A Single Amino Acid Difference between α and β Ca\(^{2+}\)/Calmodulin-dependent Protein Kinase Kinase Dictates Sensitivity to the Specific Inhibitor, STO-609*

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We recently developed STO-609, a selective inhibitor of Ca\(^{2+}\)/calmodulin-dependent protein kinase kinase (CaM-KK), and we demonstrated that CaM-KKβ is more sensitive to STO-609 than the CaM-KKα isoform (Tokumitsu H., Inuzuka H., Ishikawa Y., Ikeda M., Saji I., and Kobayashi R. (2002) J. Biol. Chem. 277, 15813–15818). By using catalytic chimera and point mutants of both isoforms, we demonstrated that Val269 in CaM-KK/Leu233 and CaM-KKα/Val269 in CaM-KKβ confer a distinct sensitivity (10-fold) to STO-609 on CaM-KK isoforms. Various mutations of Val269 in CaM-KKβ indicate that substitution by hydrophobic residues with bulky side chains significantly decreases drug sensitivity and that the V269F mutant is the most effective drug-resistant enzyme (80-fold higher IC\(_{50}\) value). These findings are consistent with a result obtained with a full-length mutant expressed in COS-7 cells. Furthermore, suppression of CaM-KK-mediated CaM-KIV activation in transfected HeLa cells by STO-609 treatment was completely abolished by the co-expression of the CaM-KKβ V269F mutant. Based on the results that the distinct sensitivity of CaM-KK isoforms to STO-609 is because of a single amino acid substitution (Val/Leu) in the ATP-binding pocket, we have generated an STO-609-resistant CaM-KK mutant, which might be useful for validating the pharmacological effects and specificity of STO-609 in vivo.

Intracellular Ca\(^{2+}\) is known to be a second messenger mediating a wide variety of physiological functions such as contraction, gene expression, and secretion. Ca\(^{2+}\)/calmodulin-dependent protein kinases (CaM-K) constitute a diverse group of enzymes involved in many cellular responses mediated by an increase in the concentration of intracellular calcium. Previous studies have demonstrated that two multifunctional CaM kinases (CaM-KI and -IV, are activated by the phosphorylation of an activation loop Thr residue by an upstream CaM kinase kinase (CaM-KK), resulting in a large increase in catalytic efficiency (1, 2). In mammals, two CaM-KK genes (CaM-KKa and CaM-KKβ) have been cloned, both of which are highly expressed in the brain and also expressed (to a low degree) in various peripheral tissues such as the thymus and spleen (3–5). CaM-KK is also known to be a Ca\(^{2+}\)/CaM-regulated enzyme (3, 5–7). Indeed, Ca\(^{2+}\)/CaM binding is absolutely required for the relief of CaM-KKs autoinhibition, which results in the activation of the enzyme (8–10). In contrast, CaM-KKβ exhibits enhanced Ca\(^{2+}\)/CaM-independent activity, because of the suppression of autoinhibition by the second regulatory segment (residues 129–151) located at the N terminus of the catalytic domain (5, 11). The CaM-KK gene has been found in Caenorhabditis elegans and Aspergillus nidulans, and the proteins they encode are components of the CaM kinase cascade of these organisms (12–15). A functional CaM kinase cascade leading to the activation of CaM-KIV in response to Ca\(^{2+}\) mobilization has been demonstrated by using transfected COS-7 cells (6), Jurkat cells (16), and cultured hippocampal neurons (17). The cascade has also been shown to be required for the activation of CaM-KI in PC-12 cells upon membrane depolarization (18). An important role has been demonstrated for the CaM-KIV cascade in the regulation of Ca\(^{2+}\)-dependent gene expression by the phosphorylation of transcription factors such as CREB (19–22). A recent study of transgenic mice carrying dominant negative CaM-KIV alleles that confer a defect in the phosphorylation of CREB indicates that these animals exhibit a disruption of late-phase long term potentiation, and that they are impaired in the consolidation/retention phase of hippocampus-dependent memory (23). We have also demonstrated that the CaM kinase cascade regulated CREB-dependent gene expression in neurons of living nematodes by using transgenic worms (24). Analysis of mice deficient in CaM-KIV revealed that the CaM-KIV-mediated pathway plays an important role in the function and development of the cerebellum and is critical for male and female fertility (25–27). In addition, a physiological role has been predicted for CaM-KK, with the suggestion that it may act as a regulatory protein kinase in the CaM kinase cascade; however, this has not been demonstrated in vivo. To evaluate the physiological functions of CaM-KK and those of the CaM kinase cascade, we recently developed a relatively selective and cell-permeable inhibitor of CaM-KK, STO-609 (28). However, a recent search for protein kinase sequences in the human genome revealed that 510 protein kinase genes are encoded in the human genome (29), indicating the practical difficulty of precisely examining the specificity of the protein kinase inhibitor in vivo as well as in vitro (35). In this study, we further characterized the inhibitory mechanism of STO-609 on CaM-KK activity; based on the results, we attempted to generate an inhibitor-resistant CaM-KK mutant that might prove

