Biochemical Pharmacology, Vol. 39, No. 5, pp. 841–849, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

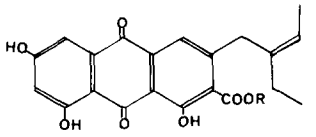
INHIBITION BY NEW ANTHRAQUINONE COMPOUNDS, K-259-2 AND KS-619-1, OF CALMODULIN-DEPENDENT CYCLIC NUCLEOTIDE PHOSPHODIESTERASE

YUZURU MATSUDA,* SATOSHI NAKANISHI, KEIKO NAGASAWA and HIROSHI KASE
Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Ltd, 3-6-6 Asahimachi, Machida-shi, Tokyo 194, Japan

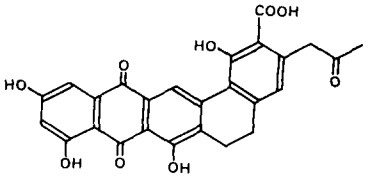
(*Received* 16 *November* 1988; *accepted* 10 *October* 1989)

Abstract—K-259-2 and KS-619-1, novel anionic anthraquinone metabolites isolated from culture broth of microorganisms, inhibited activation of bovine brain phosphodiesterase induced by calmodulin (CaM), sodium oleate, or limited proteolysis with almost equal potency. The inhibition of calmodulin-activated phosphodiesterase (CaM-PDE) by K-259-2 or KS-619-1 was overcome by a higher concentration of CaM. Direct interaction of K-259-2 and KS-619-1 with CaM was confirmed through use of hydrophobic fluorescent probes. Kinetic analysis revealed that the inhibition of the trypsin-activated phosphodiesterase was competitively inhibited by K-259-2 or KS-619-1 with respect to cAMP. Addition of a lower amount of either phosphatidylserine or sodium oleate to the reaction mixture was efficacious in attenuating the inhibition of the CaM-PDE by W-7, chlorpromazine, trifluoperazine, compound 48/80, or R-24571 but, in contrast, had little or no effect on the inhibition by K-259-2 or KS-619-1. In conclusion, K-259-2 and KS-619-1, unlike so-called CaM antagonists, do not interact with phosphatidylserine or sodium oleate and it appears that these novel anthraquinone compounds inhibit the enzyme not only via CaM antagonism but possibly also by interacting directly with the enzyme.

Calcium 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+}-binding 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]). The ability to alter the actions of these various CaM-regulated enzymes is a necessary part of any effort to dissect the physiological functions of CaM in Ca^{2+}-messenger systems in a biochemical sense. A wide range of chemically-unrelated pharmacological agents, which generally share an amphiphilic (hydrophobic plus cationic) character [4], have been shown to inhibit several CaM-dependent enzymes and cellular functions; these compounds include naphthalene sulphonamides, phenothiazines, compound 48/80 and R-24571. 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 the phosphodiesterase induced by various types of activators other than CaM [9–12]. The details of the inhibitory action, however, are not clear as yet, but it appears probable that anionic amphiphile-activated phosphodiesterase may be inhibited by binding to the activator with the inhibitor via ionic and hydrophobic interactions and for trypsin-activated enzymes by direct interaction of the inhibitor with the enzyme [9].



K-259-2 ; R=H
K-259-3 ; R=CH_3



KS-619-1

Fig. 1. The structures of K-259-2, K-259-3 and KS-619-1.

K-259-2 and KS-619-1 (Fig. 1), novel anthraquinone metabolites isolated from the culture broth

* To whom all correspondence should be addressed.

† Abbreviations used: CaM, calmodulin; CaM-PDE, Ca^{2+}/calmodulin-activated cyclic nucleotide phosphodiesterase; CaM-independent PDE, Ca^{2+}/calmodulin-independent cyclic nucleotide phosphodiesterase; EGTA, ethylene bis(oxyethylenenitrilo) tetraacetic acid; SDS, sodium dodecyl sulfate; ATP, adenosine triphosphate; TNS, 2-*p*-toluidinyl-naphthalene-6-sulfonic acid; NPN-*n*-phenyl-1-naphthylamine.

of *Micromonospora olivasterospora* and *Streptomyces californicus*, respectively, have been found to be potent inhibitors of bovine brain CaM-PDE [13, 14]. Because these compounds have both hydrophobic and anionic properties, unlike so-called CaM antagonists, we were interested in exploring the mechanism of the CaM-PDE inhibition by KS-619-1 and K-259-2, and of K-259-3, a methyl ester of K-259-2.

MATERIALS AND METHODS

Materials. K-259-2 (3-(2Z-2-ethyl-2-butenyl)-1,6,8-trihydroxyanthraquinone-2-carboxylic acid [15] and KS-619-1(8,13-dioxo-3-(2-oxopropyl)-5,6,8,13- tetrahydro- 1,7,9,11- tetrahydroxybenz[*a*]-naphtacene-2-carboxylic acid [16]) were purified from the culture broth of *M. olivasterospora* and *S. californicus*, respectively, as described previously [13, 14]. K-259-3, a methyl ester of K-259-2, was prepared and was provided by Dr C. Murakata in our laboratories. CaM (28,000 units/mg protein) used for fluorescence measurements was from Fluka Chemie AG, (Buchs, Switzerland). CaM-Sepharose 4B was purchased from Pharmacia (Uppsala, Sweden). Bovine heart CaM (*ca.* 1000 units/mg protein), and CaM-PDE and CaM-independent PDE from bovine heart were obtained from the Sigma Chemical Co. (Poole, U.K.). Chlorpromazine, trifluoperazine-2HCl, compound 48/80, verapamil-HCl, 5′-nucleotidase (from *Crotalus atrox* venom), L-α-phosphatidyl-L-serine, cAMP, trypsin (TypeIII) from bovine pancreas and trypsin inhibitor (Type 1–5) from soybean were all purchased from Sigma. W-7-HCl and R-24571 (Calmidazolium) were from the Seikagaku Kogyo Co. and Janssen Pharmaceutica, respectively. NPN(*n*-phenyl-1-naphthylamine) and TNS(2-*p*-toluidinyl-naphthalene-6-sulfonic acid) were purchased from the Tokyo Kasei Kogyo Co. (Tokyo, Japan) and Nakarai Chemical Ltd, (Tokyo, Japan), respectively. All other chemicals were analytical grade.

