements was from Fluka Chemie AG (Buchs, Switzerland).

Preparation of the 61 and 59 kDa isoenzymes of CaM-PDEs

The 61 and 59 kDa isoenzymes were purified from bovine brain and bovine heart by the method of Kincaid et al. [6] and LaPorte et al. [4], respectively. Because there are at least two major CaM-PDE isoenzymes, 61 and 63 kDa, in brain tissue, the purity of the prepared 61 kDa isoenzyme was checked by immunoprecipitation with monoclonal antibody ACAP-1, which can discriminate between the 61 and the 63 kDa isoenzymes and bind the 61 kDa isoenzyme activated by Ca²⁺/CaM [21]. The prepared 61 kDa isoenzyme was incubated at 4 °C for 60 min with 0.4 mM CaCl₂, 31 nM CaM and excess ACAP-1. Protein A-Sepharose CL-4B [10 % (w/v)] was then added and incubated at 4 °C for 2 h. After incubation, the reaction mixture was centrifuged (10000 *g* for 1 min at 4 °C) and the phosphodiesterase activity of the supernatant was measured and compared with the activity without treatment with ACAP-1. ACAP-1 monoclonal antibody adsorbed 92 % of the total phosphodiesterase activity in the presence of Ca²⁺/CaM, indicating that 92 % of the total PDE in the preparation was assigned to the 61 kDa isoenzyme. The purified 61 kDa isoenzyme preparation gave a single peak stained with Coomassie Blue on SDS/PAGE (12 % gel) (results not shown). The purified 59 kDa isoenzyme gave a major 59 kDa peak with Coomassie Blue on SDS/PAGE (12 % gel) (results not shown).

The apparent *K_m* values of the purified 61 and 59 kDa isoenzymes were as follows: 61 kDa isoenzyme, 22 μM (cAMP) and 1.3 μM (cGMP); 59 kDa isoenzyme, 20 μM (cAMP) and 1.6 μM (cGMP).

Preparation of cGMP-inhibited and cAMP-specific PDEs

cGMP-inhibited and cAMP-specific PDEs were prepared from canine heart by the methods described [22,23].

Phosphodiesterase assay

Phosphodiesterase activity was measured by a two-step procedure by the method of Kincaid and Manganiello [24]. [³H]cAMP or [³H]cGMP was used as a substrate. The reaction was performed in a standard mixture containing 50 mM Bes, pH 7.2, 1 mM MgCl₂ and 0.1 mg/ml soybean trypsin inhibitor. The concentrations of cAMP ranged from 1 to 40 μM, and those of cGMP from 1 to 4 μM. For the assay of CaM-activated CaM-PDE activity, the concentrations of CaM and CaCl₂ were respectively twice the *K_{0.5}* value for each enzyme and 0.4 mM, unless otherwise described in the text. The reaction was initiated with enzyme. The reaction mixture was then incubated at 30 °C for 10–30 min, depending on the amount of enzyme activity. The reaction was terminated by hydrochloric acid, which was thereafter neutralized with sodium hydroxide. After complete conversion of 5'-AMP product into adenosine by excess 5'-nucleotidase, samples were applied to a DEAE-Sephadex A-25 column and the [³H]adenosine or [³H]guanosine eluted by water was measured by scintillation counting with a liquid-scintillation counter (Packard Tri-Carb 4530). KS-505a was dissolved in deionized water just before use.

Preparation of trypsin-treated 61 kDa isoenzyme

The purified 61 kDa isoenzyme was incubated with 3 μg/ml trypsin in 40 mM Tris/HCl buffer, pH 7.5, containing 1 mM MgCl₂, 50 mM NaCl and 0.1 mM dithiothreitol. After incubation at 30 °C for 6 min, the reaction was terminated by addition of a 22-fold molar excess of trypsin inhibitor [25,26]. CaM sensitivity and the inhibition mode by KS-505a of the trypsin-treated isoenzyme were measured by the same method as the phosphodiesterase assay described above.