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The abbreviations used are: CaM-K, Ca\(^{2+}\)/CaM-dependent protein kinase; CaM, calmodulin; PKA, cAMP-dependent protein kinase; CREB, cAMP-response element binding protein; HA, hemagglutinin; GST, glutathione S-transferase.
Inhibitory Mechanism of CaM Kinase Kinase by STO-609

EXPERIMENTAL PROCEDURES

MATERIALS—CaM-KKs cDNA (GenBankTM accession number L43810 (3)) was obtained from a rat brain cDNA library. Rat CaM-KK was cloned by reverse transcriptase-PCR, as described previously (11). Recombinant rat CaM was expressed in the Escherichia coli strain BL21(DE3) using pET-CaM (30) (kindly provided by Dr. Nobuhiro Hayashi, Fujita Health University, Toyoake, Japan) and was purified by phenyl-Sepharose column chromatography. Rat CaM-KI-(1–293, K49E) was expressed in E. coli JM-109 as a GST fusion protein and was purified by glutathione-Sepharose (Pharmacia Biochemistry) chromatography (19). 300 ng of HA (hemagglutinin-tagged)-CaM-KIV with or without 40 ng of CaM-KKβ and 20 μg of LipofectAMINE reagent (Invitrogen) in 2.5 ml of medium. After 40 h of culture, the cells were further cultured in serum-free medium for 6 h in either the absence or presence of various concentrations of STO-609 (1 and 10 μg/ml in Me2SO at a final concentration of 0.5%) and then treated with or without 1 μM ionomycin for 5 min. Stimulation was terminated by the addition of 1 ml of lysis buffer (150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 2 mM EGTA, 1% Nonidet P-40, 10% glycerol, 0.2 mM phenylmethylsulfonyl fluoride, 10 mM MgCl2/0.5 μg/ml leupeptin, 10 μg/ml trypsin inhibitor, and 1 μg/ml microcin- R1), and the cells were lysed for 30 min on ice. The cell extract was collected and centrifuged at 15,000g for 15 min and the supernatant was precleared with 40 μl of Protein G-Sepharose (50% slurry, Amersham Biosciences) for 2 h at 4°C. The supernatant was then mixed with 4 μg of anti-HA antibody (clone 12CA5, Roche Molecular Biochemicals) for 3 h. After centrifugation, 40 μl of Protein G-Sepharose was applied to the extract and incubated overnight. The immunoprecipitated resin was washed three times with 1 ml of the lysis buffer, as described above, and then the resin was washed with 1 ml of kinase buffer (50 mM HEPES, pH 7.5, 10 mM Mg(Ac)2, 1 mM EGTA, and 1 μg/ml microcin-LR). Protein-G-Sepharose with immunoprecipitated HA-CaM-KIV was incubated with 40 μM syntide-2 at 30°C for 10 min in a solution (40 μl) containing 50 mM HEPES (pH 7.5), 10 mM Mg(Ac)2, 1 mM dithiothreitol, 1 mM MgCl2, 0.1 mM PMSF, and 100 μg/ml γ-[32P]ATP (1000 cpn/μg) and 0.1% Triton X-100, 1 mM EGTA. To estimate the amount of immunoprecipitated HA-CaM-KIV, SDS-PAGE sample buffer (50 μl) was added to immunoprecipitated samples and then heated at 95°C for 10 min. After centrifugation, 5 μl of the sample was subjected to SDS-7.5% PAGE followed by Western blotting using anti-CaM-KIV antibody (1:2000, Transduction Laboratories). To confirm the expression level of co-expressed CaM-KKβ (wild-type and V269F mutant), transfected HeLa cells were lysed with 160 μl of SDS-PAGE sample buffer after stimulation with or without ionomycin as described above, and then the sample was heated at 95°C for 10 min. After centrifugation, 20 μl of the sample was subjected to SDS-7.5% PAGE followed by Western blotting using anti-CaM-KK antibody (1:2000, Transduction Laboratories).