Assay of phosphodiesterase activity. Phosphodiesterase activity was assayed at 37° as described previously [17]. This procedure involved the coupling of the phosphodiesterase reaction with the 5′-nucleotidase reaction and measuring the P_i produced within 30 min. The standard incubation mixture (0.5 mL) contained, unless otherwise indicated, 80 mM imidazole–HCl buffer (pH 6.9), 3 mM $MgSO_4$, 0.3 mM dithiothreitol, 100 mM NaCl, 1.2 mM cAMP, and either 50 μM $CaCl_2$ plus 4 units/mL CaM or 3 mM EGTA.

Fluorescence measurements. Fluorescence measurements were carried out at room temperature using a Hitachi 650-105 Fluorescence Spectrophotometer equipped with Hitachi 561 Recorder. Samples were prepared in a total volume of 3 mL of 10 mM Tris–HCl buffer (pH 7.5), containing 0.16 μM CaM (bovine brain, Fluka AG), 0.2 mM $CaCl_2$ and either 8 μM *n*-phenyl-1-naphthylamine (NPN) or 40 μM 2-*p*-toluidinyl-naphthalene-6-sulfonate (TNS), potassium salt, and drugs to be tested. NPN was dissolved in 100% ethanol and added to cuvettes. Excitation was at 360 nm for both probes, and emission intensity was measured from 360–500 nm.

Miscellaneous methods. Bovine brain CaM and CaM-PDE were partially purified using the method of Kakiuchi *et al.* [18], and were further purified by using CaM-Sepharose 4B to obtain CaM-deficient CaM-PDE and CaM-PDE-deficient CaM [19]. Purity was judged by SDS–gel electrophoresis according to the method of Laemmli [20]. Protein concentration was determined by the dye-binding method using Bio-rad reagents. Trypsin treatment of CaM-PDE was performed according to the method of Kincaid *et al.* [21] with some modifications. In brief, purified CaM-PDE (60 μg/mL) was incubated with 3 μg/mL of trypsin in 80 mM imidazole–HCl buffer (pH 6.9), containing 3 mM $MgSO_4$, 0.3 mM dithiothreitol and 100 mM NaCl. After incubation at 30° for 12 min, the reaction was terminated by addition of a 22-fold molar excess of soy-bean trypsin inhibitor. The activity of the trypsin-treated enzyme prepared by this method was essentially identical with the value obtained with CaM and was not further increased by CaM. Phosphatidylserine microdispersions were prepared freshly by sonication (Branson Sonifier B12) for about 20 sec in 80 mM imidazole–HCl buffer (pH 6.9). Sodium oleate (1 mM) also stimulated the enzyme activity up to 65% of the CaM-dependent activation. All drugs used in the present study were solubilized in water, except for K-259-2, K-259-3, KS-619-1 and R-24571, which were first dissolved in methanol and then diluted to the appropriate concentrations with water. To determine the effect of various drugs on the enzyme activities, either the drug solution (50 μL) or its corresponding vehicle in the same volume was added to the reaction medium with vigorous mixing to give the indicated final concentration. The actual effect induced by the drug was corrected by subtracting that the amount of change caused by vehicle above. The IC_{50} value, which was determined as that concentration of drug that produced 50% inhibition of the maximally-stimulated enzyme activity, was determined using six concentrations of each inhibitor and was expressed as the mean value of three replicate experiments using a single batch of enzyme.

RESULTS

The effect of K-259-2, K-259-3 and KS-619-1 on activated CaM-PDE

Bovine brain CaM-PDE could be maximally activated 7–13-fold by CaM in the absence of inhibitory drugs. The effect of K-259-2, K-259-3 and KS-619-1 on the activation of bovine brain CaM-PDE induced by CaM, limited proteolysis or sodium oleate (1 mM) is shown in Fig. 2. These compounds are equally efficacious in inhibiting all types of activations. K-259-2, K-259-3 and KS-619-1 inhibited CaM-stimulated PDE activity in a dose-dependent manner with IC_{50} values of 6.6, 5.0 and 2.0 μM, respectively, whereas these compounds showed a lesser degree of inhibition on its basal activity with IC_{50} values of 27.5, 126 and 12.3 μM, respectively. When the ratio of the IC_{50} value of basal PDE activity to that of the CaM-stimulated activity is used to monitor the specificity

