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The effect of K-252a, a potent microbial inhibitor of protein kinase, on activated cyclic nucleotide phosphodiesterase

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K-252a, an indole carbazol compound of microbial origin, inhibited activation of bovine brain phosphodiesterase induced by calmodulin (CaM), sodium oleate, or limited proteolysis with almost equal potency. Kinetic analysis revealed that the CaM-activated phosphodiesterase (CaM-PDE) was competitively inhibited by K-252a with respect to CaM. On the other hand, inhibition of the trypsin-activated phosphodiesterase was competitive with respect to cyclic AMP. Addition of a lower amount of phosphatidylserine or sodium oleate to the reaction medium was efficacious in attenuating the inhibition of the CaM-PDE by W-7, compound 48/80, or calmidazolium but, in contrast, had no effect on the inhibition by K-252a. Furthermore, CaM-independent systems such as [^{3}H]nitrendipine receptor binding or Na^+ + K^+-ATPase were influenced less by K-252a compared with W-7, compound 48/80 and calmidazolium. In conclusion, K-252a is an inhibitor of CaM-dependent cyclic nucleotide phosphodiesterase and it appears that it inhibits the enzyme not only via CaM antagonism but possibly also by interfering with the enzyme.

INTRODUCTION

The Ca^+ ion plays a crucial role as a second messenger in various biological events [1]. Evidence has been accumulating which suggests that many of the physiological functions of Ca^{2+} may be mediated by Ca^{2+}-receptor proteins such as calmodulin (CaM). CaM indeed activates a number of enzymes in a Ca^{2+}-dependent manner [2], including a cyclic nucleotide phosphodiesterase (CaM-PDE; [3]). A wide range of chemically unrelated pharmacological agents, including naphthalene sulphonamides, phenothiazines, compound 48/80 and calmidazolium, have been shown to inhibit several CaM-dependent enzymes and cellular functions [4]. These so-called CaM antagonists share an amphiphilic (hydrophobic plus cationic) character and interact with some acidic phospholipids and fatty acids. It has been demonstrated for several CaM-dependent enzymes that unsaturated fatty acids, acidic phospholipids and limited proteolysis mimic the effect of CaM [5–8]. It is of interest also that some so-called CaM antagonists inhibit the activation of phosphodiesterase induced by various types of activators other than CaM [9–11]. It appears probable that anionic amphiphile-activated phosphodiesterase may be inhibited by complexation of the activator with the inhibitor via ionic and hydrophobic interactions and for proteolysis-activated enzyme by direct interaction of the inhibitor with the enzyme [9]. Moreover, many so-called CaM antagonists, if not all, also inhibit protein kinase C through a competitive interaction with acidic phospholipid cofactor [12–14].

K-252a, a novel indole carbazol compound isolated from the culture broths of *Nocardiopsis* sp. [15] and *Actinomadula* sp. [16], has proven to be a potent inhibitor of protein kinase. K-252a inhibits several protein kinases, including protein kinase C, cyclic nucleotide-dependent protein kinases and myosin-light-chain kinase competitively with respect to ATP with K_i values of about 20 nM [17,18]. Moreover, micromolar amounts of K-252a, which is a concentration two orders of magnitude higher than that required for the kinase inhibition, has also been shown to inhibit CaM-PDE with an IC_{50} (concentration that produces 50% inhibition of the maximally stimulated activity) value of 2.8 μM [15]. Unlike so-called CaM antagonists, a significant feature of K-252a is its neutral character. Therefore, we were interested in exploring the mechanism by which K-252a inhibits CaM-PDE.