Assessment of interaction of KS-505a and R24571 with CaM with a fluorescent probe

Fluorescence measurements were performed as previously described [27,28]. Samples were prepared in a total volume of 3 ml of 10 mM Tris/HCl buffer, pH 7.5, containing 0.16 μM CaM, 0.2 mM CaCl₂ and 8 μM NPN (potassium salt), and drugs to be tested. The excitation wavelength was 360 nm for the probe, and emission intensity was measured from 360 to 500 nm at room temperature by a fluorescence spectrophotometer.

Assessment of interaction with KS-505a and R24571 by CaM-affinity gel

A 1 ml sample of the prepared CaM-affinity gel was packed into a plastic microsyringe (4 mm × 70 mm). R24571 (10 μg) was dissolved in 1 ml of Ca²⁺ buffer I (10 mM Tris/HCl, 0.1 mM CaCl₂, pH 7.4) containing 2.0 % (v/v) methanol, and the gel was equilibrated with Ca²⁺ buffer I. KS-505a (12 μg) was dissolved in 600 μl of Ca²⁺ buffer II (10 mM Tris/HCl, 0.1 mM CaCl₂, 300 mM KCl, pH 7.4) and the gel was equilibrated with Ca²⁺ buffer II. The following chromatography procedure was performed under the same conditions for both R24571 and KS-505a

application. R24571 solution (1 ml) or KS-505a solution (600 μ l) was applied to the CaM-affinity gel. After 30 min the gel was washed with Ca^{2+} buffer II and eluted with EGTA buffer (10 mM Tris/HCl, 1.0 mM EGTA, pH 7.4). The amounts of R24571 and KS-505a in the fractions obtained (each 300 μ l) were estimated by the inhibition activities for CaM-activated 61 kDa isoenzyme on the basis of the standard inhibition curve of each inhibitor.

Measurement of intracellular cAMP concentration in rat brain slices

The cAMP concentration in rat brain slices was measured by the method of Garthwaite and Garthwaite [29], with modifications. Parahorizontal slices (300 μ m thick) of the hippocampus and the striatum were prepared from male Wistar rats (35–40 days old) with a Microslicer 1000 (Dosaka Co.). The slices were pre-incubated at 37 °C for 2 h in a Krebs–Henseleit solution, continuously gassed with O_2/CO_2 (19:1). KS-505a was then added at appropriate concentrations. After 15 min incubation the slices were withdrawn, inactivated by boiling for 5 min in 50 mM Tris/4 mM EDTA buffer, pH 7.6, and then disrupted by sonication. cAMP concentrations in the supernatants obtained by centrifugation (7000 g for 10 min at 4 °C) were measured by enzyme immunoassay with kits (Cayman Chemical). Proteins were measured by a protein assay kit (Bio-Rad) with BSA as the standard.

RESULTS

Inhibition of CaM-PDEs by KS-505a

The purified 61 and 59 kDa isoenzymes were activated by $\text{Ca}^{2+}/\text{CaM}$ up to 14 times and 8.5 times the activities in the absence of $\text{Ca}^{2+}/\text{CaM}$ (the basal activities) respectively with cAMP as a substrate. KS-505a potentially inhibited $\text{Ca}^{2+}/\text{CaM}$ -activated 61 kDa isoenzyme activity and required a much higher concentration to inhibit the 59 kDa isoenzyme (Figure 2). When

Figure 2 Typical inhibition curve of KS-505a on two CaM-PDE isoenzymes, cGMP-inhibited PDE and cAMP-specific PDE

Each data points represents the average of two independent measurements in the same preparation of enzyme. The PDE activity of the 61 kDa isoenzyme (○) was assayed with 4 μ M cGMP as a substrate and 3.1 nM CaM. The PDE activity of the 59 kDa isoenzyme (□) was assayed with 2 μ M cGMP as a substrate and 6.2 nM CaM. The concentration of CaCl_2 was 0.4 mM in both CaM-PDE assays. The PDE activities of the cGMP-inhibited PDE (△) and cAMP-specific PDE (●) were assayed with 1.0 μ M cAMP as a substrate. KS-505a was incubated with each isoenzyme.