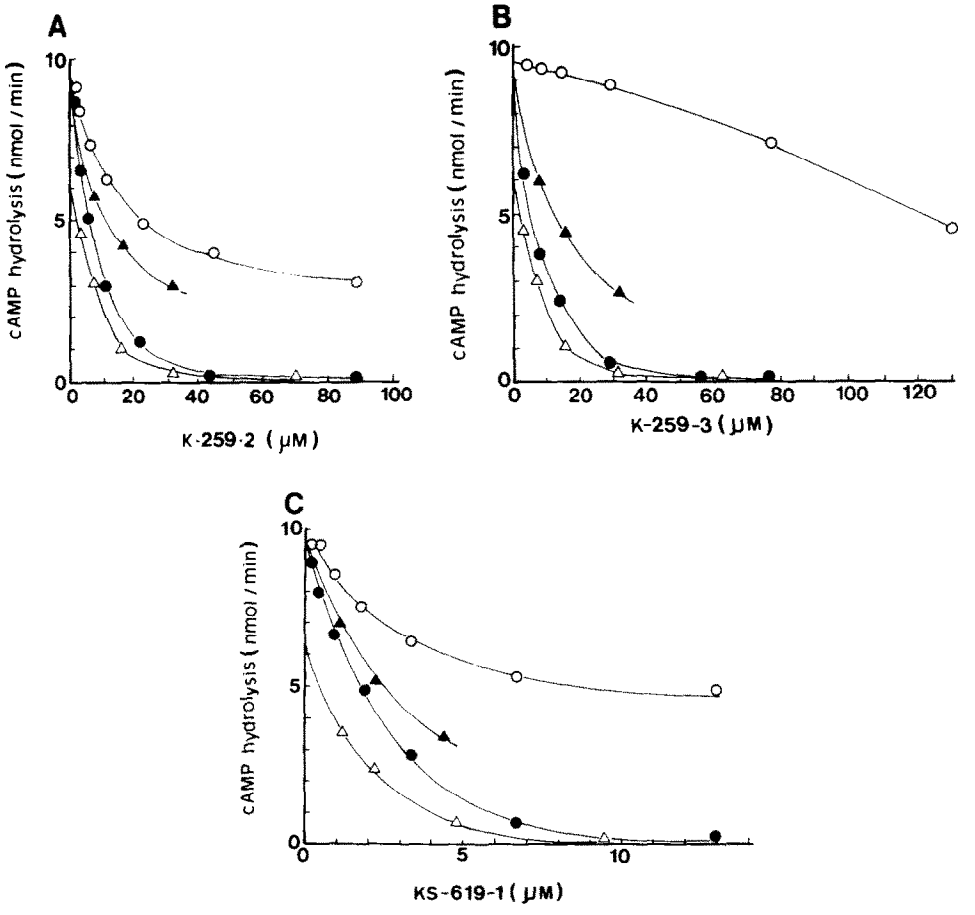


Fig. 2. Inhibition of activated phosphodiesterase by K-259-2(A), K-259-3(B) or KS-619-1(C). Bovine brain CaM-PDE (26 mUnits/mL, unit definition was done in the presence of CaM) was activated either by CaM (●, 4 units/mL), sodium oleate (△, 1 mM), or limited trypic digestion (▲, for 12 min at 30° with 50 μg of trypsin per mg of enzyme protein). With sodium oleate, the phosphodiesterase was stimulated to about 65% of the activity induced by a saturating amount of CaM. Trypsin digestion was stopped with a 22-fold molar excess of trypsin inhibitor; activity of the trypsin-treated enzyme assayed in the absence of CaM was essentially identical to that of the control enzyme assayed with CaM. The basal activity (○, 18.6 mUnits/mL) was assayed in the presence of 3 mM EGTA, but without Ca^{2+} plus CaM. Phosphodiesterase activity was measured as described in Materials and Methods with various concentrations of drugs to be tested. Each point represents the mean value of three samples from the same batch of enzyme.

of the drug used to inhibit the CaM-induced stimulation, it can be seen that the specificity ratio of 25.3 for K-259-3, a methyl ester of K-259-2, is higher than the ratios of 4.2 and 6.2 for K-259-2 and KS-619-1, respectively. K-259-2, K-259-3 and KS-619-1 inhibited oleate-activated phosphodiesterase at concentrations similar to those that inhibit CaM-stimulated activity, with IC_{50} values of 8.0, 7.8 and 1.4 μM, respectively.

Mechanism of action of K-259-2, K-259-3 and KS-619-1 on the function of CaM

To obtain evidence for the involvement of CaM in the inhibition of CaM-PDE by K-259-2, K-259-3 or KS-619-1, the enzyme activity was measured in the presence of different concentrations of CaM along with varying amounts of drugs. CaM reversed the inhibition by K-259-2, K-259-3, and KS-619-1 of CaM-PDE (Fig. 3). In the presence of 320 units/mL of CaM, these novel anthraquinone compounds inhibited the enzyme activity only weakly. However, any sound conclusions such as a competitive manner could not be drawn from the kinetic analysis of the actual data. On the other hand, increasing calcium concentration affected neither K-259-2-induced inhibition, K-259-3-induced inhibition, nor KS-619-1-induced inhibition of CaM-PDE activation (data not shown).

To obtain information regarding the binding of these compounds to CaM, we used hydrophobic fluorescent probes such as NPN and TNS. In the presence of Ca^{2+}, the intensity of the fluorescence of these probes was greatly increased in the presence of CaM (Fig. 4). The addition of 1 mM EGTA reversed the increase in the fluorescence. K-259-2 also prevented the increase in the fluorescence of

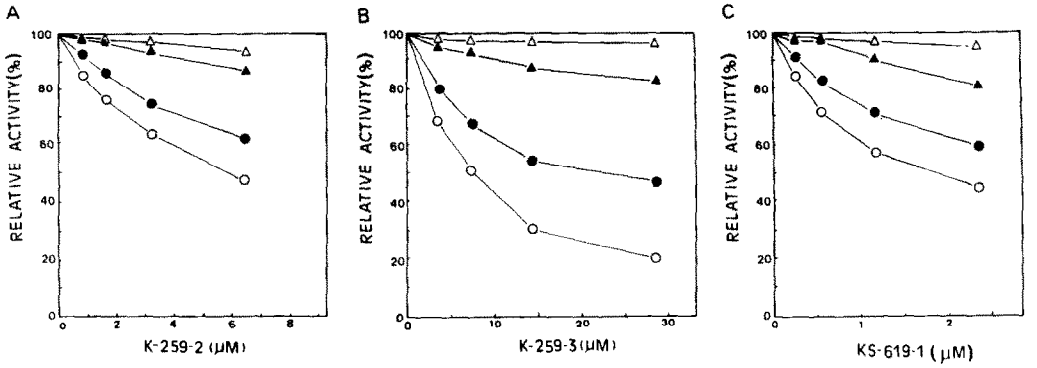


Fig. 3. Effect of CaM on the inhibition of CaM-PDE by K-259-2(A), K-259-3(B) or KS-619-1(C). Bovine brain phosphodiesterase (26 mUnits/mL) activity was measured as described in Materials and Methods in the presence of different concentrations of bovine brain CaM along with varied amounts of drugs to be tested and 1.2 mM cAMP. CaM concentrations used were: 0 units/mL (△), 4 units/mL (○), 40 units/mL (●), and 320 units/mL (▲). Each point represents the mean value of three samples from the same batch of enzyme.