MATERIALS AND METHODS

K-252a (8*R**, 9*S**, 11*S**)-(−)-9-hydroxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7*b*,11a-triazadibenzo[*a*,*g*]cyclo-octa[*c*,*d*,*e*]-trinden-1-one [19], was purified from the culture broth of *Nocardiopsis* sp. as described previously [15]. CaM–Sepharose 4B was purchased from Pharmacia. Bovine heart CaM (~1000 units/mg of protein) and its dependent and independent phosphodiesterases from bovine heart, compound 48/80, L-α-phosphatidyl-L-serine, cyclic AMP, trypsin (Type III) from bovine pancreas, soya-bean trypsin inhibitor (Type 1–5), and Na^+/K^+-ATPase from canine kidney were all obtained from Sigma Chemical Co. W-7·HCl and calmidazolium were obtained from Seikagaku Kogyo Co. (Tokyo, Japan) and Janssen Pharmaceutica respective. [^{3}H]-Nitrendipine (70–87 Ci/mmol) was from New England Nuclear. All other reagents were of analytical grade or the highest purity available.

Bovine brain CaM and CaM-PDE were partially purified using the method of Kakiuchi *et al.* [20], and

Abbreviations used: CaM, calmodulin; Cam-PDE, Ca^{2+}/calmodulin-activated cyclic nucleotide phosphodiesterase; CaM-independent PDE, Ca^{2+}/calmodulin-independent cyclic nucleotide phosphodiesterase.

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were further purified by using CaM–Sepharose 4B to obtain CaM-deficient CaM-PDE and CaM-PDE-deficient CaM [21]. Purity was judged by SDS/polyacrylamide-gel electrophoresis according to the method of Laemmli [22].

Phosphodiesterase activity was determined at 37 °C as described previously [23] using the method of Butcher & Sutherland [24]. This procedure involved coupling of the phosphodiesterase reaction with the 5′-nucleotidase reaction and measuring the P_i liberated within 30 min. The assay medium contained 80 nM-imidazole/HCl buffer, pH 6.9, 3 mM-$MgSO_4$, 0.3 mM-dithiothreitol, 100 mM-NaCl, 1.2 mM-cyclic AMP, and either 50 μM-$CaCl_2$ plus 4 units of CaM/ml or 3 mM-EGTA.

$Na^+ + K^+$-ATPase was assayed according to the method of Huang *et al.* [25]. [^{3}H]Nitrendipine-receptor-binding assay with rat brain membrane was performed basically as described by Gould *et al.* [26].

CaM-PDE was treated with 0.05 mg of trypsin/mg of protein at 30 °C for 12 min, after which the reaction was terminated by addition of a 22-fold molar excess of soyabean trypsin inhibitor. The activity of the trypsin-treated enzyme prepared by this procedure was essentially identical with the value obtained with CaM and was not further increased by CaM. Sodium oleate (1 mM) also stimulated the enzyme activity up to 65% of the CaM-dependent activation.