Table 1 Kinetic analysis of inhibition of the 61 kDa isoenzyme of CaM-PDE by KS-505a: Dixon plots for $\text{Ca}^{2+}/\text{CaM}$ against KS-505a

In each experiment triplicate PDE assays were performed and a mean value was taken. The inhibitory mode and the K_i were determined by means of Dixon plots. Only the experimental condition of cAMP as substrate is described as follows. The activity of the 61 kDa isoenzyme for 30 μ M cAMP was assayed in the presence of different concentrations of CaM (1.7, 2.0 or 16 nM) together with various amounts of KS-505a (0.085 or 0.17 μ M). The concentration of CaCl_2 was 0.4 mM in all cases.

Substrate	Inhibitory mode	K_i (μ M)
cAMP	Competitive	0.089
cGMP	Competitive	0.51

20 μ M cAMP was used as a substrate, the IC_{50} for the 61 kDa isoenzyme was 0.17 ± 0.005 μ M, and that for the 59 kDa isoenzyme was 13 ± 1.3 μ M. With cGMP as a substrate the IC_{50} for the 61 kDa isoenzyme was 0.62 ± 0.053 μ M (at 4.0 μ M cGMP), whereas that for the 59 kDa isoenzyme was 21 ± 1.6 μ M (at 2.0 μ M cGMP). The basal activities of both isoenzymes were not inhibited by KS-505a up to 50 μ M. These results show that KS-505a displays a high degree of selectivity between the 61 and the 59 kDa isoenzymes.

Effect of KS-505a on cGMP-inhibited and cAMP-specific PDEs

When assayed against other isoenzymes of PDE, KS-505a showed a considerable degree of selectivity. cGMP-inhibited and cAMP-specific PDEs were only slightly inhibited by KS-505a (approx. 30 % even at 100 μ M) (Figure 2).

Kinetic analysis of the inhibition of CaM-PDEs by KS-505a

To establish whether $\text{Ca}^{2+}/\text{CaM}$ is involved in the inhibition of the 61 kDa isoenzyme by KS-505a, we measured the enzyme activity in the presence of different concentrations of $\text{Ca}^{2+}/\text{CaM}$ together with varying amounts of KS-505a (Table 1), which shows the kinetic analysis data by means of Dixon plots [30]. In this analysis, the vertical axis was the reciprocal of PDE activity in the presence of each $\text{Ca}^{2+}/\text{CaM}$ and KS-505a concentration, and the horizontal axis was the KS-505a concentration. Three lines corresponding to three different concentrations of $\text{Ca}^{2+}/\text{CaM}$ crossed at the same point with cAMP or cGMP as substrate, demonstrating that KS-505a inhibits the 61 kDa isoenzyme activity in a competitive manner with respect to $\text{Ca}^{2+}/\text{CaM}$. K_i values of KS-505a were 0.089 μ M (cAMP) and 0.51 μ M (cGMP). KS-505a had no inhibitory activity for the basal activity of 61 kDa isoenzyme (results not shown).

We next examined the effect of various concentrations of substrate and KS-505a on the 61 kDa isoenzyme. Lineweaver–Burk plots clearly indicate uncompetitive inhibition of cAMP hydrolysis and non-competitive inhibition of cGMP hydrolysis (Table 2). These results suggest that the binding site of KS-505a on the 61 kDa isoenzyme is not the catalytic domain.

An analysis of Lineweaver–Burk plots shows that the inhibition mode of the 59 kDa isoenzyme was non-competitive with respect to cGMP (Table 2). In contrast, an analysis of Dixon plots for the 59 kDa isoenzyme indicates that the inhibition mode is independent of $\text{Ca}^{2+}/\text{CaM}$ concentrations (results not shown).