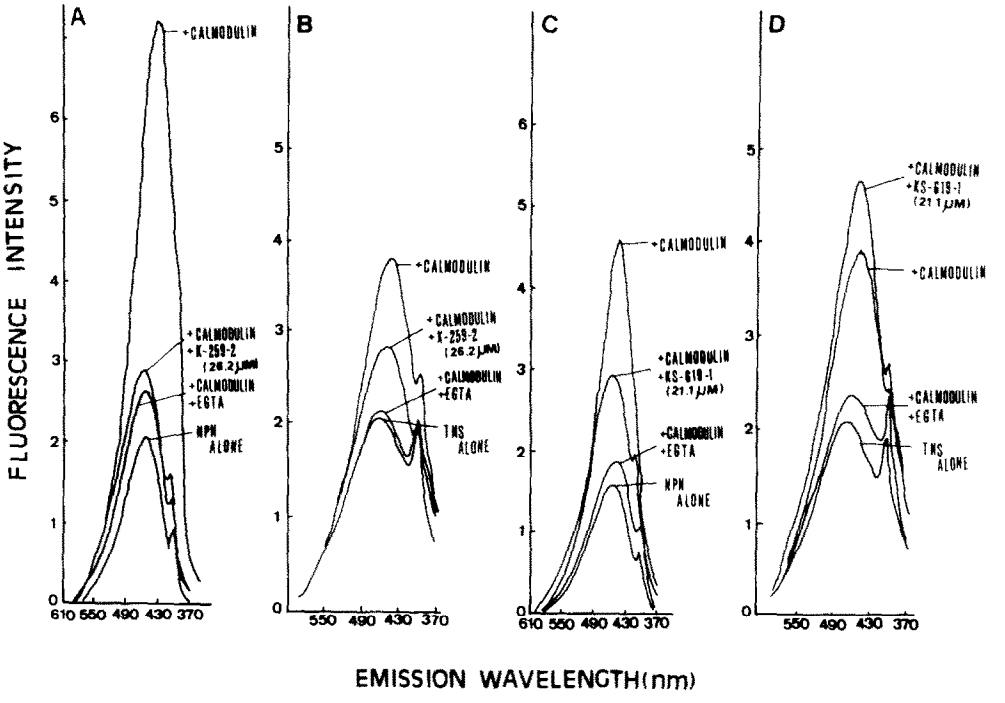


Fig. 4. Effect of K-259-2 (A and B) and KS-619-1 (C and D) on the fluorescence of NPN (A and C) and TNS (B and D) bound to CaM. CaM (0.16 μM) was incubated with 8 μM NPN or 40 μM TNS in 10 mM Tris–HCl buffer (pH 7.5) containing 0.2 mM $CaCl_2$. In some experiments, spectra were recorded in the presence of 1 mM EGTA. Fluorescent spectra were recorded as described in Materials and Methods. Excitation was at 360 nm for both probes and emission intensity was measured from 350–600 nm. The inhibitors itself had no effect on the fluorescence of NPN and TNS in the absence of CaM or in the presence of CaM and EGTA.

both NPN and TNS (Fig. 4A and B). KS-619-1 prevented the increase in NPN fluorescence (Fig. 4C); in contrast, KS-619-1 further increased TNS fluorescence (Fig. 4D). The inhibitor itself had no effect on the fluorescence of NPN and TNS in the absence of CaM or in the presence of CaM and EGTA. These results indicate that K-259-2 and KS-619-1 can interact directly with CaM although their mode of interaction may be somewhat different.

Interaction of K-259-2, K-259-3 and KS-619-1 with the enzyme

We next examined the effect of varying the amount of cAMP on the CaM-induced activation of PDE and on its inhibition by K-259-2 and KS-619-1. However, the actual phenomena could not be analysed sufficiently or accurately (data not shown). We then investigated trypsin-treated enzyme, which had lost its sensitivity to CaM. As shown in Fig. 2, K-259-2,