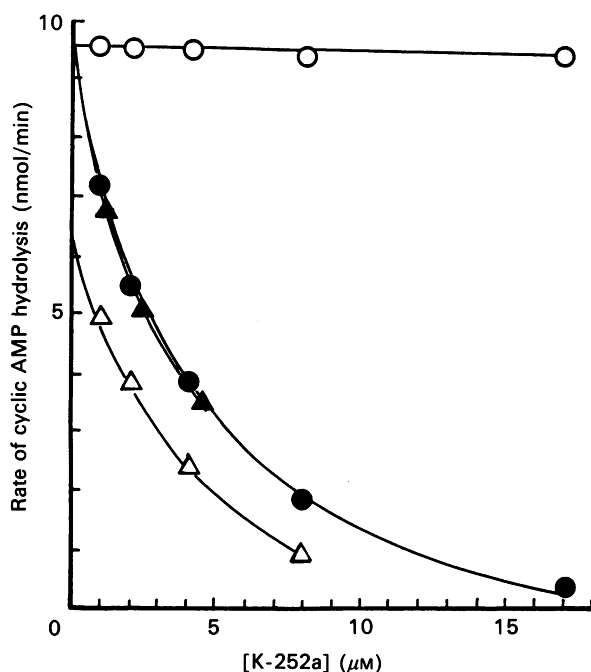

Fig. 1. Inhibition of activated phosphodiesterase by K-252a

Bovine brain CaM-PDE (26 munits/ml, unit definition was carried out in the presence of CaM) was activated either by CaM (●, 4 units/ml), sodium oleate (△, 1 mM), or limited tryptic digestion (▲, for 12 min at 30 °C with 50 μg of trypsin/mg of enzyme). The basal activity (○) was determined using a large amount of the enzyme (18.6 munits/ml) to magnify the phosphodiesterase activity in the presence of 3 mM-EGTA instead of Ca^{2+} plus CaM. For experimental details see the text. Each point represents the mean value of three samples from the same batch of enzyme. The basal and stimulated activities of phosphodiesterase by calmodulin, limited proteolysis, or sodium oleate all correspond to 9.3 nmol/min in the absence of K-252a. The sodium oleate-activated phosphodiesterase activity in the absence of K-252a corresponds to 6.3 nmol/min.

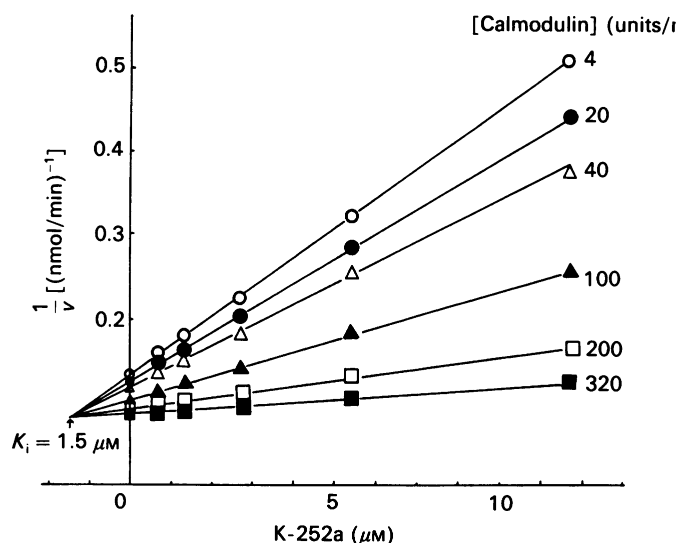

Fig. 2. Dixon plots for the inhibition of CaM-activated phosphodiesterase by K-252a

Bovine brain CaM-PDE (26 munits/ml) was assayed in the presence of different concentrations of bovine brain CaM along with varied amounts of K-252a. For experimental details see the text. [CaM] used were (units/ml): 4 (○), 20 (●), 40 (△), 100 (▲), 200 (□) and 320 (■). Each point represents the mean value of three samples from the same batch of enzyme.

Phosphatidylserine microdispersions were prepared by sonication in a Branson Sonifier B22 for 20 s in 80 mM-imidazole/HCl buffer, pH 6.9.

A methanol solution of K-252a (2 mM) was prepared freshly and was diluted with water just before use. The final concentration of methanol was $< 0.1\%$ (v/v), a concentration that had no effect on phosphodiesterase activity. Methanol at a similar concentration was used in the control.

Protein was determined by the method of Bradford [27], with bovine serum albumin as a standard. The IC_{50} was determined using six concentrations of each inhibitor and expressed as the mean value of three replicate experiments using one batch of enzyme.

RESULTS

Bovine brain CaM-PDE could be maximally activated 7–13-fold by CaM in the absence of inhibitory drugs. The effect of K-252a on the activation of bovine brain CaM-PDE induced by CaM, limited proteolysis or sodium oleate (1 mM) is shown in Fig. 1. K-252a is equally efficacious in inhibiting all types of activations. IC_{50} values for the three forms of activated enzyme were 2.8, 2.3 and 2.6 μM respectively. In contrast, the basal activity was not suppressed at all at concentrations up to 100 μM and was inhibited half-maximally by K-252a at a concentration of 260 μM. To obtain evidence for the involvement of CaM in the inhibition of CaM-PDE by K-252a, the enzyme activity was measured in the presence of different concentrations of CaM along with varying amounts of