Effect of KS-505a on trypsin-treated 61 kDa isoenzyme

Trypsin treatment of the 61 kDa isoenzyme resulted in $\text{Ca}^{2+}/\text{CaM}$ -independent activation of the enzyme to the level of

Table 2 Kinetic analysis for inhibition of CaM-PDE by KS-505a: Lineweaver–Burk plots of substrate (cAMP or cGMP) against KS-505a

In each experiment triplicate PDE assays were performed and a mean value taken. The inhibitory mode and the K_i were determined by means of Lineweaver–Burk plots. The K_i represents an average of data calculated from each case of two different concentrations of KS-505a. The activity of the 61 kDa isoenzyme was assayed in the presence or absence of different concentrations of KS-505a, together with various amounts of substrate (cAMP or cGMP) with the same preparation of enzyme. CaM (3.1 nM) and CaCl_2 (0.4 mM) were included in all cases. The activity of the 59 kDa isoenzyme was assayed in the presence or absence of different concentrations of KS-505a, together with various amounts of cGMP. CaM (6.2 nM) and CaCl_2 (0.4 mM) were included in all cases. The activity of the trypsin-treated enzyme was assayed in the presence or absence of different concentrations of KS-505a, together with various amounts of cGMP.

Enzyme	Substrate	Inhibitory mode	K_i (μM)
61 kDa isoenzyme	cAMP	Uncompetitive	0.060
61 kDa isoenzyme	cGMP	Non-competitive	0.46
59 kDa isoenzyme	cGMP	Non-competitive	19
Trypsin-treated 61 kDa isoenzyme	cGMP	Non-competitive	56

Figure 3 Effect of trypsin treatment on inhibition of 61 kDa isoenzyme by KS-505a

Tryptic digestion was performed for 6 min at 30 °C with 3 $\mu\text{g}/\text{ml}$ trypsin. Inhibition curves for KS-505a on the trypsin-treated ($\circ$) and native ($\square$) 61 kDa isoenzyme are shown. cGMP (4.0 μM) was used as substrate in both enzymes, and for the native enzyme 3.1 nM CaM and 0.4 mM CaCl_2 were used. In both cases, each data point represents the average of three independent measurements; each bar represents the S.E.M.

the fully activated native enzyme in the presence of $\text{Ca}^{2+}/\text{CaM}$ (results not shown). The resulting trypsin-treated 61 kDa isoenzyme lacked a calmodulin-binding domain [25,26]. KS-505a inhibited the native 61 kDa isoenzyme with an IC_{50} of 0.62 ± 0.053 μM , whereas the trypsin-treated 61 kDa isoenzyme was not inhibited by KS-505a up to 4.0 μM (Figure 3). Higher concentrations of KS-505a inhibited the trypsin-treated enzyme: the IC_{50} was 39 ± 3.9 μM (Figure 3). Kinetic analysis with a Lineweaver–Burk plot demonstrated that KS-505a inhibited the trypsin-treated enzyme in a non-competitive manner with respect to cGMP (Table 2).

Interaction of KS-505a with $\text{Ca}^{2+}/\text{CaM}$

Using the hydrophobic fluorescent probe NPN, we investigated whether or not KS-505a bound directly to $\text{Ca}^{2+}/\text{CaM}$. The intensity of the fluorescence of NPN was increased by adding Ca^{2+} to activate CaM (Figure 4). The addition of R24571

reversed the increase in the fluorescence in a dose-dependent manner (Figure 4A). R24571, a CaM antagonist, has been reported to bind to Ca^{2+} -activated CaM [27]. Under the same condition KS-505a had no effect on the increase of fluorescence by $\text{Ca}^{2+}/\text{CaM}$ and NPN interaction (Figure 4B).

