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POTENT AND PREFERENTIAL INHIBITION OF Ca^{2+}/ CALMODULIN-DEPENDENT PROTEIN KINASE II BY K252a AND ITS DERIVATIVE, KT5926

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SUMMARY: Effects of protein kinase inhibitors, K252a and its derivaive KT5926, on Ca^{2+}/calmodulin-dependent protein kinase II were examined. Both compounds potently inhibited Ca^{2+}/calmodulin-dependent protein kinase II. Kinetic analyses indicated that the inhibitory effect of K252a and KT5926 was competitive with respect to ATP (Ki : 1.8 and 4.4 nM, respectively) and noncompetitive with respect to the substrates. Taken together with a previous report (Nakanishi et al. Mol. Pharmacol. 37, 482, 1990) concerning the Ki values of these compounds for ATP with various protein kinases, the results suggest that K252a and KT5926 are potent and preferential inhibitors of Ca^{2+}/calmodulin-dependent protein kinase II.

Ca^{2+}/calmodulin (CaM)-dependent protein kinase II (CaM-kinase II) with a wide range of substrates is a member of the family of Ca^{2+}-regulated protein kinases and has been shown to be involved in Ca^{2+}-signal transduction in various cell types (1-4). A particular characteristic of this enzyme is its autophosphorylation which converts it from a Ca^{2+}-dependent form to a partially

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ABBREVIATIONS : CaM, calmodulin ; CaM-kinase II, Ca^{2+}/calmodulin-dependent protein kinase II; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid.

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Ca^{2+}-independent one in vitro (5-8). Generation of the partially Ca^{2+}-independent form by autophosphorylation may have important physiologic consequences by prolonging the effect of a transient elevation of intracellular Ca^{2+}. An interesting function for CaM-kinase II in cells has been suggested by the recent discovery that in vitro, several CaM-biding proteins have a phosphorylation site(s) specific for the Ca^{2+}-independent form of CaM-kinase II (9-13). Such CaM-binding proteins include CaM-kinase II itself (9), calcineurin (10,12), the 63 kDa isoenzyme of cyclic nucleotide phosphodiesterase (11), smooth muscle myosin light chain kinase (13), and phosphorylase kinase (Y. Hashimoto and T. Soderling, unpublished results). Recent reports have shown that CaM-kinase II also undergoes autophosphorylation and becomes partly Ca^{2+}-independent in the cells (14-16).

The physiologic function of CaM-kinase II remains incompletely elucidated, but there is convincing data that the enzyme phosphorylates and regulates tyrosine hydroxylase, synapsin 1 and several enzymes involved in carbohydrate metabolism in situ (3,4). Although specific inhibitors are essential tools for examining the function of CaM-kinase II in cells, to date only KN-62 (17) has been developed for use in situ. In this paper, we report additional relatively selective inhibitors of CaM-kinase II, with an inhibitory mechanism different from that of KN-62 .

MATERIALS AND METHODS

Materials: K252a was isolated from a culture broth of Nocardiopsis sp. (18,19). KT5926 was prepared as described previously (20). Smooth muscle myosin light chain was purified from chicken gizzard according to Hathaway et al.(21). CaM-kinase II(9) and syntide-2 (22) were gifts from Dr. Thomas Soderling (Vanderbilt University, Nashville). [r-^{32}P]ATP and CaM were purchased from NEN and Sigma, respectively.

Activity Assay: The basic kinase assay (20 μl) contained 50 mM HEPES (pH 7.5), 10 mM magnesium acetate, 1 mg/ml bovine serum albumin, 0.4 mM $CaCl_2$, 0.3 μM CaM, 50 μM [r-^{32}P]ATP, 20 μM syntide-2 and 1 nM (subunit concentration) CaM-kinase II. After preincubation of CaM-kinase II with K252a or KT5926 for 3 min at

30°C, the reaction was initiated by addition of [r-^{32}P]ATP. Aliquots of 5 μl were spotted on phosphocellulose paper squares at 1.5 and 3 min, which were then processed as described (23) to determine $^{32}PO_4$ incorporation into syntide-2 or myosin light chain.

RESULTS AND DISCUSSION

In previous papers, Nakanishi et al (20) have reported that K252a was a nonspecific inhibitor of protein kinases. This compound affected the functions of various cells and tissues including platelets (24, 25), mast cells (26), neutrophils (26, 27), basophils (28), PC12 cells, chick embryo dorsal root ganglion cells (30), and smooth muscle strips (31). KT5926 is a K252a derivative developed to inhibit smooth muscle myosin light chain kinase specifically and has been shown to suppress platelet aggregation and serotonin release (20). However, the effects of these compounds on CaM-kinase II have not been studied. Therefore, we first measured the activity of CaM-kinase II in the presence of various concentrations of the inhibitors under the basic conditions described in MATERIALS AND METHODS. K252a and KT5926 both potently inhibited CaM-kinase II activity with an IC_{50} of 2.8 and 5.9 nM, respectively. Similar inhibitory effects were observed when myosin light chain was used as a substrate instead of the peptide substrate syntide-2. To elucidate the mechanism involved in this inhibition of the enzyme activity, K252a and KT5926 were tested for their ability to compete with ATP, syntide-2, or Ca^{2+}/CaM binding to the enzyme. Figures 1 and 2 show double-reciprocal plots of the inhibition of CaM-kinase II activity obtained when the ATP or syntide-2 concentration was varied in the absence or presence of different concentraions of the inhibitors. Inhibition of CaM-kinase II by K252a and KT5926 was competitive with respect to ATP (Fig. 1) with Ki values of 1.8 and 4.4 nM, respectively (Table 1), but noncompetitive with respect to syntide-2 (Fig. 2). In addition, the inhibitory effect