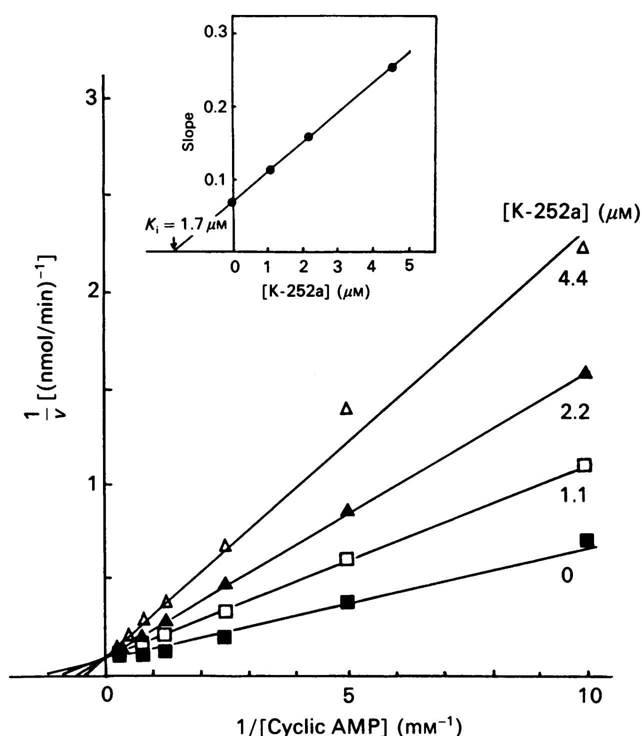

Fig. 3. Double-reciprocal plots of the inhibition of trypsin-activated phosphodiesterase by K-252a

Tryptic digestion was carried out for 12 min at 30 °C with 50 μg of trypsin/mg of enzyme. The activity of the phosphodiesterase (26 munits/ml) was assayed in the presence or absence of different concentrations of K-252a along with varied amounts of cyclic AMP. For experimental details see the text. K-252a concentrations were used: vehicle (■), 1.1 μM (□), 2.2 μM (▲), and 4.4 μM (△). Inset shows a secondary plot of the slope of the lines as a function of K-252a concentration.

K-252a. Fig. 2 shows the kinetic analysis of K-252a-induced inhibition of CaM-activated phosphodiesterase by means of Dixon plots [28]. The data are compatible with the interpretation that K-252a inhibited CaM-PDE in a competitive manner with respect to CaM; K_i value for K-252a was 1.5 μM. However, increasing [Ca^{2+}] showed no effect on K-252a-induced inhibition of CaM-activated phosphodiesterase (results not shown).

We next examined the effect of varying the amount of cyclic AMP on the trypsin-activated CaM-PDE, which had lost its sensitivity to CaM. Fig. 3 shows the kinetic analysis of K-252a-induced inhibition of trypsin-activated CaM-PDE by means of a double-reciprocal plot. As can be seen from this plot, the inhibition by K-252a of the trypsin-activated enzyme was competitive with respect to cyclic AMP, suggesting that the binding site for K-252a on the enzyme may be closely related to the active centre; K_i value for K-252a was estimated to be 1.7 μM from secondary plots. Taken together the results suggest that K-252a inhibits the CaM-induced activation of CaM-PDE by interfering directly with the enzyme as well as by CaM antagonism.

To investigate whether or not K-252a interacts with anionic amphiphiles, a smaller amount of either phosphatidylserine (100 μg/ml) or sodium oleate (15.6 μM) was added to the reaction medium. At these concentrations phosphatidylserine and sodium oleate showed no effect on the phosphodiesterase activity seen in the absence of K-252a. Since K-252a is a neutral compound, unlike so-called CaM antagonists, no interaction of K-252a with these anionic amphiles was expected. Indeed, as shown in Fig. 4, phosphatidylserine and sodium oleate had no effect on the inhibition by K-252a of CaM-PDE but, in contrast, attenuated the inhibition by W-7, compound 48/80 or calmidazolium, which share cationic amphiphilic properties. This implies that K-252a, unlike so-called CaM antagonists, does not interact with phosphatidylserine and sodium oleate. Therefore, it is suf-