Expression of CaM-KKβ in COS-7 Cells—Expression of full-length CaM-KKβ in COS-7 cells was carried out essentially as described above, using 3 μg of either pME-CaM-KKβ (wild-type or V269F mutant) or empty vector (pME18s, mock) and 20 μg of LipofectAMINE reagent (Invitrogen). After 36 h culture, cells in 6-cm dishes were lysed with 250 μg of lysis buffer, as described above, without EGTA. After centrifugation at 15,000×g for 15 min, the supernatant was collected and stored at −30°C until used for the CaM-KK activity assay. The amount of CaM-KKβ in the cell extract was estimated by Western blot analysis with anti-CaM-KK antibody (1:2000, Transduction Laboratories) using a modified recombinant wild-type CaM-KKβ (11) as a standard. The antibody was capable of recognizing both α and β isoforms of CaM-KK (11).

RESULTS AND DISCUSSION

We have recently developed a potent and relatively inhibitor of CaM-KK, STO-609, which can permeate cells and is a competitive inhibitor of ATP (28). According to previous results, the sensitivity of CaM-KKβ to the compound is >5-fold higher than that of the α isoform whereas the apparent Km values of both isoforms for ATP (~3 μM) are indistinguishable.

In Vitro Assay for CaM KK Activity—Either purified recombinant rat CaM-KKβ (30-300 ng of CaM fusion enzyme) or CaM-KK expressed in COS-7 cells (4 ng enzyme) were incubated with 10 μg of GST-CaM-KI (1–293, K49E) at 30 °C for 5 min in a solution (25 μl) containing 50 mM HEPES (pH 7.5), 10 mM Mg(Ac)2, 1 mM dithiothreitol, 50 μM γ-[32P]ATP (3000–7000 cpm/ml) with various concentrations of STO-609 (0–1 μg/ml in Me2SO at a final concentration of 4%) in the presence of either 1 μM EGTA for GST fusion enzymes or 1 μM CaCl2 for CaM-KKβ expressed in COS-7 cells. The reaction was initiated by the addition of [γ-32P]ATP and terminated by spotting aliquots (15 μl) onto phosphocellulose paper (Whatman P-81) followed by several washes with 75 mM phosphoric acid (31). Phosphate incorporation into GST-CaM-KI-(1–293, K49E) was determined by liquid scintillation counting of the filters. A 5-min reaction was chosen to determine CaM-KK activity based on a recently described time course experiment (11).