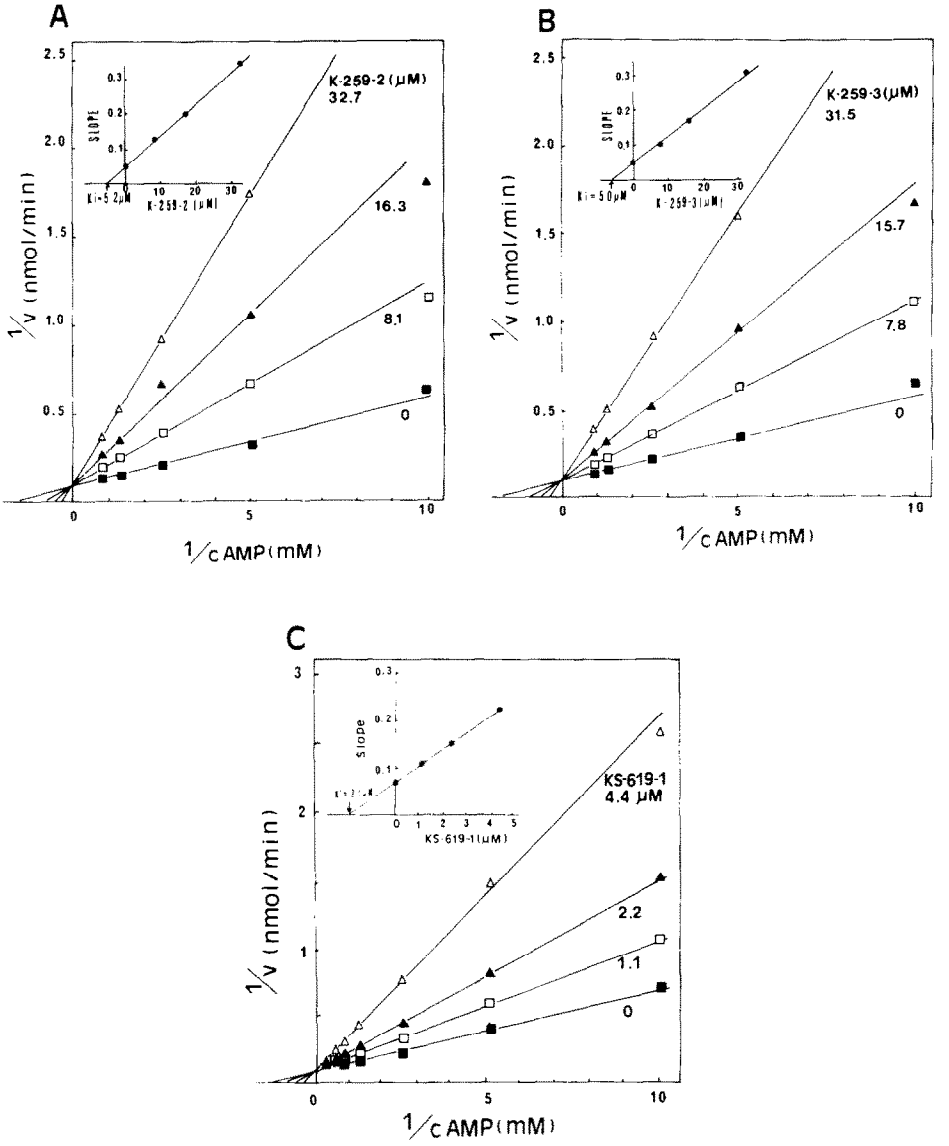


Fig. 5. Double-reciprocal plots of the inhibition of trypsin-activated phosphodiesterase by K-259-2 (A), K-259-3 (B) or KS-619-1(C). Trypsin-activated bovine brain phosphodiesterase was prepared as described in Materials and Methods. The activity of the phosphodiesterase (26 mUnits/mL) was assayed in the presence or absence of different concentrations of K-259-2(A), K-259-3(B) or KS-619-1(C), along with varied amounts of cAMP. K-259-2 concentrations used were: vehicle (■), 8.1 μM (□), 16.3 μM (▲) and 32.7 μM (△). K-259-3 concentrations used were: vehicle (■), 7.8 μM □), 15.7 μM (▲) and 31.5 μM (△). KS-619-1 concentrations used were: 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 the drug concentration.

K-259-3 and KS-619-1 also inhibited trypsin-activated enzyme in the same concentration range as they did the CaM-activated enzyme. The IC_{50} values for these compounds were 16.2, 12.6 and 2.5 μM, respectively. Figure 5 shows the kinetic analysis of the inhibition of trypsin-activated phosphodiesterase by means of double reciprocal plots. K-259-2, K-259-3 and KS-619-1 inhibited trypsin-activated enzyme in a competitive manner with respect to cAMP, and K_i values for these compounds were estimated from secondary plots to be 5.2, 5.0 and 2.1 μM respectively. These results suggest that there are binding sites for these compounds on the enzyme as well as on CaM and that these binding sites may be close to the active site. Taken together, the results suggest that these new anthraquinone compounds inhibit the CaM-induced activation of CaM-PDE by interacting directly with the enzyme as well as CaM antagonism.

Comparison of K-259-2, K-259-3 and KS-619-1 with so-called CaM antagonists

To investigate whether or not the new anthraquinone compounds interact with anionic amphiphiles, a smaller amount of either phosphatidylserine

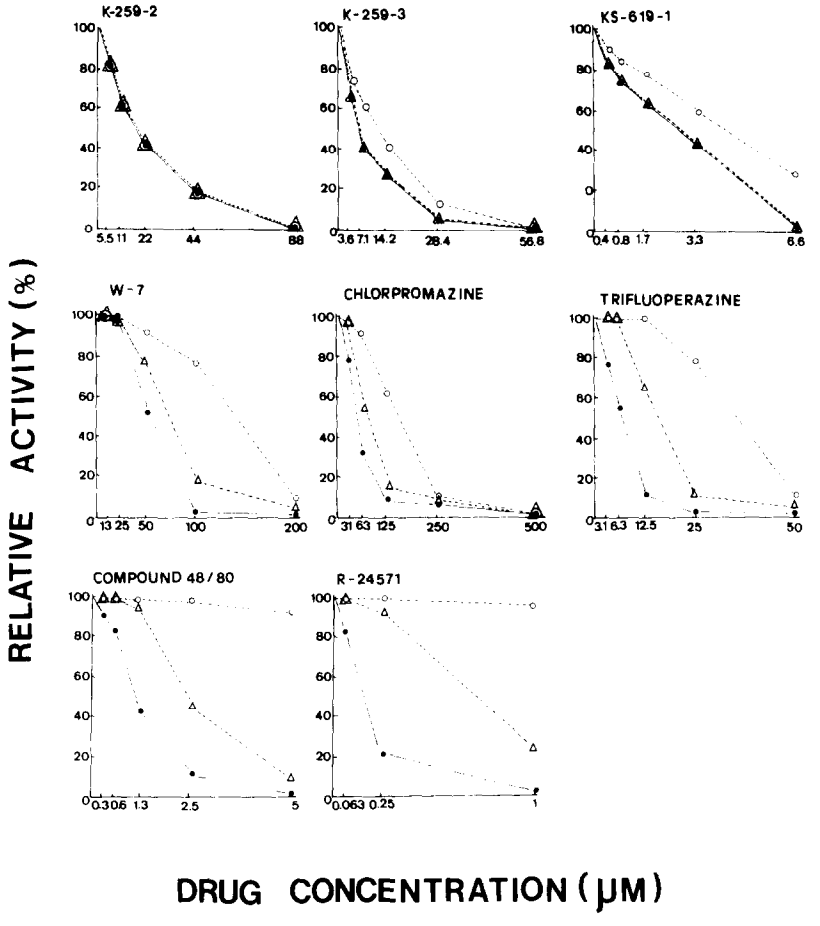


Fig. 6. Effect of phosphatidylserine and sodium oleate on the inhibition by various inhibitors of CaM-PDE. Calmodulin-activated phosphodiesterase activity was measured as described in Materials and Methods with various concentrations of one of the following drugs in the presence of either 100 μg/mL phosphatidylserine (--○--) or 15.6 μM sodium oleate (--△--): K-259-2, K-259-3, KS-619-1, chlorpromazine, trifluoperazine, compound 48/80 and R-24571. Control (—●—) experiments were conducted in a similar manner, but without phosphatidylserine and sodium oleate. Phosphatidylserine was microdispersed by sonication as described in Materials and Methods followed by the addition of reaction medium. Sodium oleate was added to reaction medium in the dissolved state. At the concentrations used, both phosphatidylserine and sodium oleate were ineffective as activators of the phosphodiesterase. Each point represents the mean value of three samples from the same batch of enzyme. Similar results were obtained using different enzyme preparations. One hundred per cent activity of the CaM-PDE corresponds to 9.3 nmol/min/tube.