Table 1. Effects of various drugs on CaM-dependent and CaM-independent systems

Bovine brain CaM-PDE (26 munits/ml) activity and bovine heart CaM-PDE (40 munits/ml) activity were assayed in the presence of 4 units and 2.5 units/ml of CaM, respectively, with 50 μM-$CaCl_2$. The basal activity of bovine brain CaM-PDE (18.6 munits/ml) and bovine heart CaM-independent PDE activity (25 munits/ml) were assayed in the presence of 3 mM-EGTA, but without CaM and $CaCl_2$. Trypsin-activated enzyme (26 munits/ml) and sodium oleate (1 mM)-activated enzyme (26 munits/ml) activities were assayed in the absence of CaM and $CaCl_2$. For the experimental details see the text. Results are means of three independent determinations using one batch of enzyme or membrane preparation and are expressed as IC_{50} (μM), except for compound 48/80 (*, μg/ml). > signifies that considerable inhibition by the drug was not observed at this final concentration. NT, not tested.

	IC_{50} (μM, * = μg/ml)							
	Bovine brain CaM-PDE							
Drug	CaM-stimulated activity	Basal activity	Trypsin-activated activity	Sodium oleate-activated activity	Bovine heart CaM-PDE	Bovine heart CaM-independent PDE	[^{3}H]-Nitrendipine receptor binding	Canine kidney $Na^+ + K^+$-ATPase
K-252a	2.8	260	2.3	2.6	1.3	97.5	> 110	> 200
W-7	42.0	> 1000	> 200	> 100	43.7	> 1000	37.8	270
Compound 48/80	1.1*	> 500*	NT	> 50*	0.7*	500*	38*	50*
Calmidazolium	0.003	> 1000	> 0.1	> 10	0.05	4.4	0.3	2.4