Transient Expression of HA-CaM-KIV with CaM-KKβ and Immuno- precipitation—HeLa cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Cells were subcul- tured in 6-cm dishes 12 h before transfection. The cells were then transferred to serum-free medium and treated with a mixture of either pME18s or pME18s plasmid DNA (10 μg) and either PHE-9161 (1000 μM) or 5 μM ionomycin as described above, and then the sample was heated at 95°C for 10 min. After centrifugation, 20 μl of the sample was subjected to SDS-7.5% PAGE followed by Western blotting using anti-CaM-KIV antibody (1:2000, Transduction Laboratories).
To elucidate the mechanism of the differential sensitivity of CaM-KK isoforms to STO-609, we examined the inhibitory effect of STO-609 on various catalytic domain chimera mutants of both isoforms, because it has been shown to be a target of the catalytic domain of CaM-KK (28). Catalytic domain mutants, including the wild-type enzyme, lack both an N-terminal autoinhibitory segment and a CaM-binding segment (8, 11). These mutants were expressed in *E. coli* and purified as GST fusion proteins. Thus, all of the recombinant enzyme phosphates (GST-CaM-KI-(1–235) and residues 251–271 in CaM-KK) is indicated by a shaded box. A schematic representation of catalytic domain chimera mutants of CaM-KK isoforms is shown. CaM-KK catalytic domain chimera mutants, including the wild-type enzyme, were constructed and expressed in *E. coli* JM-109 as a GST fusion protein and purified as described under “Experimental Procedures.” Purified CaM-KK mutants (20–30 ng) were incubated with 10 μM of GST-CaM-KI-(1–293, K49E) at 30 °C for 5 min in a solution (25 μl) containing 50 mM HEPES (pH 7.5), 10 mM Mg(Ac)2, 1 mM dithiothreitol, and 50 μM [γ-32P]ATP with various concentrations of STO-609 (0.1-μM/ml in Me2SO at a final concentration of 4%) in the presence of 1 mM EGTA. The CaM-KK activities of recombinant enzymes measured in the absence of STO-609 are shown and the IC50 values were calculated, as described under “Experimental Procedures.” Results represent the mean ± S.E. of three experiments.

(28). To elucidate the mechanism of the differential sensitivity of CaM-KK isoforms to STO-609, we examined the inhibitory effect of STO-609 on various catalytic domain chimera mutants of both isoforms, because it has been shown to be a target of the catalytic domain of CaM-KK. The IC50 values were calculated, as described under “Experimental Procedures.” Purified CaM-KK mutants (20–30 ng) were incubated with 10 μM of GST-CaM-KI-(1–293, K49E) at 30 °C for 5 min in a solution (25 μl) containing 50 mM HEPES (pH 7.5), 10 mM Mg(Ac)2, 1 mM dithiothreitol, and 50 μM [γ-32P]ATP with various concentrations of STO-609 (0.1-μM/ml in Me2SO at a final concentration of 4%) in the presence of 1 mM EGTA. The CaM-KK activities of recombinant enzymes measured in the absence of STO-609 are shown and the IC50 values were calculated, as described under “Experimental Procedures.” Results represent the mean ± S.E. of three experiments.
value of the L233V mutant of CaM-KKα-(126–434) for inhibition by STO-609 was ~8-fold lower than that of the wild-type enzyme. The catalytic activity of the L233V mutant in the absence of STO-609 was comparable with that of the wild-type CaM-KKα-(126–434). These results strongly indicate that a single amino acid difference (Val/Leu) in the catalytic domain of CaM-KK isoforms dictates sensitivity to the inhibitor. Val residue at position 269 in the rat CaM-KKβ is conserved in human CaM-KKβ (Val126) (5) and C. elegans CaM-KK (Val126) (13). According to the crystal structure of PKA, Val269 in CaM-KKβ is also conserved in subdomain V as Val123, which helps to anchor Mg-ATP by hydrogen bonding, and also contributes to a hydrophobic pocket that surrounds the adenine ring (33, 34).