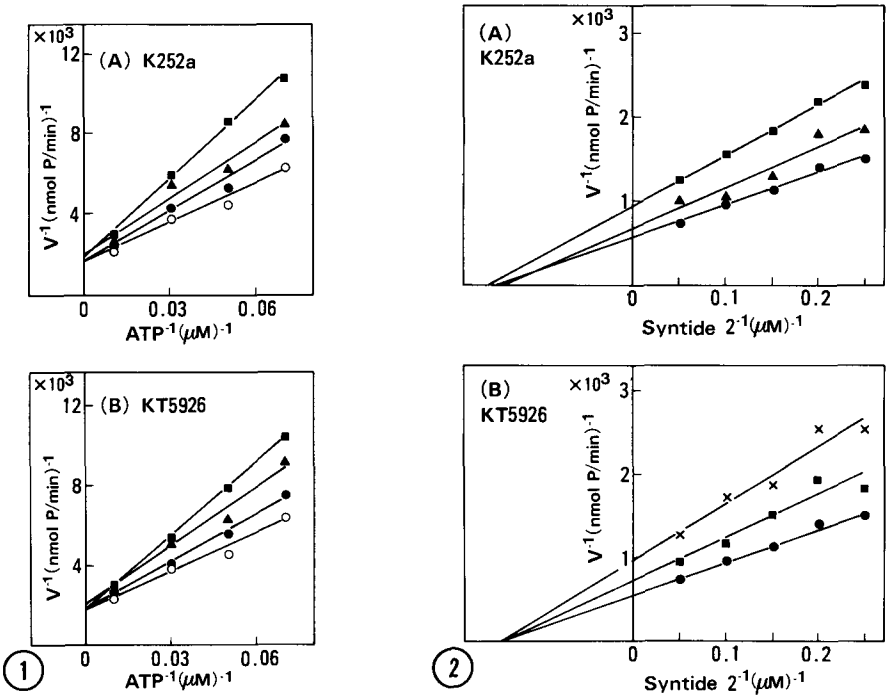

Fig. 1. Effect of the ATP concentration on the inhibition of CaM-kinase II activity by K252a and KT5926. CaM-kinase II activity was measured as described in MATERIALS AND METHODS in the absence (○) or presence of K252a (A: ●, 0.7 nM ; ▲, 1 nM ; ■, 2 nM) and KT5926 (B: ●, 1.3 nM ; ▲, 2 nM ; ■, 4 nM) in the presence of different concentrations (14.3-100 μM) of [r-^{32}P] ATP.

Fig. 2. Effect of the syntide-2 concentration on the inhibition of CaM-kinase II activity by K252a and KT5926. CaM-kinase II activity was measured as described in MATERIALS AND METHODS in the absence (●) or presence of K252a (A: ▲, 1.5 nM ; ■, 3 nM) and KT5926 (B: ■, 3 nM ; X, 6 nM) in the precence of different concentrations of syntide-2 (4-20 μM).

TABLE 1

Ki values of K252a and KT5926 for ATP with various protein kinases

Enzyme	Ki (nM)	
	K252a	KT5926
CaM-kinase II	1.8	4.4
Myosin light chain kinase	20	18
Protein kinase C	25	723
cAMP-dependent protein kinase	18	1200
cGMP-dependent protein kinase	20	158

The Ki values with CaM-kinase II were calculated from the replots of the slopes of the lines shown in Fig. 1 as a function of the K252a and KT5926 concentration. The other Ki values were quoted from Reference 20.

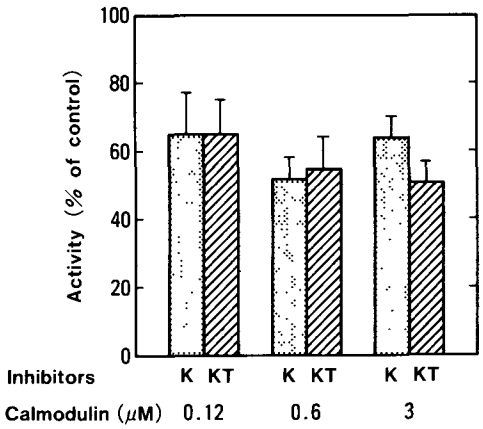

Fig. 3. Effect of the CaM concentration on the inhibition of CaM-kinase II activity by K252a and KT5926. CaM-kinase II activity was measured as described in MATERIALS AND METHODS in the absence or presence of 3 nM K252a and 6 nM KT5926 with different concentrations of CaM. The values are the means ± S.D. of three separate experiments. The enzyme activities without the compounds were 3.5 ± 0.5, 4.2 ± 0.5, and 4.3 ± 0.5 μmol $^{32}PO_4$/min/mg (means ± S.D., n=3) in the presence of 0.12, 0.6 and 3 μM CaM, respectively. K:K252a, KT:KT5926.

of both compounds was not affected by changes in the CaM concentration (Fig. 3), and thus, the inhibition was not competitive with CaM. Table 1 shows Ki values of K252a and KT5926 for ATP with various protein kinases. K252a was 10-fold selective for CaM-kinase II over other protein kinases. Although KT5926 showed only 4-fold selectivity for the enzyme over myosin light chain kinase, the selectivity over protein kinases other than myosin light chain kinase was more than 35-fold.

The only specific inhibitor of CaM-kinase II so far reported is KN-62 (17). Inhibition by KN-62 was competitive with CaM, while that by K252a and KT5926 was not competitive with CaM, but was with ATP. The observations that K252a affects functions of various cells at relatively low concentrations (24-31) suggest that it passes easily through the cell membrane. Utilization of these two inhibitors, K252a and KT5926, in addition to KN62, should therefore be useful for dissecting the the physiologic functions of CaM-kinase II.

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