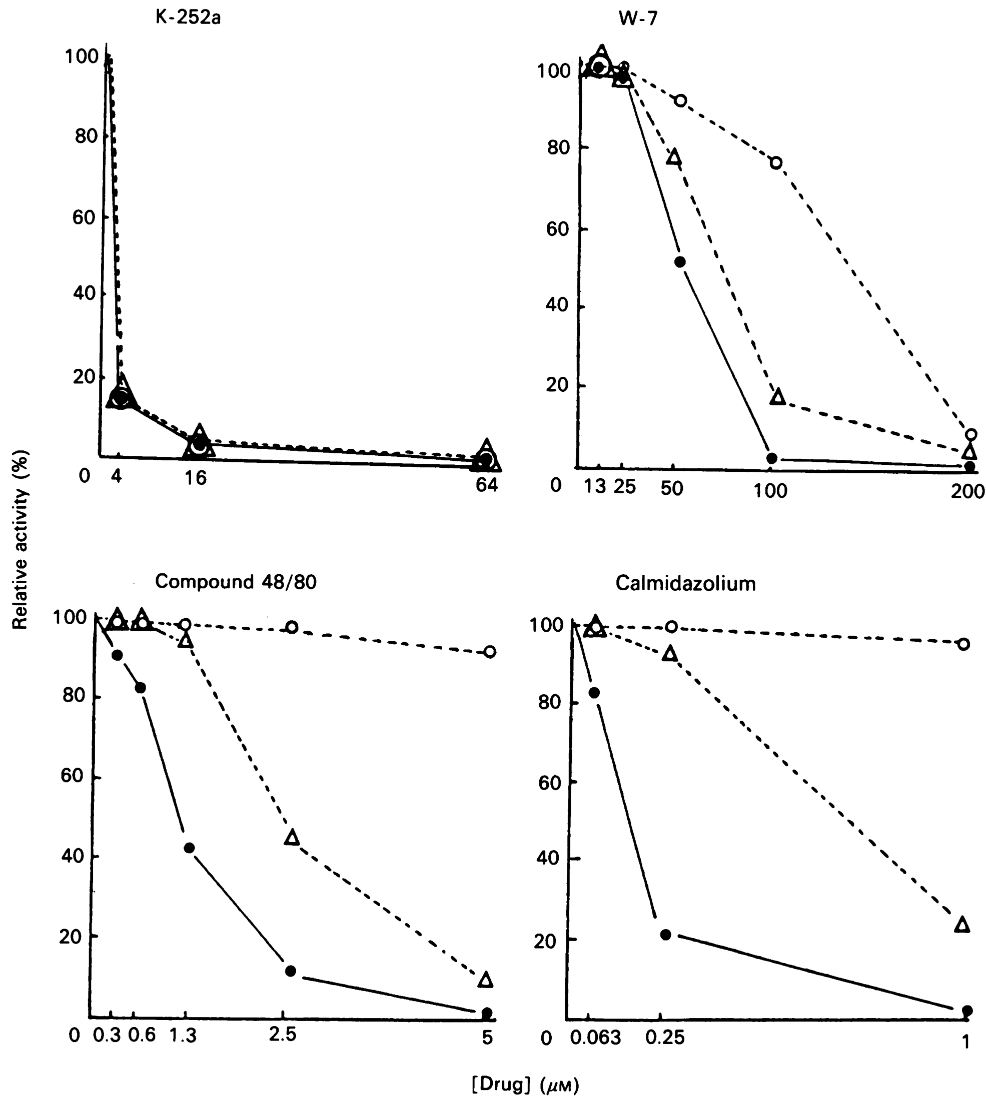

Fig. 4. Effect of phosphatidylserine and sodium oleate on the inhibition by various inhibitors of bovine brain CaM-PDE

CaM-PDE activity was measured with various concentrations of one of the following drugs in the presence of either 100 μg of phosphatidylserine/ml (○) or 15.6 μM-sodium oleate (△): K-252a, W-7, compound 48/80 and calmidazolium. Control (●) experiments were conducted in a similar manner, but without phosphatidylserine and sodium oleate. Phosphatidylserine was microdispersed by sonication. Sodium oleate was added to reaction medium in the dissolved state. For experimental details see the text. Each point represents the mean value of three samples from the same batch of enzyme. 100% activity of the CaM-stimulated phosphodiesterase activity corresponds to 9.3 nmol/min per tube.

ficient to understand that the inhibition of sodium oleate-activated CaM-PDE by K-252a, which is shown in Fig. 1, was brought about by direct interaction of K-252a with the enzyme, but not by interaction with sodium oleate.

Table 1 summarizes the effects of K-252a, W-7, compound 48/80 and calmidazolium on several CaM-dependent and -independent systems. K-252a inhibited bovine heart and brain CaM-PDE to a similar degree. However, inhibition of bovine heart CaM-independent PDE required considerably higher concentrations of K-252a. So-called CaM antagonists, such as W-7, compound 48/80 and calmidazolium, inhibited the bovine brain CaM-dependent fraction of the CaM-PDE activity without suppression of its basal activity. The IC_{50} values were in the same range as those reported previously for anti-CaM [4]. These compounds also inhibited bovine heart and brain CaM-PDE to a similar degree, but showed a lower inhibitory effect on bovine heart CaM-independent PDE. So-called CaM antagonists affected neither trypsin-activated CaM-PDE nor sodium oleate-activated CaM-PDE under the present assay conditions. Because some CaM antagonists had been shown to inhibit [^{3}H]nitrendipine receptor binding [29] and CaM-independent $Na^+ + K^+$-ATPase [30], we examined the effect of the drugs tested in the present study on these two CaM-independent systems. K-252a inhibited neither [^{3}H]nitrendipine receptor binding in rat brain membrane

nor canine kidney $Na^{+}+K^{+}$-ATPase at the concentrations indicated, whereas so-called CaM antagonists clearly did (Table 1).