Next, we mutated Val269 in CaM-KKβ-(162–470) with various amino acid residues, and in particular, hydrophobic residues, to maintain the hydrophobic ATP-binding pocket. We then examined the inhibitory potency of the inhibitor against these mutants (Fig. 3). Although the Ala mutation had little or no significant effect on the drug sensitivity of the enzyme, the mutations with hydrophilic residues with relatively bulky side chains (Leu, His, and Met) resulted in a significant increase (>10-fold) in the IC50 value for inhibition by STO-609 of the CaM-KK mutants. We found that the mutation of Val269 by Phe caused the most dramatic effect (i.e., ~8-fold increase in the IC50 value) on the drug sensitivity of CaM-KKβ. It is of note that a Pro mutation results in an inactive enzyme, probably because of the destruction of the structure of the ATP-binding pocket necessary for efficient catalysis. Because the V269A mutant was inhibited by STO-609 with the same potency as the wild-type enzyme, an amino acid residue at Val269 in CaM-KKβ/Leu233 in CaM-KKα may not be involved in the direct binding of the compound to CaM-KK. However, mutation of Val269 by hydrophilic residues with relatively larger side chains (e.g., Leu, Met, His, and Phe) can efficiently block the STO-609/CaM-KK interaction resulting in decreased drug sensitivity of CaM-KK.

Identification of Val269/Leu233 in the CaM-KK isoforms involved in the STO-609 sensitivity described above was performed by using E. coli expressing GST fusion proteins with the catalytic domain of each CaM-KK. We attempted to express the V269F mutant of full-length CaM-KKβ in mammalian cells to confirm the drug sensitivity of the mutant enzyme. As shown in Fig. 4, the protein kinase activity of wild-type CaM-KKβ expressed in COS-7 cells was inhibited by ~80% in the presence of 0.1 μg/ml STO-609 with an IC50 value of 20–30 ng/ml. This result is consistent with that of a previous report using E. coli expressing full-length CaM-KKβ (28). In contrast, although the CaM-KK activities of COS-7 cells expressing the wild-type CaM-KKβ (504 ± 15 nmol/min/mg) and the V269F mutant (656 ± 22 nmol/min/mg) in the absence of STO-609 were comparable, 1 μg/ml STO-609 suppressed only 40% of the V269F mutant activity. And the IC50 value for inhibition by STO-609 of the mutant was approximately 2 orders of magnitude higher than that of the wild-type enzyme. This finding is in good agreement with the results obtained with catalytic domain mutants, as shown in Fig. 3.

Finally, we examined whether or not ectopic expression of the CaM-KKβ V269F mutant induces STO-609 resistance in the kinase-expressing cells. After we co-transfected HA-tagged CaM-KIV with an empty vector, wild-type CaM-KKβ, or V269F mutant cDNA into HeLa cells, the cells were treated with (1 and 10 μg/ml) or without STO-609, and then the cells were stimulated with ionomycin for 5 min, thereby increasing the intracellular Ca2+ concentration. Then, HA-CaM-KIV was immunoprecipitated with anti-HA antibody followed by measurement of its Ca2+/CaM-independent activity using syntide-2 as a substrate (Fig. 5). Western blot analysis of the cell extract using anti-CaM-KK antibody revealed that HeLa cells contain endogenous CaM-KKα and the expression levels of overexpressed wild-type CaM-KKβ and V269F mutant were indistinguishable (Fig. 5, upper inset). The amount of immunoprecipitated HA-CaM-KIV was also confirmed by Western blot analysis (Fig. 5, lower inset). The protein kinase activity of immunoprecipitated CaM-KIV from cells co-expressed with or without wild-type CaM-KKβ or V269F mutant was largely

| CaM-KKα | 126–434 | CaM-KKβ | 162–470 |
|---------|---------|---------|---------|
| V2511   | 128 ± 2 | N258A   | 95 ± 6  |
| H261N   | 91 ± 1  | M264L   | 113 ± 1 |
| E267D   | 55 ± 1  | N270R   | 179 ± 8 |
| V269L   | 139 ± 4 | Q271K   | 157 ± 3 |
| CaM-KKα | 126–434 | CaM-KKβ | 162–470 |
| L233V   | 163 ± 6 | L233V   | 163 ± 6 |