Interaction of KS-505a with CaM was further tested by using CaM-conjugated Affigel-15. R24571 was not detected in the effluent with Ca^{2+} -containing buffer II, but it was with EGTA buffer as eluent. This behaviour of R24571 indicates that R24571 binds to Ca^{2+} -activated CaM and dissociates from CaM in the absence of Ca^{2+} . KS-505a passed through the CaM-conjugated affinity gel in the presence of Ca^{2+} and its recovery rate was approximately 100 %. None of the KS-505a was detected in the EGTA-eluted fractions. These results demonstrate clearly that KS-505a does not interact with or bind to Ca^{2+} -activated CaM.

Effect of KS-505a on cAMP level in rat brain slices

To test whether KS-505a could discriminate between the functional differences of the CaM-PDE isoenzymes *in vivo*, we examined the effect of the compounds on cAMP levels in different regions of rat brain slices. In rat hippocampal slices, KS-505a at 10 μM increased the cAMP concentration to approximately three times the basal level (Table 3). In contrast, in rat striatal slices no increase in the cAMP concentration by KS-505a was observed at concentrations between 1.0 and 10 μM (Table 3).

DISCUSSION

The important findings of this study are that KS-505a is a potent and highly selective inhibitor of the brain 61 kDa isoenzyme of CaM-PDE and that it apparently acts by selective binding to the CaM-binding domain of the enzyme.

The following evidence supports the interaction between KS-505a and the CaM-binding domain of the 61 kDa isoenzyme. First, kinetic analysis (Dixon plot) revealed that KS-505a competed with $\text{Ca}^{2+}/\text{CaM}$ but not with the substrates (Table 1). Secondly, it was shown that KS-505a had no interaction with the $\text{Ca}^{2+}/\text{CaM}$ by the experiments with a fluorescent probe for CaM and a CaM-conjugated gel. KS-505a also has no interaction with Ca^{2+} itself because its enzyme-inhibition activity was independent of Ca^{2+} concentration (results not shown). Thirdly, trypsin-activated 61 kDa isoenzyme, which lacked the $\text{Ca}^{2+}/\text{CaM}$ -binding domain, was not potently inhibited by KS-505a (Figure 3). Kinetic analysis with the trypsin-activated enzyme demonstrated that the inhibition mode of KS-505a was non-competitive (Table 2). Fourthly, KS-505a did not inhibit the basal activity of the 61 kDa isoenzyme (results not shown). Taken together, KS-505a inhibits CaM-activated 61 kDa isoenzyme activity by binding directly to its CaM-binding domain and interferes with the interaction between $\text{Ca}^{2+}/\text{CaM}$ and the enzyme.

KS-505a inhibited the 59 kDa isoenzyme non-competitively with respect to cGMP (Table 2). The results suggest the existence of a low-affinity KS-505a binding site located elsewhere than the catalytic site in the 59 kDa isoenzyme. From the Dixon plot analysis, KS-505a did not compete with $\text{Ca}^{2+}/\text{CaM}$ in the 59 kDa isoenzyme (results not shown). The binding site of KS-505a in the 59 kDa isoenzyme thus shows different properties in its affinity and kinetics from the binding site of KS-505a in the 61 kDa isoenzyme. KS-505a also had no inhibitory activity towards other $\text{Ca}^{2+}/\text{CaM}$ -independent PDE isoenzymes, including cAMP-specific and cGMP-inhibited PDE (Figure 2). Therefore KS-505a is a potent and highly selective inhibitor of the 61 kDa isoenzyme and distinguishes between members of the CaM-PDE subfamilies. It has been reported recently that steroid glycoside ginsenosides also distinguish between members of the

Figure 4 Effect of R24571 (A) and KS-505a (B) on the fluorescence of NPN bound to CaM

CaM (0.16 μ M) was incubated with 8 μ M NPN in 10 mM Tris/HCl buffer, pH 7.5, containing 0.2 mM CaCl_2 . Fluorescence spectra were recorded as described in the Experimental section. Excitation was at 360 nm and emission intensity was measured over the range 350–600 nm. (A) Curve 1, NPN alone; curve 2, NPN plus CaM; curve 3, NPN plus CaM and 50 ng/ml R24571; curve 4, NPN plus CaM and 200 ng/ml R24571. (B) Curve 1, NPN alone; curve 2, NPN plus CaM; curve 3, NPN plus CaM and 50 ng/ml KS-505a; curve 4, NPN plus CaM and 200 ng/ml KS-505a.