(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 inhibitory drugs. Since K-259-2 and KS-619-1 are anionic amphiphiles, unlike so-called CaM antagonists, no interactions of these compounds with these anionic amphiphiles were expected. Indeed, as shown in Fig. 6, phosphatidylserine and sodium oleate had little or no effect on the inhibition by K-259-2, K-259-3 and KS-619-1 of CaM-PDE but, in contrast, attenuated the inhibition by so-called CaM antagonists, which share cationic amphiphilic properties.

These results indicate that new anthraquinone compounds, unlike so-called CaM antagonists, do not interact with phosphatidylserine or sodium oleate. Therefore, it is sufficient to understand that the inhibition of sodium oleate-activated CaM-PDE by K-259-2, K-259-3 and KS-619-1, which is shown in Fig. 2, was brought about by direct interaction of these new anthraquinone compounds with the enzyme, but not by interaction with sodium oleate.

Table 1 provides a list of the IC_{50} values of various drugs for CaM-dependent and CaM-independent PDE activities. K-259-2, K-259-3 and KS-619-1 inhibited bovine heart and brain CaM-PDE to a similar degree. However, inhibition of bovine heart CaM-independent PDE required higher concentrations of these new anthraquinone compounds. So-called CaM antagonists, such as W-7, chlorpromazine, trifluoperazine, compound 48/80 and R-24571 inhibited the bovine brain CaM-dependent fraction of the CaM-PDE activity without suppression of its

Table 1. Effects of various drugs on calmodulin-dependent and calmodulin-independent enzyme activities

Drug	IC_{50} (μM,* μg/mL)					
	Bovine brain CaM-PDE					
	CaM-stimulated activity	Basal activity‡	Trypsin-activated activity§	Sodium oleate-activated activity‖	Bovine heart CaM-PDE¶	Bovine heart CaM-independent PDE**
K-259-2	6.6	27.4	16.2	8.0	2.9	40.7
K-259-3	5.0	126	12.6	7.8	3.2	28.4
KS-619-1	2.0	12.3	2.5	1.4	1.5	25.9
W-7	42.0	>1000	>200	>100	43.7	>1000
Chlorpromazine	51.0	>500	>250	>500	58.0	>500
Trifluoperazine	8.6	>500	>200	>500	14.3	270
Compound 48/80	1.1*	>500*	NT	>50*	0.7*	500*
R-24571	0.03	>1000	>0.1	>10	0.05	4.4

† The activity of phosphodiesterase (26 mUnits/mL) was assayed in the presence of 4 units/mL CaM plus 50 μM $CaCl_2$.

‡ The basal activity of phosphodiesterase (18.6 mUnits/mL) was assayed in the presence of 3 mM ethylene bis(oxyethylenenitrilo)tetraacetic acid (EGTA), without CaM and $CaCl_2$.

§ The activity of proteolysed phosphodiesterase (26 mUnits/mL) was assayed in the absence of CaM and $CaCl_2$.

‖ The activity of phosphodiesterase (26 mUnits/mL) activated with 1 mM sodium oleate was assayed in the absence of CaM and $CaCl_2$.

¶ The activity of phosphodiesterase (40 mUnits/mL) was assayed in the presence of 2.5 units/mL CaM plus 50 μM $CaCl_2$.

** The activity of phosphodiesterase (25 mUnits/mL) was assayed in the presence of 3 mM EGTA, without CaM and $CaCl_2$.

Phosphodiesterase activity was assayed according to the method described in Materials and Methods. The IC_{50} value, which was defined as a drug concentration which produced 50% inhibition of the maximally-stimulated enzyme activity, was determined using six concentrations of each inhibitor. Results are means of three independent determinations using a single batch of enzyme and expressed in IC_{50} (μM) except compound 48/80, which is in IC_{50} (μg/mL). The figures expressed with > signifies considerable inhibition by the drug was not observed at this final concentration.

NT, not tested; CaM, calmodulin; CaM-PDE, Ca^{2+} and calmodulin-dependent cyclic nucleotide phosphodiesterase.

basal activity (Table 1). The IC_{50} values were in the same range as those reported previously for anti-CaM activity [22]. These so-called CaM antagonists inhibited bovine heart and brain CaM-PDE to a similar degree, but showed 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 (Table 1).

DISCUSSION

K-259-2, K-259-3 and KS-619-1, which are novel anthraquinone compounds having anionic amphiphilic properties, unlike so-called calmodulin antagonists, inhibit bovine brain CaM-PDE not solely by binding to CaM, but may also interfere directly with the enzyme, indicating that the binding site for these compounds on the enzyme may be structurally similar to that on CaM itself. A similar conclusion has been drawn by Itoh and Hidaka [11] and Matsuda *et al.* [23, 24] from a study of the inhibition of CaM-PDE by trifluoperazine, chlorpromazine and a series of W-7 analogues, indole carbazol compound and isoflavonoid compounds. Furthermore, penfluridol and trifluoperazine seem to bind, in addition to CaM, also to erythrocyte Ca^{2+}, Mg^{2+}-ATPase, since activation by both tryptic digestion and CaM can be completely inhibited in a similar concentration range [9]. More recently, Xu *et al.* [12] reported that a derivative of bisbenzylisoquinoline also inhibited trypsin-activated Ca^{2+}, Mg^{2+}-ATPase at concentrations similar to those that inhibited CaM activation. Kemp *et al.* [25] showed that synthetic peptides corresponding to both the skeletal muscle and smooth muscle myosin light chain kinase CaM binding regions were also potent competitive inhibitors with respect to substrate. On the basis of these findings from several laboratories, including ours, we can say that the binding site for the CaM antagonists, irrespective of their cationic and anionic characters, on the enzyme may have similar features to that on CaM and that similar features seem to exist among different CaM-regulated enzymes.