Table of IC50 values

| CaM-KK Activity (nmol/min/mg) | IC50 value (ng/ml) |
|------------------------------|--------------------|
| 130 ± 2                      | 10.8 ± 0.8        |
| 128 ± 2                      | 14.1 ± 1.0        |
| 95 ± 6                       | 9.6 ± 1.5         |
| 91 ± 1                       | 10.8 ± 0.8        |
| 113 ± 1                      | 10.6 ± 0.1        |
| 55 ± 1                       | 12.6 ± 0.6        |
| 139 ± 4                      | 140.3 ± 5.3       |
| 179 ± 8                      | 12.7 ± 0.3        |
| 157 ± 3                      | 11.2 ± 0.4        |
| 148 ± 3                      | 133.2 ± 6.5       |
| 163 ± 6                      | 17.6 ± 0.7        |
activated by Ca^{2+} mobilization. Maximum activity of the immunoprecipitated CaM-KIV induced by ionomycin was not affected by co-expression of either wild-type CaM-KKβ or V269F mutant. Suppression of Ca^{2+}-dependent induction of CaM-KIV activity by STO-609 treatment indicates an endogenous CaM-KKβ/CaM-independent activity measured in the millimolar range. Therefore, a much higher concentration of STO-609 appears to be required to suppress CaM-KK activity in these cells, as compared with the concentration required for the inhibition of CaM-KK activity in vitro. Although the overexpression of wild-type CaM-KKβ significantly reduced the inhibitory potency of STO-609 for the activi-

Figure 3. Effect of various mutations at Val^{269} in CaM-KKβ on STO-609 sensitivity. Schematic representation of point mutants at Val^{269} of GST-fused CaM-KKβ(162–470) is shown. Point mutants of GST-fused CaM-KKβ(162–470), including the wild-type enzyme, were expressed and purified as described under “Experimental Procedures” and 20–30 ng of each mutant was assayed to measure the inhibitory effect of STO-609, as described in the legend to Fig. 1. The CaM-KK activities of recombinant enzymes measured in the absence of STO-609 are shown and the IC_{50} values were calculated as described under “Experimental Procedures.” The results represent the mean ± S.E. of three experiments. ND, not detected.

Figure 4. Effect of STO-609 on the activities of full-length CaM-KKβ wild-type and V269F mutant expressed in COS-7 cells. Wild-type CaM-KKβ (CaM-KKβ WT, closed circle) and V269F mutant (CaM-KKβ V269F, open circle) cDNA was transfected into COS-7 cells and the cell extracts (4 ng of CaM-KK) were assayed to measure the inhibitory effects of STO-609 in the presence of 1 mM CaCl_2. The results are expressed as a percentage of the mean activity of the enzyme treated with 0 mM STO-609. Each extract, including the extract from cells transfected with an empty vector, were subjected to SDS-10% PAGE followed by Western blot analysis using anti-CaM-KK antibody (inset). The arrow indicates CaM-KKβ.