DISCUSSION

K-252a inhibits protein kinases at nanomolar concentrations by competing for ATP in a rather non-specific way [17,18], whereas it inhibits CaM-PDE at micromolar concentrations [15]. The data in this paper clearly show that K-252a, under the present conditions, does not inhibit CaM-PDE solely by CaM antagonism, but may also interfere directly with the enzyme. Moreover, the binding site on the enzyme is at, or near to, the active centre, since K-252a inhibited trypsin-activated phosphodiesterase in a competitive manner with respect to cyclic AMP.

There have been several papers on the direct interaction of CaM antagonists with CaM-regulated enzymes. Itoh & Hidaka [11] showed that trypsin-treated CaM-PDE was inhibited by various CaM antagonists, such as trifluoperazine, chlorpromazine and a series of W-7 analogues. Furthermore, penfluridol, calmidazolium and trifluoperazine seem to bind, as well as CaM, to erythrocyte $Ca^{2+}+Mg^{2+}$-ATPase, a CaM-regulated enzyme, since activation by both tryptic digestion and CaM can be completely inhibited in a similar concentration range [9,10]. More recently, Xu *et al.* [31] reported that a derivative of bisbenzylisoquinoline also inhibited trypsin-activated $Ca^{2+}+Mg^{2+}$-ATPase at concentrations similar to those that inhibit the activation by CaM. These results suggest that there are binding sites for CaM antagonists on trypsin-activated CaM-regulated enzymes which have similar features to those on CaM.

Although K-252a inhibits CaM-PDE in a competitive manner with respect to CaM, the precise mechanism for this CaM antagonism is still a matter of speculation and study. There are two possible mechanisms that might explain how K-252a could competitively inhibit the CaM-induced activation of CaM-PDE. K-252a could either interact directly with CaM itself or it could compete with CaM for the CaM-recognition site on the CaM-sensitive enzyme. We used hydrophobic fluorescent probes [32] to obtain information regarding the binding properties of K-252a to CaM; however, K-252a could not be studied because of its intrinsic fluorescence. Studies of the direct interaction of K-252a with CaM using other techniques will be undertaken. Of the above two explanations, the former appears to be a possibility since preliminary results, obtained using the equilibrium-binding technique on gel filtration, suggest that K-252a clearly binds to CaM (results not shown). For comparison, we examined the effect of K-252a along with the reference CaM antagonists W-7, compound 48/80 and calmidazolium on several CaM-independent systems. In contrast with K-252a, so-called CaM antagonists, under the present assay conditions, did not inhibit the activation of CaM-PDE by limited proteolysis. Our results agree with previous findings reported by Gietzen *et al.* [9], but not with those by Itoh & Hidaka [11]. Based on the explanation by Itoh & Hidaka [11], this discrepancy might be due to differences in the concentration of the substrate.

Unlike so-called CaM antagonists, a significant feature of K-252a is its neutral character. K-252a does not interact with anionic amphiphiles, such as phosphatidylserine and sodium oleate. Although it is uncertain whether this property of K-252a is related to cell-membrane permeability, K-252a could probably penetrate cell membranes very well. Indeed, K-252a shows the following pharmacological activities in some intact cells or tissues: inhibition of secretory responses in platelets, mast cells and neutrophils [33–35]; inhibition of contractile response in smooth muscle [36]; and specific inhibition of the action of nerve growth factor on PC12 cells [37] and on chick embryo dorsal-root-ganglion cells [38].

In conclusion, K-252a was shown to be an inhibitor of CaM-PDE in that: (1) unlike so-called CaM antagonists, K-252a is a neutral compound; (2) it does not interact with phosphatidylserine and sodium oleate; (3) it is equally effective at antagonizing all types of activation (CaM or sodium oleate mediated or tryptic digestion), suggesting that it inhibits CaM-PDE phosphodiesterase not only by CaM antagonism but possibly also by interfering directly with the enzyme; and (4) and importantly, in contrast with so-called CaM antagonists, it does not inhibit [^{3}H]nitrendipine binding and CaM-independent $Na^{+}+K^{+}$-ATPase.

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