Although the inhibition of CaM-PDE by K-259-2, K-259-3 or KS-619-1 was overcome by a higher concentration of CaM, the precise mechanism for this CaM antagonism is still a matter of speculation and study. We used hydrophobic fluorescent probes [26] to obtain information regarding the binding properties of these new anthroquinone compounds to CaM. K-259-2 and KS-619-1 showed different effects on the fluorescent emission of hydrophobic probes bound to CaM. K-259-2 depressed this enhanced fluorescence, in contrast, however, KS-619-1 decreased NPN-fluorescence but enhanced TNS-fluorescence. It has also been shown that bepridil exhibits differential effects on two hydrophobic probes: it decreases 9-anthroylchloride-fluorescence, but increases the fluorescence intensity of TNS bound to CaM [27]. Anyway, the results obtained from a study with fluorescent probes clearly show

that K-259-2 and KS-619-1 bind to CaM and, further, that KS-619-1 can interact with CaM in a manner different from that of K-259-2.

For comparison, we examined the effect of new anthraquinone compounds along with the reference CaM antagonists W-7, chlorpromazine, trifluoperazine, compound 48/80 and calmidazolium on several CaM-independent systems. In contrast with new anthraquinone compounds, 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 and Hidaka [11]. Based on the explanation by Itoh and Hidaka [11], this discrepancy might be due to differences in the concentration of the substrate.

Stimulation of brain phosphodiesterase by limited proteolysis and by acidic and hydrophobic compounds confirms the results from several laboratories [5, 9, 11, 19]. Because the preparation of small vesicles of hydrophobic compounds is of crucial importance [28], in the present study we used sodium oleate in place of oleic acid and added it into the reaction mixture in a solubilized state. A high concentration of sodium oleate (1 mM) also stimulates phosphodiesterase up to about 65% of the activity of the enzyme maximally stimulated by CaM. So-called CaM antagonists, such as W-7, chlorpromazine, trifluoperazine, compound 48/80 and R-24571, in the present assay conditions, failed to inhibit the activity of brain phosphodiesterase induced by sodium oleate, but the new anthraquinone compounds do so effectively. However, in contrast with our results, Gietzen *et al.* [9] presented data showing that CaM antagonists, such as trifluoperazine and penfluridol inhibited the activation of phosphodiesterase by oleic acid (50 μM) as well as by CaM. This discrepancy might be due to different concentrations (1 mM sodium oleate and 50 μM oleic acid) and different conditions (solubilized state and dispersed micellar form) of the fatty acid in the assay mixture, but further studies are needed to clarify this point.

Unlike so-called CaM antagonists, a significant feature of the present new anthraquinone compounds is their anionic amphiphilic character. The lack of interaction of these compounds with anionic amphiphiles was confirmed by the finding that phosphatidylserine and sodium oleate did not overcome the inhibition of CaM-stimulated phosphodiesterase by K-259-2, K-259-3 and KS-619-1. Although it is uncertain whether this property of these compound is related to cell membrane permeability, we can say, at least, this property of the new anthraquinone compounds is advantageous in pharmacological studies. Indeed, experiments to be described elsewhere have shown that these compounds strongly inhibit the secretory responses of rat mast cells.

Because extensive work of Tanaka *et al.* [29] on the structure–activity relationship of W-7 and its naphthalensulfonamide derivatives has shown that the length of the hydrocarbon chain is an important determinant of anti-CaM activity, we obtained a methyl ester of K-259-2, K-259-3. K-259-3, in fact, inhibited more specifically CaM-stimulated phosphodiesterase. The present results suggest that future work should involve derivatives of K-259-2 substituted with longer hydrocarbon chains.

In conclusion, we can say from the results presented here that K-259-2, K-259-3 and KS-619-1 are a unique type of inhibitor of CaM-PDE, and that these novel anthraquinone compounds, under the present conditions, cause inhibitory effects on this enzyme not solely through their binding to CaM, but may also interact directly with the enzyme itself. To confirm these results, more experiments, including an evaluation of which interaction with CaM or with enzyme is the more important component of the inhibition of CaM-PDE by these novel anthraquinones, are needed. Finally, K-259-2, K-259-3 and KS-619-1 may be useful tools with which to clarify the mechanism of interaction between calmodulin and calmodulin-dependent phosphodiesterase.

Acknowledgement—We acknowledge with thanks the expert assistance of Miss Tomoko Itoh and Mrs Miyoko Funayama-Kojima. We also gratefully thank Dr Gordon Guroff (NICHD, National Institutes of Health, Bethesda) for kindly correcting the manuscript.