Figure 5. CaM-KKβ V269F mutant induces STO-609 resistance in HeLa cells. After transfection of either HA-CaM-KIV cDNA (2.7 μg, +) or empty vector (left lane, −) without (−) or with either 40 ng of wild-type CaM-KKβ (WT) or V269F mutant cDNA (V269F) into HeLa cells, the cells were either untreated (−) or treated with various concentrations of STO-609 (1 and 10 μg/ml), as indicated, for 6 h in serum-free medium. The cells were then stimulated with 1 μM ionomycin for 10 min (–), or not stimulated (−). HA-CaM-KIV was immunoprecipitated and its Ca^{2+}-CaM-independent activity was measured at 30 °C for 10 min in the presence of 1 mM EGTA, as described under “Experimental Procedures.” The results represent the mean of two independent transfections. Similar results were obtained in three separate experiments. Transfected HeLa cell extracts (1/10 volume) and immunoprecipitated samples (1/10 volume), including a sample prepared from cells transfected with an empty vector, were subjected to Western blot analysis with an anti-CaM-KK antibody (upper inset) and an anti-CaM-KIV antibody (lower inset), respectively, as described under “Experimental Procedures.” The upper and lower arrows in the upper inset indicate overexpressed CaM-KKβ and endogenous CaM-KKα, respectively. The upper and lower arrows in the lower inset indicate HA-CaM-KIV and the anti-HA antibody (IgG), respectively.

### Table 1: CaM-KK Activity IC_{50} Value (ng/ml)

| CaM-KK Activity (nmol/min/mg) | IC_{50} Value (ng/ml) |
|-----------------------------|----------------------|
| CaM-KKβ 162–470             | 130 ± 2               |
| CaM-KKβ V269A               | 124 ± 6               |
| CaM-KKβ V269L               | 139 ± 4               |
| CaM-KKβ V269I               | 88 ± 2                |
| CaM-KKβ V269F               | 79 ± 3                |
| CaM-KKβ V269M               | 71 ± 3                |
| CaM-KKβ V269P               | 127 ± 4               |

ND, not detected.
µg/ml STO-609 in the presence of overexpressed wild-type CaM-KKβ. In contrast, activation of CaM-KIV from the cells co-expressed with the V269F mutant was completely resistant to the inhibitor at concentrations up to 10 µg/ml. This result establishes that the suppression of ionomycin-induced CaM-KIV activity by STO-609 was because of direct inhibition of endogenous CaM-KK in these cells, as effected by the inhibitor. We have observed a similar result with the same transfection experiment using COS-7 cells (data not shown). Taken together, these results support the idea that the V269F mutant of CaM-KKβ functions as an STO-609-resistant mutant in intact cells as well as in vitro.

In summary, we have characterized the inhibitory mechanism of a recently developed inhibitor of CaM-KK, STO-609, with regard to the distinct sensitivity of CaM-KK isoforms to the inhibitor. We have shown in this report that a single amino acid difference (Val269 in CaM-KKβ/Leu258 in CaM-KKα) in the ATP-binding catalytic domain of CaM-KK isoforms is directly involved in the distinct sensitivity to the inhibitor. This conclusion is well correlated with findings from the kinetic analysis of STO-609 inhibition, which revealed that the compound is ATP-competitive (28). Although Val269 is conserved in PKA (Val123) as an important residue that contributes to a hydrophobic pocket and also helps to anchor Mg-ATP, STO-609 has a less effective inhibitory potency (<100-fold) against PKA than does CaM-KK (28). This suggests that the reduced inhibitory potency of this compound against PKA appears to proceed in a totally different manner from that of CaM-KK. According to the present results with various point mutants of Val269 in CaM-KKβ, substitution of Val269 by residues with bulky side chains resulted in effectively increasing IC50 values of the mutants. This result is probably because of interference with the binding of STO-609 to CaM-KK by bulky side chains, because the substitution of Val269 by a residue with a relatively small side chain such as Ala had no impact on inhibition by STO-609. Based on these results, we generated an STO-609-resistant CaM-KKβ mutant (V269F), which was able to induce STO-609 resistance in transfected HeLa cells in terms of the activation of CaM-KK-mediated CaM-KIV activation. Because 510 protein kinases are likely encoded in the human genome (29), it is very difficult to evaluate the specificity of a protein kinase inhibitor, in vitro as well as in vivo. Therefore, the availability of an STO-609-resistant CaM-KKβ mutant could be useful to validate the pharmacological effect(s) and the specificity of a compound in vivo with regard to elucidation of the physiological function(s) mediated by the CaM kinase cascade.

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