Table 3 Effect of KS-505a on cAMP concentration in rat brain slices

Each result represents the mean $\pm$ S.E.M. The rat brain slices (300 μ m) were incubated at 37 $^\circ\text{C}$ in a Krebs–Henseleit solution, continuously gassed with O_2/CO_2 (19:1) in the presence or absence of KS-505a. After incubation the slices were homogenized and the cAMP content of the supernatant was measured by ELISA. Statistical significance: ** $P < 0.01$ compared with control ($n = 3$) (Dunnett multiple comparison procedure).

[KS-505a] (μ M)	[cAMP] (pmol per mg of protein)	
	Hippocampus	Striatum
0	7.90 $\pm$ 0.45	3.35 $\pm$ 0.20
1.0	7.35 $\pm$ 0.66	3.84 $\pm$ 0.51
10	23.0 $\pm$ 1.8**	4.15 $\pm$ 0.59

CaM-PDE family; they inhibit both the 59 and 61 kDa isoenzymes but not the 63 kDa isoenzyme [31]. The mechanism of the inhibition by ginsenosides has not yet been clarified.

The 61 and 63 kDa isoenzymes are found predominantly in cells of the central nervous system. From the results of the Northern analysis, the 61 kDa isoenzyme mRNA is widely distributed in brain regions (the hippocampus, the cerebellum, the cerebral cortex and the basal ganglia) [9]. Bentley et al. [10] have reported that the 63 kDa isoenzyme mRNA is detected in several regions (the hippocampus, the cerebellum and the cerebral cortex) of the rat central nervous system and is especially highly expressed in the basal ganglia region. Polli and Kincaid [32] and Yan et al. [33] have further indicated that the 63 kDa isoenzyme is enriched in the mouse striatum, which is a major region in the basal ganglia. Hybridization *in situ* demonstrated high levels of 63 kDa isoenzyme mRNA in the striatum, nucleus accumbens and olfactory tubercle [32,33]. Blockade of the isoenzymes by KS-505a should be a valuable means to test whether CaM-PDE isoenzymes function differentially in different regions of brain. In rat hippocampal slices the cAMP concentration was increased approx. 3-fold by 10 μ M KS-505a, whereas in rat striatal slices no increase was observed at KS-505a concentrations between 1.0 and 10 μ M (Table 3). The results indicate that KS-505a distinguishes the functions and distributions of CaM-PDE isoenzymes in these regions *in vivo*. Although no results on the inhibition of the 63 kDa isoenzyme by KS-505a have been obtained yet, the

fact that KS-505a has no effect on cAMP concentration in the striatum region implies that KS-505a hardly affects the activity of the 63 kDa isoenzyme. However, in rat hippocampus the inhibition of the 61 kDa isoenzyme may contribute to the elevation of the cAMP concentration.

We observed that KS-505a administered intraperitoneally had an ameliorative effect on amnesia induced in rat by electroconvulsive shock [16]. Hippocampal long-term potentiation is thought to serve as an elementary mechanism for the establishment of memory, and cAMP stimulates long-term potentiation in hippocampal CA1 neurons [34]. In this respect it is fascinating to hypothesize that the anti-amnesia activity of the compound is due to the elevation of the cAMP concentration in the hippocampus by inhibiting the 61 kDa isoenzyme. It will be worth investigating the relationship between anti-amnesia activity and selective inhibition for CaM-PDE in brain from the viewpoint of the therapeutic application of this compound.

Further studies on the regulation of specific CaM-PDE isoenzyme by KS-505a should provide clues not only to the role of the isoenzymes in neuronal function and differentiation but to the potential therapeutic use of the compound for amnesia.

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