REFERENCES

1. Rubin RP, Historical and biological aspects of calcium action. In: *Calcium in Biological Systems* (Eds. Rubin RP, Weiss GB and Putney Jr JW), pp. 5–11. Plenum Press, New York, 1985.
2. Klee CB, Crouch TH and Richman PG, Calmodulin. *Annu Rev Biochem* **49**: 489–515, 1980.
3. Lin YM and Cheung WY, Ca^{2+}-dependent cyclic nucleotide phosphodiesterase. In: *Calcium and Cell Function Vol. 1: Calmodulin* (Ed. Cheung WY), pp. 231–237. Academic Press, New York, 1980.
4. Weiss B, Sellinger-Barnette M, Winkler JD, Schechter LE and Prozialeck WC, Calmodulin antagonists: structure–activity relationships. In: *Calmodulin Antagonists and Cellular Physiology* (Eds. Hidaka H and Hartshorne DJ), pp. 45–62. Academic Press, New York, 1985.
5. Wolff DJ and Brostrom CO, Calcium-dependent cyclic nucleotide phosphodiesterase from brain: identification of phospholipids as calcium-independent activators. *Arch Biochem Biophys* **173**: 720–731, 1976.
6. Taverna RD and Hanahan DJ, Modulation of human erythrocyte Ca^{2+}/Mg^{2+} ATPase activity by phospholipase A_2 and proteases: a comparison with calmodulin. *Biochem Biophys Res Commun* **94**: 652–659, 1980.
7. Niggli V, Adunyah ES and Carafoli E, Acidic phospholipids, unsaturated fatty acids, and limited proteolysis mimic the effect of calmodulin on the purified erythrocyte Ca^{2+}-ATPase. *J Biol Chem* **256**: 8588–8592, 1981.
8. Ikebe M, Stepinska M, Kemp BE, Means AR and Hartshorne DJ, Proteolysis of smooth muscle myosin light chain kinase: formation of inactive and calmodulin-independent fragments. *J Biol Chem* **262**: 13828–13834, 1987.
9. Gietzen K, Sadorf I and Bader H, A model for the regulation of the calmodulin-dependent enzymes erythrocyte Ca^{2+}-transport ATPase and brain phosphodiesterase by activators and inhibitors. *Biochem J* **207**: 541–548, 1982.
10. Adunyah ES, Niggli V and Carafoli E, The anti-calmodulin drugs trifluoperazine and R24571 remove the activation of the purified erythrocyte Ca^{2+}-ATPase by acidic phospholipids and by controlled proteolysis. *FEBS Lett* **143**: 65–68, 1982.
11. Itoh H and Hidaka H, Direct interaction of calmodulin antagonists with Ca^{2+}/calmodulin-dependent cyclic

nucleotide phosphodiesterase. *J Biochem* **96**: 1721–1726, 1984.
12. Xu Y, Liu J, Zhang S and Liu L, The effect of berbamine derivatives on activated Ca^{2+}-stimulated Mg^{2+}-dependent ATPase in erythrocyte membranes. *Biochem J* **248**: 985–988, 1987.
13. Matsuda Y, Asano K, Kawamoto I and Kase H, K-259-2, a new inhibitor of Ca^{2+} and calmodulin-dependent cyclic nucleotide phosphodiesterase from *Micromonospora olivasterospora*. *J Antibiotics* **40**: 1092–1100, 1987.
14. Matsuda Y and Kase H, KS-619-1, a new inhibitor of Ca^{2+} and calmodulin-dependent cyclic nucleotide phosphodiesterase from *Streptomyces californicus*. *J Antibiotics* **40**: 1104–1110, 1987.
15. Yasuzawa T, Yoshida M, Shirahata K and Sano H, Structure of a novel Ca^{2+} and calmodulin-dependent cyclic nucleotide phosphodiesterase inhibitor K-259-2. *J Antibiotics* **40**: 1101–1103, 1987.
16. Yasuzawa T, Yoshida M, Shirahata K and Sano H, Structure of a novel Ca^{2+} and calmodulin-dependent cyclic nucleotide phosphodiesterase inhibitor KS-619-1. *J Antibiotics* **40**: 1111–1114, 1987.
17. Kubo K, Matsuda Y, Kase H and Yamada K, Inhibition of calmodulin-dependent cyclic nucleotide phosphodiesterase by flunarizine, a calcium-entry blocker. *Biochem Biophys Res Commun* **124**: 315–321, 1984.
18. Kakiuchi S, Yamazaki R, Teshima Y, Uenishi K and Miyamoto E, Multiple cyclic nucleotide phosphodiesterase activities from rat tissues and occurrence of calcium-plus-magnesium-ion-dependent phosphodiesterase and its protein activator. *Biochem J* **146**: 109–120, 1975.
19. Klee CB, Crouch TH and Krinks MH, Subunit structure and catalytic properties of bovine brain Ca^{2+}-dependent cyclic nucleotide phosphodiesterase. *Biochemistry* **18**: 722–729, 1979.
20. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685, 1970.
21. Kincaid RL, Stith-Coleman IE and Vaughan M, Proteolytic activation of calmodulin-dependent cyclic nucleotide phosphodiesterase. *J Biol Chem* **260**: 9009–9015, 1985.
22. Roufogalis BD, Calmodulin antagonism. In: *Calcium and Cell Physiology* (Ed. Marme D), pp. 148–169. Springer, Berlin, 1985.
23. Matsuda Y, Nakanishi S, Nagasawa K, Iwahashi K and Kase H, The effect of K-252a, a potent microbial inhibitor of protein kinase, on activated cyclic nucleotide phosphodiesterase. *Biochem J* **256**: 75–80, 1988.
24. Matsuda Y, Asano K, Kawamoto I, Yasuzawa T, Shirahata K, Sano H and Kase H, K-251 compounds, inhibitors of Ca^{2+} and calmodulin-dependent cyclic nucleotide phosphodiesterase from *Streptomyces album*. *Agric Biol Chem* **52**: 3211–3213, 1988.
25. Kemp BE, Pearson RB, Guerriero Jr V, Bagchi IC and Means AR, The calmodulin binding domain of chicken smooth muscle myosin light chain kinase contains a pseudosubstrate sequence. *J Biol Chem* **262**: 2542–2548, 1987.
26. Epstein PM, Fiss K, Hachisu R and Andrenyak DM, Interaction of calcium antagonists with cyclic AMP phosphodiesterases and calmodulin. *Biochem Biophys Res Commun* **105**: 1142–1149, 1982.
27. Schaeffer P, Luginer C, Follenius-Wund A, Gerard D and Stoclet J-C, Comparative effects of calmodulin inhibitors on calmodulin's hydrophobic sites and on the activation of cyclic nucleotide phosphodiesterase by calmodulin. *Biochem Pharmacol* **36**: 1989–1996, 1987.
28. Gietzen K, Xü Y-H, Galla H-J and Bader H, Multimers of anionic amphiphiles mimic calmodulin stimulation of cyclic nucleotide phosphodiesterase. *Biochem J* **207**: 637–640, 1982.
29. Tanaka T, Ohmura T and Hidaka H, Hydrophobic interaction of the Ca^{2+}-calmodulin complex with calmodulin antagonists: naphthalenesulfonamide derivatives. *Mol Pharmacol* **22**: 403–407, 1982.